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Application Number: 17395534 Document Date: 08/06/2021

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NanoString v. Harvard
IPR2024-00526

PTO/AIA/15 (10-17)
Approved for use through 11/30/2020. OMB 0651-0032
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	010498.01437\US
First Named Inventor	Evan R. Daugharthy
Title	Method and Apparatus for Volumetric Imaging
Priority Mail Express® Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [ ] **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. [ ] **Applicant asserts small entity status.** See 37 CFR 1.27
3. [ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. [X] **Specification** [*Total Pages* 59]
   Both the claims and abstract must start on a new page.
   (See MPEP § 608.01(a) for information on the preferred arrangement)
5. [X] **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 13]
6. **Inventor's Oath or Declaration** [*Total Pages*]
   (including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))
   a. [ ] Newly executed (original or copy)
   b. [X] A copy from a prior application (37 CFR 1.63(d))
7. [X] **Application Data Sheet** ** See note below.*
   See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program (*Appendix*)
   [ ] Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** (*if applicable, items a. – c. are required*)
   a. [ ] Computer Readable Form (CRF)
   b. [ ] Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

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ACCOMPANYING APPLICATION PAPERS

10. [ ] **Assignment Papers** (cover sheet & document(s))
    Name of Assignee
11. [ ] **37 CFR 3.73(c) Statement** (*when there is an assignee*) [X] **Power of Attorney**
12. [ ] **English Translation Document** (*if applicable*)
13. [X] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
14. [ ] **Preliminary Amendment**
15. [ ] **Return Receipt Postcard** (*MPEP § 503*) (*Should be specifically itemized*)
16. [ ] **Certified Copy of Priority Document(s)** (*if foreign priority is claimed*)
17. [ ] **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. [ ] **Other:**

***Note:** (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

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Country		Telephone		Email	

Signature	/John P. Iwanicki/	Date	August 5, 2021
Name (Print/Type)	John P. Iwanicki	Registration No. (Attorney/Agent)	34,628

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Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82A (07-13)
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Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Evan R. Daugharthy
Title	Method and Apparatus for Volumetric Imaging
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

SIGNATURE of Applicant or Patent Practitioner

Signature	/John P. Iwanicki/	Date (Optional)	August 5, 2021
Name	John P. Iwanicki	Registration Number	34,628
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			

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21388721

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

U.S.PATENTS						Remove
Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	7473767		2009-01-06	Dimitrov	
	2	5563056		1996-10-08	Swan et al.	
	3	7323305		2008-01-29	Leamon et al.	
	4	9217151		2015-12-22	Yin et al.	
	5	8551710		2013-10-08	Bernitz et al.	
	6	4123610		1978-10-31	Summerton et al.	
	7	4981985		1991-01-01	Kaplan et al.	
	8	5830708	A	1998-11-03	Naughton	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	First Named Inventor	Evan R. Daugharthy
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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	9	5594235	A	1997-01-14	Lee	
	10	4844617	A	1989-07-04	Kelderman et al.	
	11	8501459		2013-08-06	Chen et al.	
	12	5151189		1992-09-29	Hu et al.	
	13	6083726		2000-07-04	Mills, Jr. et al.	
	14	7745129	B1	2010-06-29	Schatz	
	15	5871921		1999-02-16	Landegren et al.	
	16	6632655		2003-10-14	Mehta et al.	
	17	10494662		2019-12-03	Church et al.	
	18	10138509		2018-11-27	Church et al.	
	19	5834758		1998-11-10	Trulson et al.	

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	20	10266888		2019-04-23	Daugharthy et al.	
	21	8697359		2014-04-15	Zhang	
	22	9257135		2016-02-09	Ong et al.	
	23	9267135		2016-02-23	Church et al.	
	24	8993233		2015-03-31	Zhang et al.	
	25	8999641		2015-04-07	Zhang et al.	
	26	8932814		2015-01-13	Cong et al.	
	27	8871445		2014-10-28	Cong et al.	
	28	8906616		2014-12-09	Zhang et al.	
	29	10494667		2019-12-03	Chee	
	30	8460865		2013-06-11	Chee et al.	

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	31	8268554		2012-09-18	Schallmeiner	
	32	8124751		2012-02-28	Pierce et al.	
	33	9201063		2015-12-01	Sood et al.	
	34	10267808		2019-04-23	Cai	
	35	6534266		2003-03-18	Singer	
	36	5695940		1997-12-09	Drmanac et al.	
	37	7255994		2007-08-14	Lao	
	38	6306597		2001-10-23	Macevicz	
	39	7906285		2011-03-15	Drmanac	
	40	7427479		2008-09-23	Karger et al.	
	41	10227639		2019-03-12	Levner et al.	

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	42	4886741		1989-12-12	Schwartz	
	43	10545075		2020-01-28	Deisseroth et al.	
	44	8329404		2012-12-11	McKernan et al.	
	45	6586176		2003-07-01	Trnovsky et al.	
	46	6068979		2000-05-30	Akhavan-Tafti	
	47	11021737		2021-06-01	Church et al.	
	48	9371598		2016-06-21	Chee	
	49	10501791		2019-12-10	Church et al.	

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	1	20110092376	A1	2011-04-21	Colston, JR. et al.	

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	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	2	20140087378	A1	2014-03-27	Chatre et al.	
	3	20060292611	A1	2006-12-28	Berka et al.	
	4	20100047924	A1	2010-02-25	WEBSTER et al.	
	5	20040248144	A1	2004-12-09	Mir	
	6	20100268478	A1	2010-10-21	Andregg et al.	
	7	20090246879	A1	2009-10-01	Drmanac et al.	
	8	20050147981	A1	2005-07-07	Yamakawa et al.	
	9	20120122712	A1	2012-05-17	Goldstein	
	10	20070117109	A1	2007-05-24	Rothemund	
	11	20100009868	A1	2010-01-14	Yan et al.	
	12	20050233318	A1	2005-10-20	Chee et al.	

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	13	20060177833	A1	2006-08-10	Brenner	
	14	20110086774	A1	2011-04-14	Dunaway	
	15	20110294135	A1	2011-12-01	Carlson	
	16	20090208965	A1	2009-08-20	Tafas et al.	
	17	20130245096	A1	2013-09-19	Abitbol	
	18	20060024711	A1	2006-02-02	Lapidus et al.	
	19	20070117177	A1	2007-05-24	LUO et al.	
	20	20120040397	A1	2012-02-16	Luo et al.	
	21	20020015952	A1	2002-02-07	Anderson et al.	
	22	20090005259	A1	2009-01-01	Drmanac	
	23	20060248349	A1	2006-11-02	Rathjen et al.	

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	24	20100223276	A1	2010-09-02	Al-Shameri et al.	
	25	20070087362	A1	2007-04-19	Church et al.	
	26	20140220587	A1	2014-08-07	Green, JR. et al.	
	27	20160265046	A1	2016-09-15	ZHANG et al.	
	28	20060234261	A1	2006-10-19	Pierce et al.	
	29	20150133319	A1	2015-05-14	Fu et al.	
	30	20170212983	A1	2017-07-27	CAI et al.	
	31	20070020650	A1	2007-01-25	Kahvejian	
	32	20110245111	A1	2011-10-06	Chee	
	33	20030148335	A1	2003-08-07	Shen et al.	
	34	20110257031	A1	2011-10-20	BODEAU et al.	

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	35	20040077014	A1	2004-04-22	Becker	
	36	20080176769	A1	2008-07-24	Rank et al.	
	37	20160024555	A1	2016-01-28	Church et al.	
	38	20130017229	A1	2013-01-17	Mooney et al.	
	39	20110020291	A1	2011-01-27	Banerjee et al.	
	40	20050106629	A1	2005-05-19	McGrath et al.	
	41	20040259190	A1	2004-12-23	Naughton	
	42	20020155989	A1	2002-10-24	Efimov et al.	
	43	20140049632	A1	2014-02-20	Hemmer	
	44	20060077536	A1	2006-04-13	Bromage et al.	
	45	20070206275	A1	2007-09-06	Hemmer et al.	

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	46	20020172950	A1	2002-11-21	Kenny et al.	
	47	20050191687	A1	2005-09-01	Wang et al.	
	48	20070231823	A1	2007-10-04	McKernan et al.	
	49	20080269068	A1	2008-10-30	CHURCH et al.	
	50	20090220968	A1	2009-09-03	Issadore et al.	
	51	20100151472	A1	2010-06-17	Nolan et al.	
	52	20180010166	A1	2018-01-11	Pierce et al.	
	53	20180282787	A1	2018-10-04	WALTER et al.	
	54	20120330636	A1	2012-12-27	Albou	
	55	20140087427	A1	2014-03-27	Bujnicki et al.	
	56	20080050718	A1	2008-02-28	Gesteland et al.	

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	57	20110104693	A1	2011-05-05	SELIGMANN	
	58	20060183107	A1	2006-08-17	Melkonyan et al.	
	59	20110033520	A1	2011-02-10	Mather et al.	
	60	20180051322	A1	2018-02-22	Church et al.	
	61	20070292877	A1	2007-12-20	Dimitrov	
	62	20100087325	A1	2010-04-08	Buermann	
	63	20140200146	A1	2014-07-17	Xie et al.	
	64	20140270435	A1	2014-09-18	DUNN	
	65	20160253584	A1	2016-09-01	Fodor et al.	
	66	20160289740	A1	2016-10-06	Fu et al.	
	67	20170176338	A1	2017-06-22	Wu et al.	

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	68	20100076057	A1	2010-03-25	Sontheimer et al.	
	69	20110189776	A1	2011-08-04	Terns et al.	
	70	20110223638	A1	2011-09-15	Wiedenheft et al.	
	71	20130130248	A1	2013-05-23	Haurwitz et al.	
	72	20130253040	A1	2013-09-26	Miller et al.	
	73	20140068797	A1	2014-03-06	Doudna et al.	
	74	20140179770	A1	2014-06-26	ZHANG et al.	
	75	20140273226	A1	2014-09-18	Wu	
	76	20140315985	A1	2014-10-23	May et al.	
	77	20140342457	A1	2014-11-20	MALI et al.	
	78	20140273233	A1	2014-09-18	Chen et al.	

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	79	20160024524	A1	2016-01-28	Joung et al.	
	80	20140310830	A1	2014-10-16	ZHANG et al.	
	81	20140179006	A1	2014-06-26	ZHANG	
	82	20150247150	A1	2015-09-03	Zhang et al.	
	83	20160153006	A1	2016-06-02	ZHANG et al.	
	84	20160355795	A1	2016-12-08	Ran et al.	
	85	20170191078	A1	2017-07-06	ZHANG et al.	
	86	20160340662	A1	2016-11-24	Zhang et al.	
	87	20160160210	A1	2016-06-09	Mali et al.	
	88	20110223585	A1	2011-09-15	GULLBERG et al.	
	89	20060228733	A1	2006-10-12	Pierce et al.	

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	90	20120252686	A1	2012-10-04	Umbarger et al.	
	91	20160358326	A1	2016-12-08	Sarachan et al.	
	92	20200090786	A1	2020-03-19	QUIROZ ZARATE et al.	
	93	20120126142	A1	2012-05-24	Matsui et al.	
	94	20140220578	A1	2014-08-07	Bohannon et al.	
	95	20060216339	A1	2006-09-28	Ambron et al.	
	96	20150004598	A1	2015-01-01	Gao et al.	
	97	20150267251	A1	2015-09-24	Cai et al.	

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	1	2008069973	WO	A2	2008-06-12	Univ Columbia		

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	2	2009/046149	WO	A1	2009-04-09	Applied Biosystems		
	3	2007/121489	WO	A2	2007-10-25	Applera Corp		
	4	2010080134	WO	A1	2010-07-15	Harvard College		
	5	9746704	WO	A1	1997-12-11	Lynx Therapeutics, Inc		
	6	2012150035	WO	A1	2012-11-08	Cherkasov, Dmitry		
	7	2012/058638	WO	A2	2012-05-03	President And Fellows Of Harvard College,		
	8	2013/055995	WO	A2	2013-04-18	Harvard College		
	9	20080003402	KR	A	2008-01-07	Mycosol, Inc		
	10	2007086900	WO	A2	2007-08-02	Mycosol, Inc		
	11	2012005595	WO	A2	2012-01-12	De Laat, Wouter		
	12	2014/0163886	WO	A1	2014-10-09	Harvard College		

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	13	2014/182528	WO	A2	2014-11-13	California Inst Of Techn		
	14	2015/118029	WO	A1	2015-08-13	Olink AB		
	15	2011/143583	WO	A1	2011-11-17	Illumina, Inc		
	16	01/26708	WO	A1	2001-04-19	Univ Ohio State		
	17	01/37266	WO	A1	2001-05-25	Research Foundation of State University of New Yor		
	18	2004/104645	WO	A2	2004-12-02	Lucid, Inc.		
	19	2007/001986	WO	A2	2007-01-04	Luo, Yuling		
	20	2012/110899	WO	A2	2012-08-23	Bernitz, Mats		
	21	2015/127183	WO	A2	2015-08-27	Massachusetts Inst Technology		
	22	2008157696	WO	A2	2008-12-24	Kokoris, Mark		
	23	2016081740	WO	A1	2016-05-26	Nanostring Technologies, Inc		

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	24	2878671	EP	A1	2015-06-03	LSI Medience Corporation		
	25	98/56955	WO	A1	1998-12-17	Bioseparations, Inc.		
	26	2017/161251	WO	A1	2017-09-21	President and Fellows of Harvard College		
	27	2014/048083	WO	A1	2014-04-03	School Of Ophthalmology And Optometry Wenzhou Medi		
	28	2007076128	WO	A2	2007-07-05	Nanostring Technologies, Inc		
	29	2007/123744	WO	A2	2007-11-01	Solexa, Inc		
	30	2012-170337	JP	A	2012-09-10	Hitachi High Tech Corp		
	31	2009/046348	WO	A1	2009-04-09	Applied Biosystems		
	32	2010/087325	WO	A1	2010-08-05	Sharp KK		
	33	2007/149696	WO	A1	2007-12-27	Applera Corp		
	34	2006/138257	WO	A2	2006-12-28	Callida Genomics, Inc		

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	Attorney Docket Number	010498.01437\US

	35	2008/108989	WO	A2	2008-09-12	Danisco		
	36	2010/054108	WO	A2	2010-05-14	University Of Georgia Research Foundation, Inc		
	37	2012/164565	WO	A1	2012-12-06	Yeda Res & Dev		
	38	2013/098244	WO	A1	2013-07-04	Univ Wageningen		
	39	2013176772	WO	A1	2013-11-28	Univ California		
	40	2013/142578	WO	A1	2013-09-26	Vilnius University,		
	41	2013/141680	WO	A1	2013-09-26	Univ Vilnius		
	42	2013/126794	WO	A1	2013-08-29	Hutchinson Fred Cancer Res		
	43	2011/143124	WO	A2	2011-11-17	Univ California		
	44	2014/022702	WO	A2	2014-02-06	Univ California		
	45	2014/113493	WO	A1	2014-07-24	Univ Emory		

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Attorney Docket Number	010498.01437\US

	46	2014/191518	WO	A1	2014-12-04	Cellectis		
	47	2014/150624	WO	A1	2014-09-25	Caribou Biosciences, Inc		
	48	2014/093694	WO	A1	2014-06-19	The Broad Institute, Inc		
	49	2014/099744	WO	A1	2014-06-26	Harvard College		
	50	2014/089290	WO	A1	2014-06-12	Sigma Aldrich Co LLC		

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	1	Office Action issued for corresponding European Patent Application No. 12780609.9, dated September 23, 2015.	
	2	SEO, et al. Four-color DNA sequencing by synthesis on a chip using photocleavable fluorescent nucleotides. Proceeding of the National Academy of Sciences, 2005 April, 102 (17) 5926-5931.	
	3	BRENNER, et al. "Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays". Nature Biotechnology volume 18, pages 630-634 (2000) doi:10.1038/76469	
	4	2014 Dec 18 (PCT) International Preliminary Report - App PCT/US2013/044241	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	5	Shendure Jay et al., "Accurate multiplex polony sequencing of an evolved bacterial genome," Science, American Association for the Advancement of Science, Washington, DC; US, vol. 309, no. 5741, 1 September 2005, pages 1728-1732, XP002427180, ISSN: 0036-8075, DOI: 10.1126/SCIENCE.1117839	
	6	Extended European Seach Report issued in corresponding European Application No. 12860433.7, dated August 13, 2015.	
	7	International Search Report and Written Opinion issued in corresponding International Application No. PCT/US2012/071398, mailed April 8, 2013.	
	8	BENNER et al. "Gene Expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays". Nature Biotechnology, Vol. 18, pp. 630-634 (31 June 2000).	
	9	HAN et al. "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules". Nature Biotechnology, Vol. 19, 99. 631-635 (31 July 2001).	
	10	LEE, JH et al. Highly Multiplexed Subcellular RNA Sequencing In Situ. Science. 21 March 2014, Vol. 343, No. 6177; pages 1360-1363; abstract; page 1360, second column, second paragraph to third column, first paragraph; page 1361, first column, first paragraph; page 1363, first column, second paragraph to second column, first paragraph; DOI: 10.1126/science.1250212.	
	11	ASCANO, M et al. Identification Of RNA-Protein Interaction Networks Using PAR-CLIP. Wiley Interdiscip Rev RNA. March 2012, Vol. 3, No. 2; pages 159-177; page 3, third paragraph; page 16, figure 1; page 25, figure 6; DOI: 10.1002/wrna.1103.	
	12	GINART, P et al. RNA Sequencing In Situ. Nat Biotechnol. June 2014, Vol. 32, No. 6; pages 543-544; DOI: 10.1038/nbt.2921.	
	13	SALIBA, AE et al. Single-Cell RNA-Seq: Advances And Future Challenges. Nucleic Acids Res. 22 July 2014, Vol. 42, No. 14; pages 8845-8860; DOI: 10.1093/nar/gku555.	
	14	Eliscovich et al. mRNA on the move: The road to its biological destiny. Journal of Biological Chemistry, Vol. 288, No. 28, pages 20361-20368, July 2013, in press May 2013 (Year: 2013)	
	15	Weis et al. Protein targeting to subcellular organelles via mRNA localization. Biochimica et Biophysica Acta, Vol. 1833, pages 260- 273, 2013, available online April 2012 (Year: 2012)	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	16	Jambhekar et al. Cis-acting determinants of asymmetric, cytoplasmic RNA transport. RNA, Vol. 13, pages 625-642, 2007 (Year: 2007)	
	17	Singer-Kruger et al. Here, there, everywhere. RNA Biology, Vol. 11, No. 8, pages 1031-1039, August 2014. (Year: 2014)	
	18	Matlin et al. Spatial expression of the genome: the signal hypothesis at forty. Nature Reviews. Molecular Cell Biology, Vol. 12, No. 5, pages 333-340, May 2011, Epub April 2011. (Year: 2011)	
	19	Polidoros et al. Rolling circle amplification-RACE: a method for simultaneous isolation of 5' and 3' cDNA ends from amplified cDNA templates. Bio Techniques, Vol. 41, No. 1, pages 35, 36, 38 and 40, July 2006, including page 1/1 of Supplementary Material. (Year: 2006)	
	20	Tsaftaris et al. Isolation of three homologous AP1-like MADS-box genes in crocus (Crocus sativus L.) and characterization of their expression. Plant Science, Vol. 166, No. 5, pages 1235-1243, May 2004. (Year: 2004)	
	21	Meeks et al. Characterization of genes encoding poly(A) polymerases in plants: Evidence for duplication and functional specialization. PLoS ONE, Vol. 4, No. 11, e8082, November 2009, printed as pages 1/10-10/10. (Year: 2009)	
	22	Kalivas et al. famRCA-RACE: A rolling circle amplification RACE for isolating a family of homologous cDNAs in one reaction ... Preparative Biochemistry and Biotechnology, Vol. 40, No. 3, pages 177-187, July 2010. (Year: 2010)	
	23	Thisse et al. "High-Resolution in situ hybridization to whole-mount zebrafish embryos" 2008. Nature Protocols. Vol.3 No.1 pp 59-69. Doi:10.1038/nprot.2007.514	
	24	Grompe (1993) Nature Genetics DOI: 10.1038/ng1093-111.	
	25	Thisse et al. 2008 Nature protocols vol.3 No 1 pp 59-69. Doi:10.1038/nprot.2007.514	
	26	Doillon et al. "Actin Filaments in Normal Dermis and During Wound Healing" The American Journal of Pathology, Vol 126 Issue 1 (1987): pages 164-170; page 164 col 1 para 1, page 170 col 1 para 2, fig. 4A-C	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Attorney Docket Number	010498.01437\US

	27	International Search Report and Written Opinion based on PCT/US2018/027583 issued 29 June 2018	
	28	Soderberg, Ola et al.,"Direct observation of individual endogenous protein complexes in situ by proximity ligation," Nature Methods, December 2006, pp. 995-1000, vol. 3, no. 12, Nature Publishing Group	
	29	Schweitzer, Barry et al., "Immunoassays with rolling circle DNA amplification: A versatile platform for ultrasensitive antigen detection" PNAS, August 29, 2000, pp. 10113-10119, vol. 97, no. 18.	
	30	Cao, Yi et al.," In-situ immuno-PCR to detect antigens," The Lancet, September 16, 2000, pp. 1002-1003, vol. 356.	
	31	Sano, Takeshi et al. "Immuno-PCR: Very Sensitive Antigen Detection by Means of Specific Antibody-DNA Conjugates," Science, October 2, 1992, pp. 120-122, vol. 258	
	32	DASARI, Vivek et al., "Platform for Spatial Molecular Data by Vivek Dasari 1-7 Sig nature redacted Thesis Supervisor", 20 August 2015 (2015-08-20), XP055559164, Retreived from the Internet: URL:http://dspace.mit.edu/bitstream/handle/1721.1/107103/971494098-MIT.pdf?sequence=1 [retreived on 2019-02-20]	
	33	Extended European Search Report issued 13 MAY 2019 for EP Application No. 16862929.3	
	34	LEE, Je Hyuk et al., "Fluorescent in situ sequencing (FISSEQ) or RNA for gene expression profiling in intact cells and tissues", Nature Protocols, vol. 10, no. 3, 12 February 2015 (2015-02-12), pages 442-458. XP055272042, GB ISSN: 1754-2189, DOI: 10.1038/nprot.2014.191	
	35	Extended European Search Report issued May 21, 2019 for European Application No. 16862945.9	
	36	CHOI, Harry M.T. et al., "Next-Generation in Situ Hybridization Chain Reaction: Higher Gain, Lower Cost, Greater Durability" ACS NANO, vol. 8, no. 5, 27 May 2014 (2014-05-27), pages 4284-4294, XP055409053, US	
	37	RAVAN, Hadi, et al. "Isothermal RNA detection through the formation of DNA concatemers contiaining HRP-mimicking DNAzymes on the surface of gold nanoparticles", BIOSENSORS AND BIOELECTRONICS, ELSEVIER SCIENCE LTD. UK, AMSTERDAM, NL, vol. 80, 18 January 2016 (2016-01-18), pages 67-73, XP029441324	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	38	Extended European Search Report issued for EP Application No. 17790240.0 on September 4, 2019	
	39	Brown et al., Review Article : In situ Hybridization with Riboprobes : An Overview for Veterinary Pathologists. Veterinary Pathology 35 : 159-167 (Year: 1998)	
	40	Choi et al.,Programmable in situ amplification for multiplexed imaging of mRNA expression. Nature Biotechnology 28 (11): 1208 (Year: 2010)	
	41	Choi & Love et al., Immuno-Hybridization Chain Reaction for Enhancing Detection of Individual Cytokine-Secreting Human Peripheral Mononuclear Cells. Analytical Chemistry 83 : 6890-6895 (Year: 2011)	
	42	Hansen et al., Sensitive ligand-based protein quantification using immuno-PCR: A critical review of single-probe and proximity ligation assays. Biotechniques 56:217-228 (Year: 2014)	
	43	Kuimelis et al., Cleavage properties of an oligonucleotide containing a bridged internucleotide 5 -phosphorothioate RNA linkage. Nucleic Acids Research 23 (23) : 4753-4760 (Year: 1999)	
	44	Mag et al., Synthesis and selective cleavage of an oligodeoxynucleotide containing a bridged internucleotide 5'-phosphorothioate linkage. Nucleic Acids Research 19(7): 1437 (Year: 1991)	
	45	Richardson et al., Experimental and Theoretical Studies of Light-to-Heat Conversion and Collective Heating Effects in Metal Nanoparticle Solutions. Nano Letters 9(3) : 1139-1146 (Year: 2009)	
	46	Song et al., Hybridization chain reaction-based aptameric system for the highly selective and sensitive detection of protein. Analyst 137: 1396 (Year: 2012)	
	47	Xiao et al., Single-step electronic detection of femtomolar DNA by target-induced strand displacement in an electrode-bound duplex. PNAS 103(45): 16677-16680 (Year: 2006)	
	48	Zhang et al., Dynamic DNA nanotechnology using strand-displacement reactions. Nature Chemistry 3 : 103-113 (Year: 2011)	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	49	Zhao et al., An electrochemical aptasensor based on hybridization chain reaction with enzyme-signal amplification for interferon-gamma detection. Biosensors and Bioelectronics 36: 129-134 (Year: 2012)	
	50	Srinivas et al., On the biophysics and kinetics of toehold-mediated DNA strand displacement. Nucleic Acids Research 41 (22) : 10641-10658 (Year: 2013)	

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	1	2014/197568	WO	A2	2014-12-11	Harvard College		
	2	2014/093661	WO	A2	2014-06-19	The Broad Institute, Inc		
	3	2014/093595	WO	A1	2014-06-19	The Broad Institute, Inc		

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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	4	2014/093622	WO	A2	2014-06-19	The Broad Institute, Inc		
	5	2014/065596	WO	A1	2014-05-01	Toolgen Inc		
	6	112015012375	BR	A2	2017-09-26	Sigma Aldrich Co LLC		X
	7	112015014425	BR	A2	2017-10-10	Harvard College		☒
	8	112015022061	BR	A2	2017-11-14	Caribou Biosciences, Inc		☒
	9	112015013784	BR	A2	2017-07-11	Massachusetts Inst Technology		☒
	10	112015013785	BR	A2	2017-07-11	Massachusetts Inst Of Technolgy		☒
	11	112015008708	BR	A2	2017-09-26	Toolgen Incorporated		☒
	12	2891347	CA	A1	2014-06-12	Sigma Aldrich Co LLC		☐
	13	2014/144288	WO	A1	2014-09-18	Gen Hospital Corp		☐
	14	2003044229	WO	A1	2003-05-30	Gullberg, Mats		☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
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	15	1580283	CN	A	2005-02-16	Univ Tsinghua		☒
	16	1959384	CN	A	2007-05-09	Henan Provincial Bioengineerin		☒
	17	101553306	CN	A	2009-10-07	Clondiag GmbH		☒
	18	2013096851	WO	A1	2013-06-27	Harvard College		☐
	19	2016007839	WO	A1	2016-01-14	Harvard College		☐
	20	2017079406	WO	A1	2017-05-11	Harvard College		☐
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	1	WEIBRECHT, Irene et al., "Simultaneous Visualization of Both Signaling Cascade Activity and End-Point Gene Expression in Single Cells", PLOS ONE, vol. 6, no. 5, 25 May 2011 (2011-05-25)	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Attorney Docket Number	010498.01437\US

	2	Larsson, Chatarina; Grundberg, Ida; Sbderberg, Ola; Nilsson, Mats: 11 In situ detection and genotyping of individual mRNA molecules", Nature Methods, vol . 7, no. 5 11 April 2010 (2010-04-11), pages 395-397, XP055035168, DOI: 10.1038/nmeth.1448 Retrieved from the Internet: URL:http://www.nature.com/nmeth/journal/v7/n5/pdf/nmeth.1448.pdf [retrieved on 2012-08-09] * the whole document *	
	3	NUOVO: "Co-labeling Using In Situ PCR: A Review "JOURNAL OF HISTOCHEMISTRY & CYTOCHEMISTRY, vol . 49, no. 11, 1 November 2001 (2001-11-01), pages 1329-1339, XP055164942, ISSN: 0022-1554, DOI: 10.1177/002215540104901101 * the whole document *	
	4	MITRA R. D. ET AL: 11 In situ localized amplification and contact replication of many individual DNA molecules 11 NUCLEIC ACIDS RESEARCH, INFORMATION RETRIEVAL LTD, GB, vol . 27, no. 24, 15 December 1999 (1999-12-15), page e34, XP002292358, ISSN: 0305-1048, DOI: 10.1093/NAR/27.24.E34 * abstract *	
	5	KE ET AL: 11 In situ sequencing for RNA analysis in preserved tissue and cells 11 NATURE METHODS, vol. 10, no. 9, 14 July 2013 (2013-07-14), pages 857-860, XP055163946, ISSN: 1548-7091, DOI: 10.1038/nmeth.2563 * the whole document *	
	6	LEE ET AL: "Highly Multiplexed Subcellular RNA Sequencing in Situ", SCIENCE, vol. 343, no. 6177, 27 February 2014 (2014-02-27), pages 1360-1363, XP055305772, us ISSN: 0036-8075, DOI: 10.1126/science.1250212	
	7	CLAUSSON ET AL: "Compaction of rolling circle amplification products increases signal integrity and signal-to-noise ratio", SCIENTIFIC REPORTS, vol. 5, 23 July 2015 (2015-07-23), page 12317, XP055305777, DOI: 10.1038/ srep12317	
	8	NADJI et al.,"Photochemically and Photoenzymatically Cleavable DNA," J. Am. Chem. Soc. 1992, 114, 9266-9269.	
	9	Extended European Search Report and Written Opinion issued December 17, 2019 for EP 19180827.8	
	10	Supplementary European Search Report and Written Opinion issued March 18, 2020	
	11	CHEN et al., "Expansion microscopy," SCIENCE, Vol. 347, no. 6221, pages 543-548 (January 30, 2015)	
	12	CHOZINSKI et al., "Expansion microscopy with conventional antibodies and fluorescent proteins," NATURE METHODS, vol. 13, no. 6, pages 485-488 (June 1, 2016)	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	13	CHEN et al., "Nanoscale imaging of RNA with expansion microscopy," NATURE METHODS, vol. 133, no. 8, pages 679-684 (August 1, 2016)	
	14	Supplementary European Search Report issued April 9, 2020 for EP 17847555	
	15	Amasino, "Acceleration of nucleic acid hybridization rate by polyethylene glycol," ANALYTICAL BIOCHEMISTRY, Vol. 152, No. 2, pages 304-307 (February 1, 1986).	
	16	Bouché et al., "The effect of spermidine on endonuclease inhibition by agarose contaminants," ANALYTICAL BIOCHEMISTRY, Vol. 115, No. 1, pages 42-45 (July 15, 1981).	
	17	Kuznetsova et al., "What Macromolecular Crowding Can Do to a Protein," Int. J. Mol. Sci., Vol. 15, No. 12, pages 23090-23140 (December 1, 2014).	
	18	Oupicky et al., "Laterally stabilized complexes of DNA with linear reducible polycations: Strategy for triggered intracellular actication of DNA delivery vectors," Journal of the American Chemical Society, Vol. 124, No. 1, pages 8-9 (January 9, 2002).	
	19	Nguyen, Son C., "Strategies for Studying Chromatin Regulation and Organization," Doctoral Dissertation, Harvard University (May 1, 2018); retrieved from https://dash.harvard.edu/bitstream/handle/1/33493431/NGUYEN-DISSERTATION-2016.pdf?sequence=4&isAllowed=y on April 8, 2020.	
	20	ZHOU et al. "In Situ Detection of Messenger RNA Using Digoxigenin-Labeled Oligonucleotides and Rolling Circle Amplification" Experimental and Molecular Pathology 70, 281-288 (2001)	
	21	2020 May 29 - Examination Report issued for EP 18173059.9	
	22	2020 June 1 - Examination Report issued for GB 1809029.0	
	23	Wright et al., "Dynamic closed-loop system for focus tracking using a spatial light modulator and a deformable membrane mirror," Optics Express, Vol. 14, No. 1, pp. 222-228 (January 9, 2006).	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Attorney Docket Number	010498.01437\US

	24	Wang et al., "The method of axial drift compensation of laser differential confocal microscopy based on zero-tracking," Proc. of SPIE, Vol. 9618, 96180X (2015).	
	25	Ohata et al., "Confocal Imaging Analysis of Intracellular Ions in Mixed Cellular Systems or in Situ Using Two Types of Confocal Microscopic Systems," Methods in Enzymology, Vol. 307, pp. 425-441 (1999), particularly p. 437.	
	26	Supplemental Material for Schweitzer et al. (PNAS 2000; 97(18):10113-10119) (Year: 2000)	
	27	2020 Aug 03 - (US) Non-Final Office Action - US Appl. 16/157,243	
	28	2020 Aug 03 (US) Non-Final Office Action - Appl. No. 16/393,215	
	29	2020 Jul 02 - (US) Non-Final Office Action - US Appl. 16/255,920	
	30	2020 Aug 10 - (GB) Examination Report - GB App. No. 1809029.0	
	31	2020 Sept 24 - (US) Final Office Action - App. 15/772,652	
	32	Markaki et al. "Fluorescence In Situ Hybridization Applications for Super-Resolution 3D Structured Illumination Microscopy" Methods in Microbiology, January 2013	
	33	Achim et al. "High-throughput spatial mapping of single-cell RNA-seq data to tissue of origin" Nature Biotechnology, April 13, 2015	
	34	2020 Sept 25 - (US) Non-Final Office Action - Appl 16/386,337	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	35	2020 Aug 25 - (JP) Notice of Reasons for Rejection - App. No. 2018-522985	X
	36	PI: Piezo Nano Positioning, 2008 (online), retrieved on 2020.08.12, pp. 1-6 <https://www.pi-usa.us/fileadmin/user_upload/pi_us/files/product_datasheets/N725_Piezo_Focus_Positioner.pdf>	☐
	37	2020 Sept 14 - (CA) Examination Report - App. No. 2,850,509	☐
	38	MALI, P. et al. RNA-Guided Human Genome Engineering Via Cas9. Science. 03 January 2013,Vol. 339; pages 823-826; abstract; page 823, second column, second to third paragraph; page 823, third column, second paragraph to third paragraph; figure 1; Supplementary material, page 4, first paragraph; page 7, first paragraph; Supplementary figures S1, S3. DOI: 10.1126/science.1232033.	☐
	39	TILEY, LS et al. The VP16 Transcription Activation Domain Is Functional When Targeted To A Promoter-Proximal RNA Sequence. Genes and Development. 1992. Vol. 6; pages 2077-2087; abstract; page 2077, first column, first paragraph.	☐
	40	TRAFTON, A. Editing The Genome With High Precision [online]. MIT News office. 03 January 2013 [retrieved on 2014-12-04). Retrieved from the Internet: <URL:http:/lnewsoffice. Trafton .edut20 13/editing-the-genome-with-high-precision-01 03 >;pages 1-3; page 3, third paragraph.	☐
	41	LEMAN, AR et al. The Replication Forie Understanding The Eukaryotic Replication Machinery And The Challenges To Genome Duplication. Genes. 29 January 2013. Vol. 4; pages 1-32; figure 1; DOI: 10.3390/genes4010001.	☐
	42	QI, L et al. Repurposing CRISPR As An RNA-Guided Platform For Sequence-Specific Control Of Gene Expression. Cell. 28 February 2013. Vol. 152; pages 1173-1183; figures 2, 4. DOI: 10.1 016/j.cell.2013.02.022.	☐
	43	GASIUNAS, G et aL Cas9-crRNA Ribonucleoprotein Complex Mediates Specific DNA Cleavage For Adaptive Immunity In Bacteria. PNAS. 04 September 2012. VoL 109, No. 39; pages E2579-E2586; page E2583, first column, first paragraph. DOI: 1 0.1073/pnas.1208507109.	☐
	44	CONG, Let al. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science. 03 January 2013, Vol. 339; pages 819-823; abstract; page 821, third column; page 822, first column, first paragraph; figure 4. DOI: 10.1126/science.1231143.	☐
	45	JINEK, M et al. A Programmable Dual-RNA-Guided DNA Endonuclease In Adaptive Bacterial Immunity. Science. 28 June 2012. Vol. 337; pages 816-821; DOI: 10.1126/science.1225829.	☐

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	46	CRISPR In The Lab: A Practical Guide [online]. Addgene. 04 September 2014. Retrieved on 2014-12-04. Retrieved from the Internet: <URL: https://www.addgene.org/CRISPR/guide/>.	☐
	47	CHENG, AW et al. Multiplexed Activation Of Endogenous Genes By CRISP R-on, An RNA-Guided Transcriptional Activator System. Cell Research. 27 August 2013. Vol. 23; pages 1163-1171. DOI: 10.1038/cr.2013.122.	☐
	48	MALI, P. et al. CAS9 Transcriptional Activators For Target Specificity Screening And Paired Nickases For Cooperative Genome Engineering. Nature Biotechnology. 01 August 2013. Vol.31; pages 833-838; entire document. DOI: 10.1038/nbt.2675. ·	☐
	49	RAN, FA et al. Double Nicking By RNA-Guided CRISPR Cas9 For Enhanced Genome Editing Specificity. Cell. 12 September 2013. Vol. 154; pages 1380-1389. DOI: 10.1016/j.cell.2013.08.021.	☐
	50	International Search Report and Written Opinion issued in corresponding International Application No. PCT/US14/40868, mailed December 31, 2014.	☐

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	1	AL-ATTAR et al., Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs): The Hallmark of an Ingenious Antiviral Defense Mechanism in Prokaryotes, Bioi Chern. (20 11) vol. 392, Issue 4, pp. 277-289.	
	2	CARROLL, "A CRISPR Approach to Gene Targeting" 20(9) Molecular Therapy 1658-1660 (Sep. 2012).	
	3	JINEK , et al. 'RNA-programmed genome editing in human cells.' eLite 2013;2:e00471 . [retrieved 1-3, 6, 7, 10-12 on 06.03 .2014). Retrieved from the Internet. <URL: http://elife .elifesciences.org/content/2/e00471 >. entire document.	
	4	HALE et al., Essential Features and Rational Design of CRISPR RNAs That Function With the Cas RAMP Module Complex to Cleave RNAs, Molecular Cell, (20 12) vol. 45, Issue 3, 292-302.	
	5	HATOUM-ASLAN, et al. 'Mature clustered, regularly interspaced, short palindromic repeats RNA 5,9, 14 (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site.' Proceedings of the National Academy of Sciences. Vol. 108, No. 52. Pages 21218-21222. December 2011. entire document.	
	6	MAKAROVA et al., "Evolution and classification of the CRISPR-Cas systems" 9(6) Nature Reviews Microbiology 467-477 (1-23) (Jun. 2011).	
	7	RHO, Mina et al. 'Diverse CRISPRs Evolving in Human Microbiomes.' PLoS Genetics. Vol. 8, No. 6. 1-14 Pages 1-12. June 2012. entire document.	
	8	SONTHEIMER Erik, Project 7: Establishing RNA-Directed DNA Targeting in Eukaryotic Cells; Project dates: Nov. 16, 2011 to Dec. 31, 2012 (Feb. 4, 2012).	
	9	WIEDENHEFT eta!., "RNA -guided genetic silencing systems in bacteria and archaea" 482 Nature 331-338 (Feb. 16, 2012).	
	10	Liu et al, Cell-Penetrating Peptide-Mediated Delivery of TALEN Proteins via Bioconjugation for Genome Engineering, PLOS ONE, 2014, Vol9(1), pages 1-7.	
	11	Ramakrishna et al, Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA, Genome Res. published online April 2, 2014, pages 1-20 plus figures.	

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	12	The Delivery Problem, Nature Biotechnology, 2006, Vol 24(3), pages 305-306	
	13	Ansari et al, Riboactivators: Transcription activation by non-coding RNA, Grit Rev Biochem Mol Bioi. 2009 ; 44(1): 50-61.	
	14	Sapranauskas et al (Nucleic Acids Research, 2011, 39:9275-9282)	
	15	US Application No. 61/794,422, filed March 15, 2013, DAVIS, G. et al.	
	16	US Application No. 61/781,598, filed March 14, 2013, HAURWITZ, R.	
	17	GILBERT, Luke A., et al.,"CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes," CELL, vol. 154, no. 2, 1 July 2013 (2013-07-01), pages 442-451	
	18	MALI, P. et al., "Supplementary Materials for RNA-Guided Human Genome Engineering via Cas9," SCIENCE, vol. 339, no. 6121, 3 January 2013 (2013-01-03), pages 1-36	
	19	MAEDER, Morgan L., et al.," Robust, synergistic regulation of human gene expression using TALE activators," HHS PUBLIC ACCESS AUTHOR MANUSCRIPT, vol. 10, no. 3, 10 February 2013 (2013-02-10), pages 243-245	
	20	PEREZ-PINERA, Pablo, et al., "Synergistic and tunable human gene activation by combinations of synthetic transcription factors," NATURE METHODS, vol . 10. no. 3, 3 February 2013 (2013-02-03), pages 239-242	
	21	Preliminary Office Action issued by Brazilian Patent Office on April 7, 2020	☒
	22	Official Notification issued May 24, 2020 for IL 242959	☒

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	23	2020 June 2 - (JP) Notice of Reasons for Rejection for App. No. 2019-039027	☒
	24	2020 July 3 - (AU) Examination Report for App. No. 20202039777	☐
	25	2020 Aug 19 - (MX) Office Action - App. No. MX/a/2015/016798	☒
	26	2020 Sept 10 - (CA) Office Action - App. No. 2,914,638	☐
	27	2020 Sept 21 - (NZ) First Examination Report - App. No. 715280	☐
	28	2020 Sept 21 - (NZ) First Examination Report - App. No. 753950	☐
	29	Fu et al., "Improving CRISPR-Cas nuclease specificity using truncated guide RNAs," Nature Biotechnology, Vol. 32, pp. 249-284 (January 26, 2014).	☐
	30	Lin et al., "CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences," Nucleic Acids Research, Vol. 42, No. 11, pp. 7473-7485 (May 16, 2014).	☐
	31	2020 Sept 21 - (NZ) First Examination Report - App. No. 753951	☐
	32	2020 Sept 25 - (RU) Office Action - App. No. 2019114706	☒
	33	DiCarlo et al., "Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems," Nucleic Acids Research, Vol. 41, No. 7, pp. 4336-4343 (2013).	☐

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	34	Gusev et al. "Rolling Circle Amplification: A New Approach to Increase Sensitivity for Immunohistochemistry and Flow Cyometry" American Journal of Pathology, Vol. 159, No. 1, July 2001, pp. 63-69	☐
	35	2020 Nov 10 - (US) Non-Final Office Action - Appl. No. 16/285,292	☐
	36	Pihlak et al. "Rapid genome sequencing with short universal tiling probes" Nature Biotechnology, Vol. 26, No. 6, June 2008, pp. 676-684	☐
	37	Lizardi "Next-generation sequencing-by-hybridization" Nature Biotechnology, Vol. 26, No. 6, June 2008, pp. 649-650	☐
	38	Mignardi et al. "Fourth-generation sequencing in the cell and the clinic" Genome Medicine, 2014, 6:31	☐
	39	2020 Dec 24 (US) - Notice of Allowance - App 16/393,215	☐
	40	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155459/http://www.polonator.org/index.htm; Wayback Machine (August 7, 2008) "Open, Affordable, Sequencing..."	☐
	41	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155600/http://www.polonator.org;/vision.aspx; Wayback Machine (August 7, 2008) "The Vision"	☐
	42	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155250/http://www.polonator.org/ecosystem; Wayback Machine (August 7, 2008) "The Polonator Ecosystem"	☐
	43	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155759/http://www.polonator.org/instrument; Wayback Machine (August 7, 2008) "Instrument Overview"	☐
	44	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155857/http://www.polonator.org; Wayback Machine (August 7, 2008) "Protocols"	☐

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	45	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133362/http://www.polonator.org/protocols/pet.aspx; Wayback Machine (September 5, 2008) "PET (Paired End-Tag) Genomic Shotgun Library Construction Protocol"	☐
	46	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133855/http://www.polonator.org/protocols/pcr.aspx; Wayback Machine (September 5, 2008) "Emulsion PCR Protocol"	☐
	47	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133913/http://www.polonator.org; Wayback Machine (September 5, 2008) "Emulsion Breaking Protocol"	☐
	48	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133837/http://www.polonator.org/protocols/beadenrichment.aspx; Wayback Machine (September 5, 2008) "Bead Enrichment Protocol"	☐
	49	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133372/http://www.polonator.org/protocols.beadcapping.aspx; Wayback Machine (September 5, 2008) "Bead Capping Protocol"	☐
	50	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133372/http://www.polonator.org/protocols/coverslip.aspx; Wayback Machine (September 5, 2008) "Coverslip Aminosilanation and Arraying Protocol"	☐

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

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Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-08-05
Name/Print	John P. Iwanicki	Registration Number	34,628

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First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
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	1	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133800/http://www.polonator.org; Wayback Machine (September 5, 2008) "Polony Sequence by Ligation Protocol"	
	2	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080705172604/http://www.polonator.org; Wayback Machine (July 5, 2008) "Polony Sequence Protocols"	
	3	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133818/http://www.polonator.org; Wayback Machine (September 5, 2008) "Help Wanted"	
	4	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155529/http://www.polonator.org/software.aspx; Wayback Machine (August 7, 2008) "Software"	
	5	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155404/http://www.polonator.org; Wayback Machine (August 7, 2008) "Reagent Kits"	
	6	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133541/http://www.polonator.org/reagentkits/run.aspx; Wayback Machine (September 5, 2008) "Run Kits"	
	7	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133601/http://www.polonator.org/reagentkits.pairedtag.aspx; Wayback Machine (September 5, 2008) 'Paired-Leg Library Kits"	
	8	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133741/http://www.polonator.org/reagentkits.emulsion.aspx; Wayback Machine (September 5, 2008) 'Emulsion PCR/Bead Capping Kits"	
	9	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133702/http://www.polonator.org/reagentkits/enrichment.aspx; Wayback Machine (September 5, 2008) 'Enrichment Kits"	
	10	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155316/http://www.polonator.org/flowcells.aspx; Wayback Machine (August 7, 2008) "Flow Cells"	
	11	Church GM. 2006. "Genomes for all" Sci Am 294: 46-54	

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	12	de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. 2005. "Efficiency and power in genetic association studies" Nat Genet 37: 1217-23.	
	13	Dixon AL, Liang L, Moffatt MF, Chen W, Heath S, Wong KC, Taylor J, Burnett E, Gut I, Farrall M, Lathrop GM, Abecasis GR, Cookson WO. 2007. "A genome-wide association study of global gene expression" Nat Genet 39: 1202-7	
	14	Emilsson V, Thorleifsson G, Zhang B, Leonardson AS, Zink F, Zhu J, Carlson S, Helgason A, Walters GB, Gunnarsdottir S, Mouy M, Steinthorsdottir V, Eiriksdottir GH, Bjornsdottir G, Reynisdottir I, Gudbjartsson D, Helgadottir A, Jonasdottir A, Jonasdottir A, Styrkarsdottir U, Gretarsdottir S, Magnusson KP, Stefansson H, Fossdal R, Kristjansson K, Gislason HG, Stefansson T, Leifsson BG, Thorsteinsdottir U, Lamb JR, Gulcher JR, Reitman ML, Kong A, Schadt EE, Stefansson K. 2008; "Genetics of gene expression and its effect on disease" Nature 452: 423-8.	
	15	Risch N, Merikangas K. 1996. "The future of genetic studies of complex human diseases" Science 273: 1516-7	
	16	Schadt EE, Monks SA, Drake TA, Lusis AJ, Che N, Colinayo V, Ruff TG, Milligan SB, Lamb JR, Cavet G, Linsley PS, Mao M, Stoughton RB, Friend SH. 2003. "Genetics of gene expression surveyed in maize, mouse and man" Nature 422: 297-302	
	17	Altshuler D, Daly MJ, Lander ES. 2008. "Genetic mapping in human disease" Science 322: 881-8	
	18	Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M. 2009. "Mapping complex disease traits with global gene expression" Nat Rev Genet 10: 184-94	
	19	International HapMap C. 2005. "A haplotype map of the human genome" Nature 437: 1299-320. PMC ID: PMC1880871	
	20	Klein RJ. 2007. "Power analysis for genome-wide association studies" BMC Genet 8: 58. PMC ID: PMC2042984	
	21	Kwan T, Benovoy D, Dias C, Gurd S, Provencher C, Beaulieu P, Hudson TJ, Sladek R, Majewski J. 2008. "Genome-wide analysis of transcript isoform variation in humans" Nat Genet 40: 225-31	
	22	McCarroll SA. 2008. "Extending genome-wide association studies to copy-number variation" Hum Mol Genet 17: R135-42	

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	23	Morley M, Molony CM, Weber TM, Devlin JL, Ewens KG, Spielman RS, Cheung VG. 2004. "Genetic analysis of genome-wide variation in human gene expression" Nature 430: 743-7	
	24	Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D, International SNPMWG. 2001. "A map of human genome sequence variation containing 1.42 million	
	25	Schadt EE, Molony C, Chudin E, Hao K, Yang X, Lum PY, Kasarskis A, Zhang B, Wang S, Suver C, Zhu J, Millstein J, Sieberts S, Lamb J, GuhaThakurta D, Derry J, Storey JD, Avila-Campillo I, Kruger MJ, Johnson JM, Rohl CA, van Nas A, Mehrabian M, Drake TA, Lusis AJ, Smith RC, Guengerich FP, Strom SC, Schuetz E, Rushmore TH, Ulrich R. 2008. "Mapping the genetic architecture of gene expression in human liver" PLoS Biol 6: e107. PMC ID: PMC2365981	
	26	Serre D, Gurd S, Ge B, Sladek R, Sinnett D, Harmsen E, Bibikova M, Chudin E, Barker DL, Dickinson T, Fan JB, Hudson TJ. 2008. "Differential allelic expression in the human genome: a robust approach to identify genetic and epigenetic cis-acting mechanisms regulating gene expression" PLoS Genet 4: e1000006. PMC ID: PMC2265535	
	27	Ball MP, Li JB, Gao Y, Lee J, LeProust E, Park I-H, Xie B, Daley GQ, Church GM. 2009. "Targeted and whole-genome methylomics reveals gene-body signatures in human cell lines" Nat Biotechnol 27: 361-8	
	28	Brenner S, Williams SR, Vermaas EH, Storck T, Moon K, McCollum C, Mao JI, Luo S, Kirchner JJ, Eletr S, DuBridge RB, Burcham T, Albrecht G. 2000. "In vitro cloning of complex mixtures of DNA on microbeads: physical separation of differentially expressed cDNAs" Proc Natl Acad Sci U S A 97: 1665-70. PMC ID: PMC26493	
	29	Chiang DY, Getz G, Jaffe DB, O'Kelly MJ, Zhao X, Carter SL, Russ C, Nusbaum C, Meyerson M, Lander ES. 2009. "High-resolution mapping of copy-number alterations with massively parallel sequencing" Nat Methods 6: 99-103. PMC ID: PMC2630795	
	30	Choy E, Yelensky R, Bonakdar S, Plenge RM, Saxena R, De Jager PL, Shaw SY, Wolfish CS, Slavik JM, Cotsapas C, Rivas M, Dermitzakis ET, Cahir-McFarland E, Kieff E, Hafler D, Daly MJ, Altshuler D. 2008. "Genetic analysis of human traits in vitro: drug response and gene expression in lymphoblastoid cell lines" PLoS Genet 4: e1000287. PMC ID: PMC2583954	
	31	Christian AT, Pattee MS, Attix CM, Reed BE, Sorensen KJ, Tucker JD. 2001. "Detection of DNA point mutations and mRNA expression levels by rolling circle amplification in individual cells" Proc Natl Acad Sci U S A 98: 14238-43. PMC ID: PMC64666	
	32	Church GM, Porreca GJ, Terry RC, Lares M. 2008. "High-Speed Imaging for DNA Sequencing" Biophotonics (<http://www.photonics.com/Content/ReadArticle.aspx?ArticleID=33989>)	
	33	Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K. 2009. "Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming" Nat Biotechnol 27: 353-60	

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	34	Eberwine J, Kacharmina JE, Andrews C, Miyashiro K, McIntosh T, Becker K, Barrett T, Hinkle D, Dent G, Marciano P. 2001. "mRna expression analysis of tissue sections and single cells" J Neurosci 21: 8310-4	
	35	Kolb HC, Finn MG, B. SK. 2001. "Click Chemistry: Diverse Chemical Function from a Few Good Reactions" Angew. Chem. Int. 40: 2004-21	
	36	Kwiatkowski M, Fredriksson S, Isaksson A, Nilsson M, Landegren U. 1999. "Inversion of in situ synthesized oligonucleotides: improved reagents for hybridization and primer extension in DNA microarrays" Nucleic Acids Res 27: 4710-4. PMC ID: PMC148770	
	37	Li JB, Gao Y, Aach J, Zhang K, Kryukov GV, Xie B, Ahlford A, Yoon J-K, Rosenbaum AM, Wait-Zaranek A, LeProust E, Sunyaev S, Church GM. 2009. "Multiplex padlock capture and sequencing reveal human hypermutable CpG variations" Genome Res in press	
	38	Mitra RD, Butty VL, Shendure J, Williams BR, Housman DE, Church GM. 2003. "Digital genotyping and haplotyping with polymerase colonies" Proc Natl Acad Sci U S A 100: 5926-31. PMC ID: PMC156303	
	39	Pan X, Urban AE, Palejev D, Schulz V, Grubert F, Hu Y, Snyder M, Weissman SM. 2008. "A procedure for highly specific, sensitive, and unbiased whole-genome amplification" Proc Natl Acad Sci U S A 105: 15499-504. PMC ID: PMC2563063	
	40	Stougaard M, Lohmann JS, Zajac M, Hamilton-Dutoit S, Koch J. 2007. "In situ detection of non-polyadenylated RNA molecules using Turtle Probes and target primed rolling circle PRINS" BMC Biotechnol 7: 69. PMC ID: PMC2203993	
	41	Wang Z, Gerstein M, Snyder M. 2009. "RNA-Seq: a revolutionary tool for transcriptomics" Nat Rev Genet 10: 57-63	
	42	Wu J, Zhang S, Meng Q, Cao H, Li Z, Li X, Shi S, Kim DH, Bi L, Turro NJ, Ju J. 2007. "3'-O-modified nucleotides as reversible terminators for pyrosequencing" Proc Natl Acad Sci U S A 104: 16462-7. PMC ID: PMC2034218	
	43	Zhang K, Li JB, Gao Y, Egli D, Xie B, Deng J, Li Z, Lee J, Aach J, Leproust E, Eggan K, Church GM. 2009. "Digital RNA Allelotyping Reveals Tissue-specific and Allele-specific Gene Expression in Human" (submitted to Nature Methods)	
	44	Bakal C, Aach J, Church G, Perrimon N. 2007. "Quantitative morphological signatures define local signaling networks regulating cell morphology" Science 316: 1753-6	

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	45	Bang D, Church GM. 2008. "Gene synthesis by circular assembly amplification" Nat Methods 5: 37-9
	46	Bell J. 2004. "Predicting disease using genomics" Nature 429: 453-6
	47	Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, Peluso P, Rank D, Baybayan P, Bettman B, Bibillo A, Bjornson K, Chaudhuri B, Christians F, Cicero R, Clark S, Dalal R, Dewinter A, Dixon J, Foquet M, Gaertner A, Hardenbol P, Heiner C, Hester K, Holden D, Kearns G, Kong X, Kuse R, Lacroix Y, Lin S, Lundquist P, Ma C, Marks P, Maxham M, Murphy D, Park I, Pham T, Phillips M, Roy J, Sebra R, Shen G, Sorenson J, Tomaney A, Travers K, Trulson M, Vieceli J, Wegener J, Wu D, Yang A, Zaccarin D, Zhao P, Zhong F, Korlach J, Turner S. 2009. "Real-time DNA sequencing +
	48	Harris TD, Buzby PR, Babcock H, Beer E, Bowers J, Braslavsky I, Causey M, Colonell J, Dimeo J, Efcavitch JW, Giladi E, Gill J, Healy J, Jarosz M, Lapen D, Moulton K, Quake SR, Steinmann K, Thayer E, Tyurina A, Ward R, Weiss H, Xie Z. 2008. "Single-molecule DNA sequencing of a viral genome" Science 320: 106-9
	49	Kim JB, Porreca GJ, Song L, Greenway SC, Gorham JM, Church GM, Seidman CE, Seidman JG. 2007. "Polony multiplex analysis of gene expression (PMAGE) in mouse hypertrophic cardiomyopathy" Science 316: 1481-4
	50	Kurimoto K, Yabuta Y, Ohinata Y, Saitou M. 2007. "Global single-cell cDNA amplification to provide a template for representative high-density oligonucleotide microarray analysis" Nat Protoc 2: 739-52

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CERTIFICATION STATEMENT

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Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

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	1	Li JB, Levanon EY, Yoon J-K, Aach J, Xie B, LeProust E, Zhang K, Gao Y, G.M. C. 2009. "Genome-wide Identification of Human RNA Editing Sites by Parallel DNA Capturing and Sequencing" Science in press	
	2	Meng Q, Kim DH, Bai X, Bi L, Turro NJ, Ju J. 2006. "Design and synthesis of a photocleavable fluorescent nucleotide 3'-O-allyl-dGTP-PC-Bodipy-FL-510 as a reversible terminator for DNA sequencing by synthesis" J Org Chem 71: 3248-52	
	3	Mitra RD, Shendure J, Olejnik J, Edyta Krzymanska O, Church GM. 2003. "Fluorescent in situ sequencing on polymerase colonies" Anal Biochem 320: 55-65	
	4	Porreca GJ, Shendure J, Church GM. 2006. "Polony DNA sequencing" Curr Protoc Mol Biol Chapter 7: Unit 7 8	
	5	Porreca GJ, Zhang K, Li JB, Xie B, Austin D, Vassallo SL, LeProust EM, Peck BJ, Emig CJ, Dahl F, Gao Y, Church GM, Shendure J. 2007. "Multiplex amplification of large sets of human exons" Nat Methods 4: 931-6	
	6	Shendure J, Mitra RD, Varma C, Church GM. 2004. "Advanced sequencing technologies: methods and goals" Nat Rev Genet 5: 335-44	
	7	Shendure JA, Porreca GJ, Church GM. 2008. "Overview of DNA sequencing strategies" Curr Protoc Mol Biol Chapter 7: Unit 7 1	
	8	Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, Wang X, Bodeau J, Tuch BB, Siddiqui A, Lao K, Surani MA. 2009. "mRNA-Seq whole-transcriptome analysis of a single cell" Nat Methods 6: 377-82	
	9	Vigneault F, Sismour AM, Church GM. 2008." Efficient microRNA capture and bar-coding via enzymatic oligonucleotide adenylation" Nat Methods 5: 777-9	
	10	Zhang K, Martiny AC, Reppas NB, Barry KW, Malek J, Chisholm SW, Church GM. 2006. "Sequencing genomes from single cells by polymerase cloning" Nat Biotechnol 24: 680-6	
	11	Zhang K, Zhu J, Shendure J, Porreca GJ, Aach JD, Mitra RD, Church GM. 2006. "Long-range polony haplotyping of individual human chromosome molecules" Nat Genet 38: 382-7	

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	12	Church et al.; Center for Casual Consequences of Variation (CCV) "An NHGRI Center for Excellence in Genomic Science" https://web.archive.org/web/20110703211120/http://ccv.med.harvard.edu/; Wayback Machine (July 3, 2011)	
	13	Church et al.; Center for Casual Consequences of Variation (CCV) "Our four Specific Aims" https://web.archive.org/web/20110813071548//http://ccv.med.harvard.edu/specific_aims.htm; Wayback Machine (August 13, 2011)	
	14	Church; "Proposal for a Center for the determination of the Casual Transcriptional Consequences of Human Genetic Variation (CTCHGV)"" http://ccv.med.harvard.edu/CEGS09_Complete_Proposal_minus_Admin_Sections.09May21.final.pdf; Wayback Machine (August 13, 2011)	
	15	J. H. Lee, M.D. Ph.D. presentation entitled "Population-wide Tissue-specific Functional Analysis of Human iPS Cells Using Single-Cell In Situ Sequencing" George Church Laboratory, Wyss Institute for Biology Inspired Engineering, Harvard Medical School, Boston, January 10, 2010	
	16	2021 May 17 (US) Notice of Allowance - Appln No 17/122,168	
	17	2021 June 18 (US) Non-Final Office Action - Appln No 15/772,652	
	18	2021 May 8 - (CN) Office Action - App. No. 201680077501.7	X
	19	2021 July 2 - (US) Non-Final Office Action - Appln No 17/238,642	☐
	20	2021 July 21 (US) Non-Final Office Action - Appl No. 16/693,611	☐
	21	HO et al. "Sequencing by ligation variation with endonuclease V digestion and deoxyinosine-containing query oligonucleotides" BMC Genomics, 2011, 12:598	☐
	22	JIANG et al. "Solar thermal polymerase chain reaction for smartphone-assisted molecular diagnostics" Scientific Reports, 4:4137, 2014	☐

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	23	JU et al. "Four-color DNA sequencing by synthesis using cleavable fluorescent nucleotide reversible terminators" PNAS, December 26, 2006, vol. 103, no. 52, pp. 19635-19640	☐
	24	LUBECK et al. "Single cell in situ RNA profiling by sequential hybridization" Nature Methods, April 2014, 11(4), pp. 360-361	☐
	25	PARINOV et al. "DNA sequencing by hybridization to microchip octa- and decanucleotides extended by stacked pentanucleotides" Nucleic Acids Research, 1996, vol. 24, no. 15, pp. 2998-3004	☐
	26	SCHOUTEN et al. "Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification" Nucleic Acids Research, 2002, vol. 30, no. 12, e57	☐
	27	2021 July 23 (US) Non-Final Office Action - Appl No 16/170,751	☐

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-08-05
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 22910

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

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[ ] The address associated with Customer Number:

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City		State		Zip	
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I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

President and Fellows of Harvard College

[ ] Inventor or Joint Inventor (title not required below)

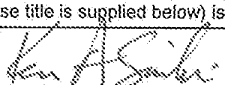
[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	
Name	Karen A Sinclair		
Title	Director of Intellectual Property, Harvard Office of Technology Development		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	Method and Apparatus for Volumetric Imaging of a Three-Dimensional Nucleic Acid Containing Matrix

As the below named inventor, I hereby declare that:

This declaration is directed to:

- [ ] The attached application, or
- [x] United States application or PCT international application number 15/969,118 filed on 05/02/2018 .

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

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LEGAL NAME OF INVENTOR

Inventor: Evan R. Daugharthy Date (Optional): 5.22.18

Signature:

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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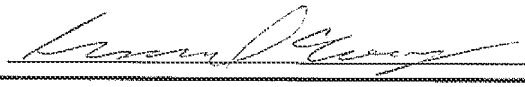
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LEGAL NAME OF INVENTOR

Inventor: Richard C. Terry Date (Optional) : _____

Signature: 

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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LEGAL NAME OF INVENTOR

Inventor: Je-Hyuk Lee Date (Optional): 5/21/2018

Signature: Je-Hyuk Lee

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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LEGAL NAME OF INVENTOR

Inventor: George M. Church Date (Optional): May 17th 2018

Signature: George Church

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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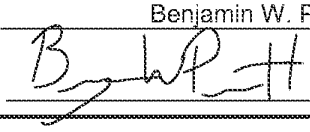
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LEGAL NAME OF INVENTOR

Inventor: Benjamin W. Pruitt Date (Optional): 5/17/2018

Signature: 

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

FIG. 1

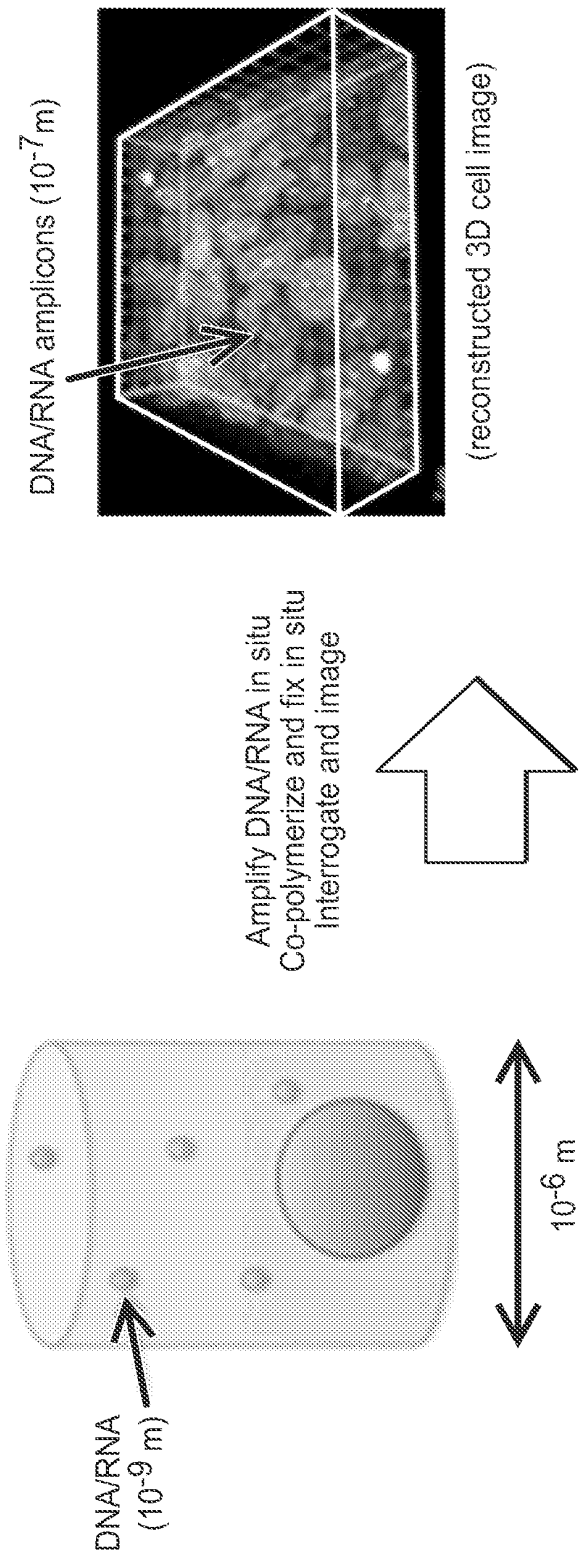

DNA/RNA amplicons (10^{-7} m)
(reconstructed 3D cell image)
Amplify DNA/RNA in situ
Co-polymerize and fix in situ
Interrogate and image
DNA/RNA (10^{-9} m)
10^{-6} m

FIG. 2

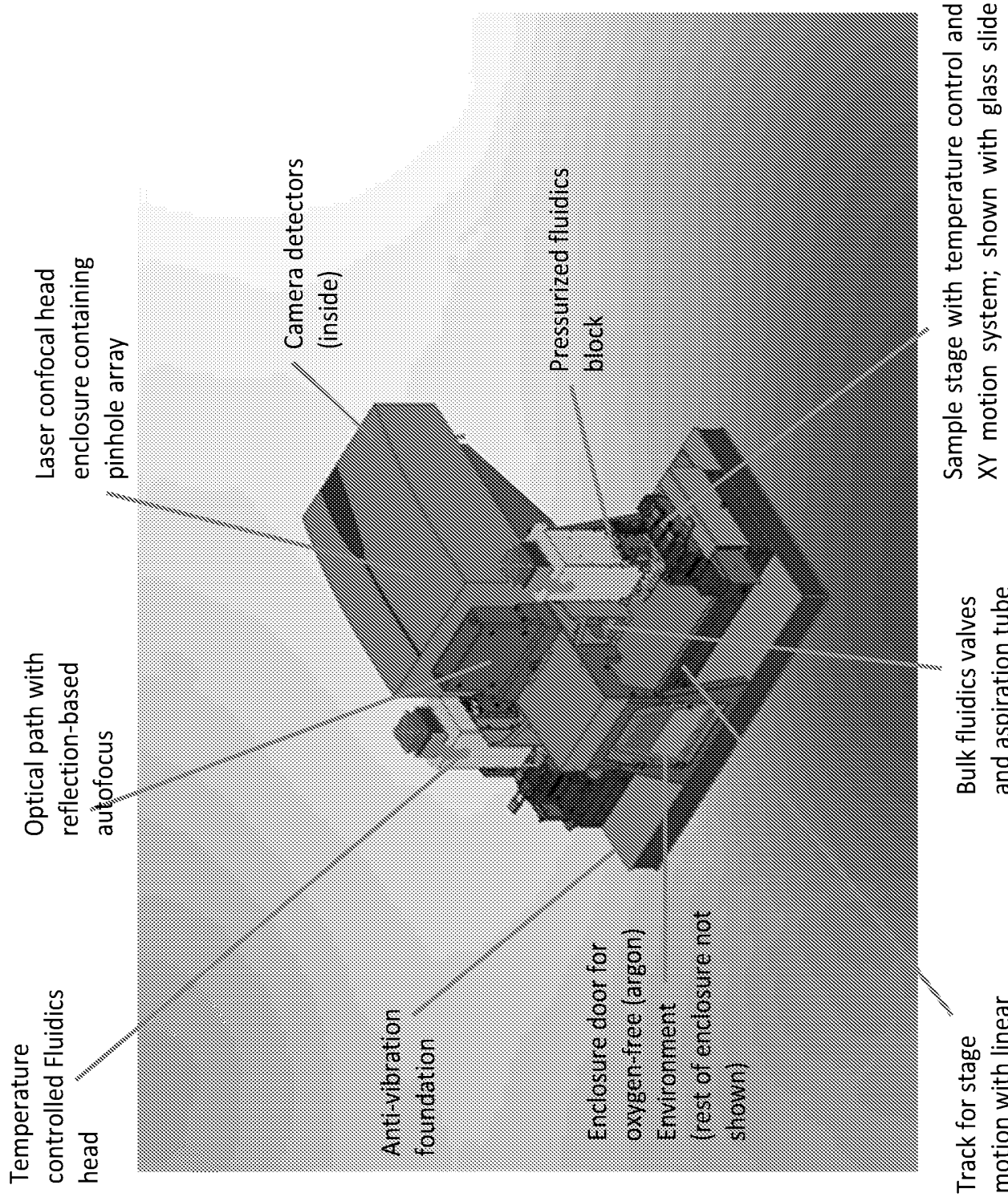

Temperature controlled Fluidics head
Optical path with reflection-based autofocus
Laser confocal head enclosure containing pinhole array
Camera detectors (inside)
Pressurized fluidics block
Sample stage with temperature control and XY motion system; shown with glass slide sample holder
Anti-vibration foundation
Enclosure door for oxygen-free (argon) Environment (rest of enclosure not shown)
Bulk fluidics valves and aspiration tube
Track for stage motion with linear encoders

FIG. 3

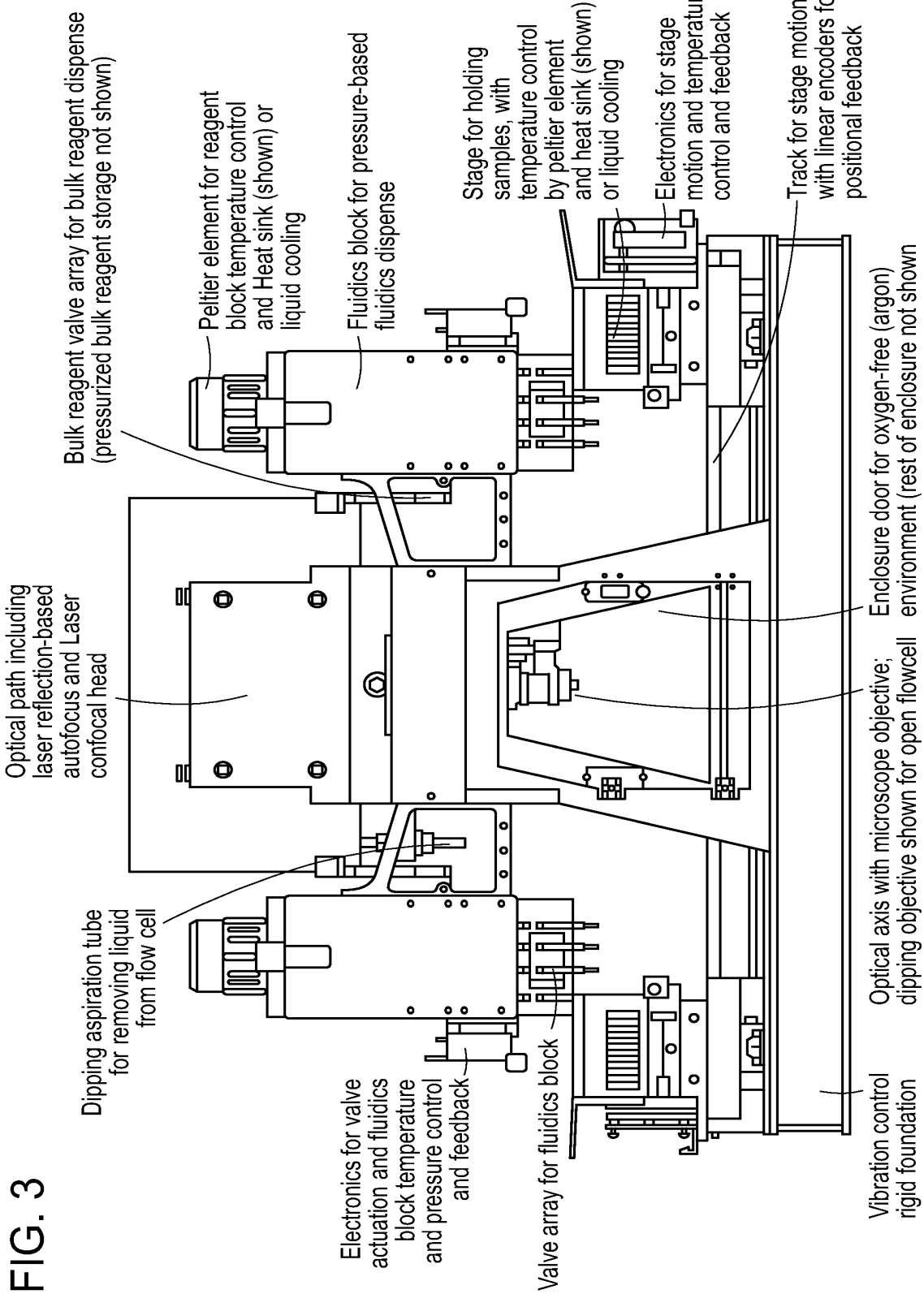

Bulk reagent valve array for bulk reagent dispense (pressurized bulk reagent storage not shown)
Peltier element for reagent block temperature control and Heat sink (shown) or liquid cooling
Fluidics block for pressure-based fluidics dispense
Stage for holding samples, with temperature control by peltier element and heat sink (shown) or liquid cooling
Electronics for stage motion and temperature control and feedback
Track for stage motion with linear encoders for positional feedback
Optical path including laser reflection-based autofocus and Laser confocal head
Enclosure door for oxygen-free (argon) environment (rest of enclosure not shown
Dipping aspiration tube for removing liquid from flow cell
Optical axis with microscope objective; dipping objective shown for open flowcell
Electronics for valve actuation and fluidics block temperature and pressure control and feedback
Valve array for fluidics block
Vibration control rigid foundation

FIG. 4

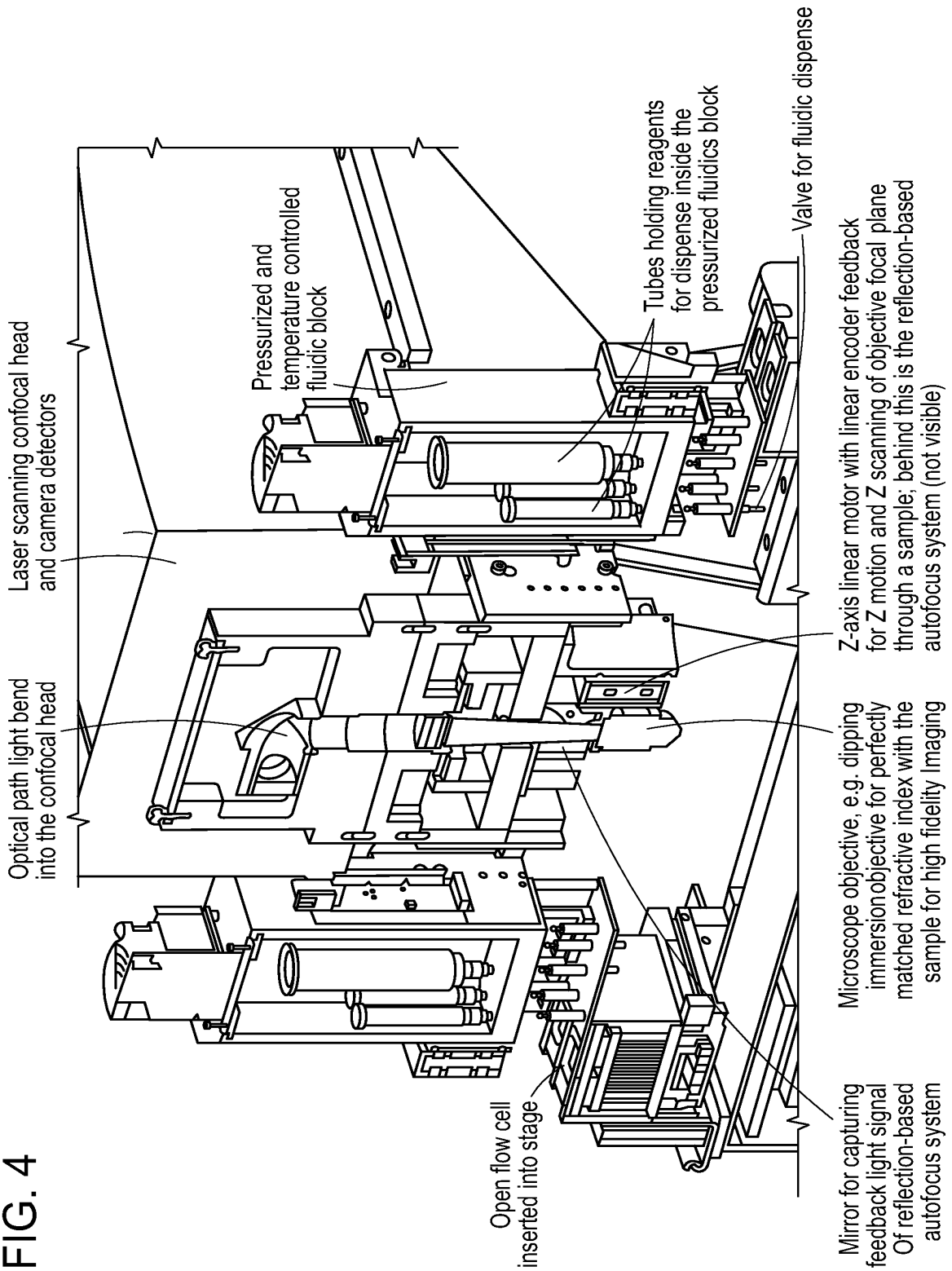

Optical path light bend into the confocal head
Laser scanning confocal head and camera detectors
Pressurized and temperature controlled fluidic block
Tubes holding reagents for dispense inside the pressurized fluidics block
Valve for fluidic dispense
Z-axis linear motor with linear encoder feedback for Z motion and Z scanning of objective focal plane through a sample; behind this is the reflection-based autofocus system (not visible)
Microscope objective, e.g. dipping immersion objective for perfectly matched refractive index with the sample for high fidelity Imaging
Open flow cell inserted into stage
Mirror for capturing feedback light signal Of reflection-based autofocus system

FIG. 5

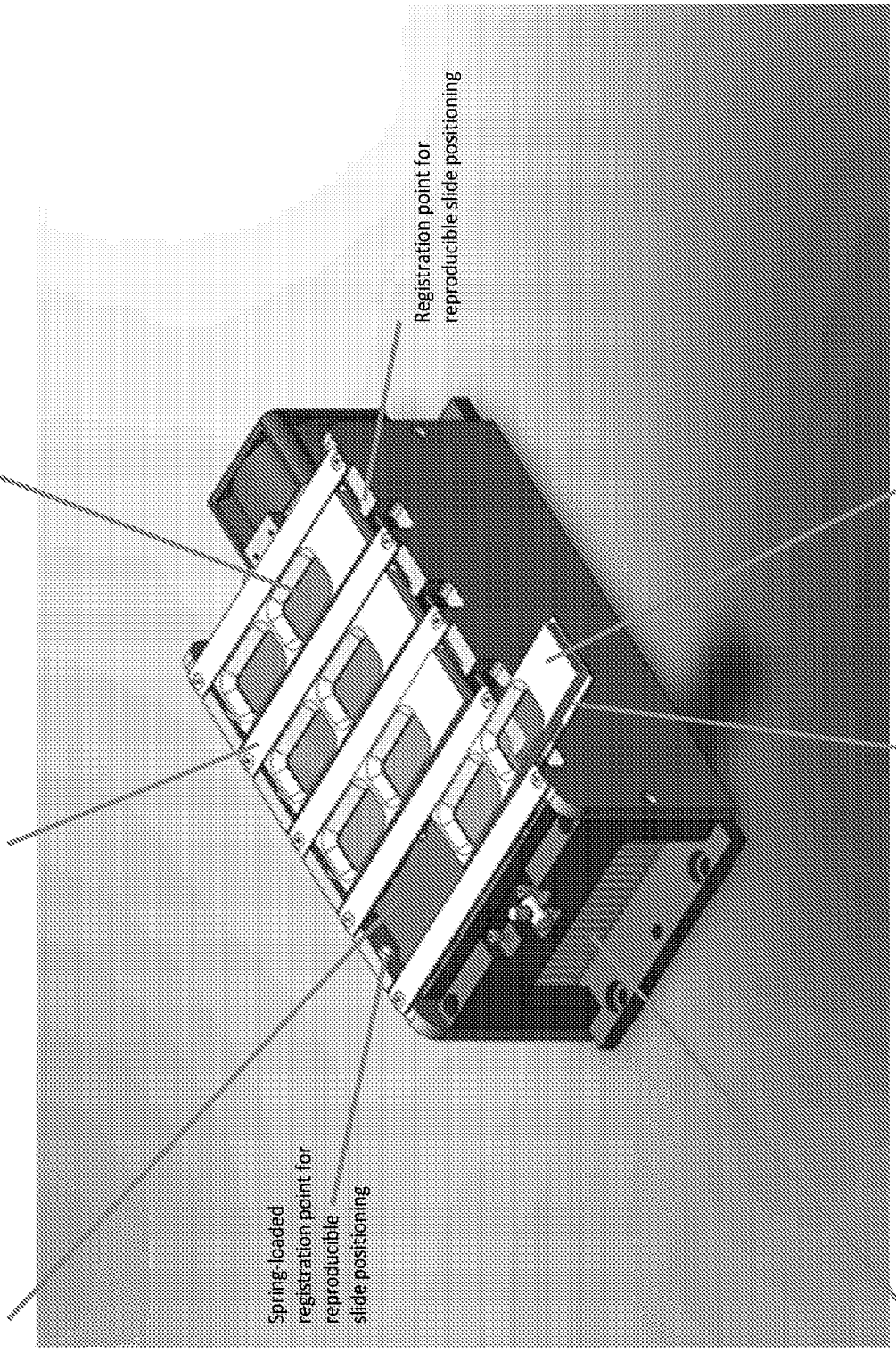

3D sequencing library matrix attached inside the well
Rail system for reproducible slide positioning provides 2D directional force onto the slide (downward and along one of the long edges of the slide) and secures the sample
Thermo-cycling stage top
Spring-loaded registration point for reproducible slide positioning
Registration point for reproducible slide positioning
Fluidic well component of the open-format flowcell; 2-well format shown. Open-format flowcell avoids problems with bubbles and laminar flow over irregular 3D matrix that would cause uneven diffusion of reagents into and out of the 3D sequencing library matrix
Flow cell foundation, shown is a standard 1x3 inch 1mm thick glass microscopy slide onto which the 3D sequencing library matrix is attached
Heat sink for peltier for stage temperature control

FIG. 6

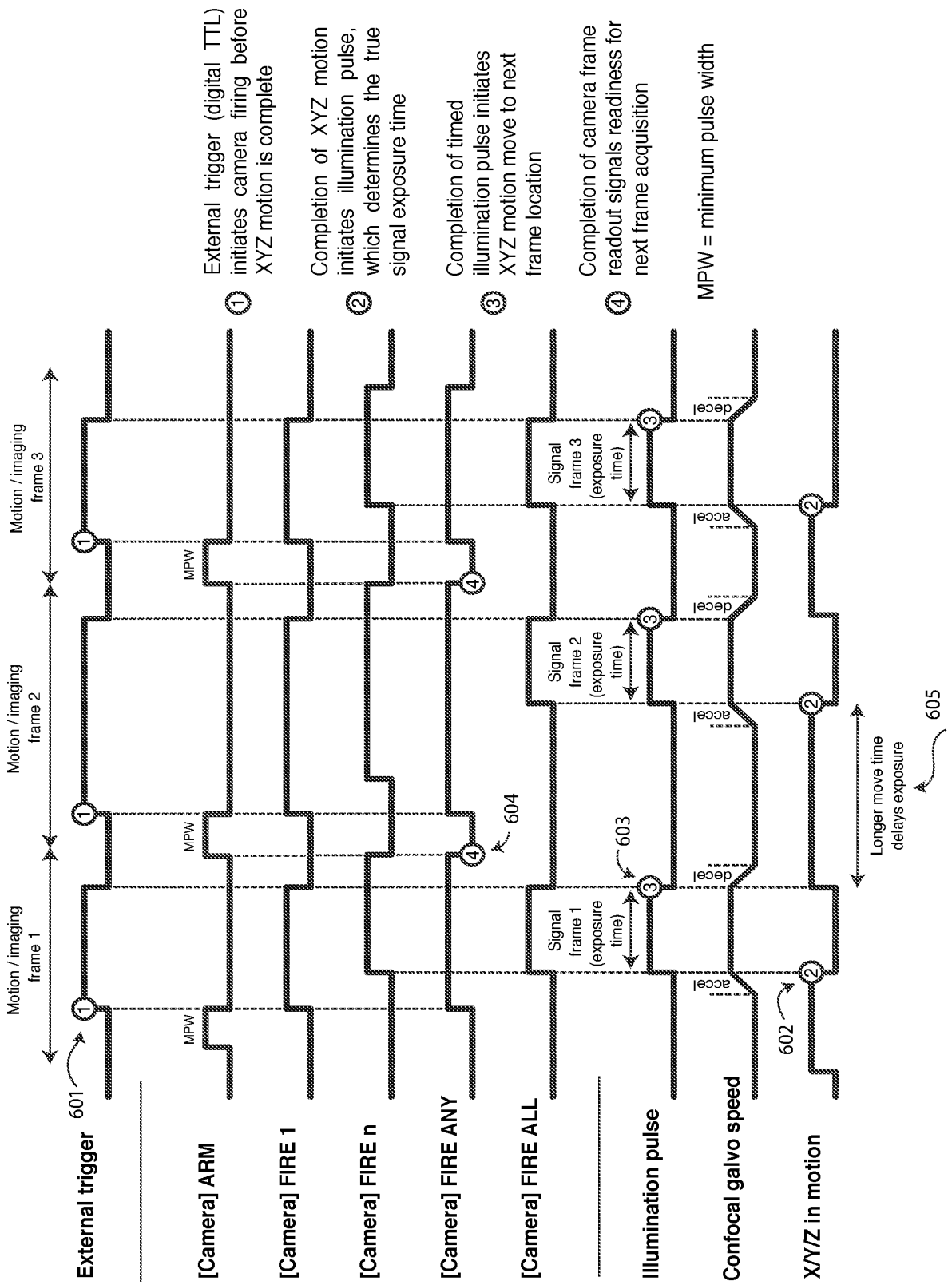

External trigger
[Camera] ARM
[Camera] FIRE 1
[Camera] FIRE n
[Camera] FIRE ANY
[Camera] FIRE ALL
Illumination pulse
Confocal galvo speed
X/Y/Z in motion
Motion / imaging frame 1
Motion / imaging frame 2
Motion / imaging frame 3
MPW
Signal frame 1 (exposure time)
Signal frame 2 (exposure time)
Signal frame 3 (exposure time)
accel
decel
601
602
603
604
605
Longer move time delays exposure
1 External trigger (digital TTL) initiates camera firing before XYZ motion is complete
2 Completion of XYZ motion initiates illumination pulse, which determines the true signal exposure time
3 Completion of timed illumination pulse initiates XYZ motion move to next frame location
4 Completion of camera frame readout signals readiness for next frame acquisition
MPW = minimum pulse width

FIG. 7

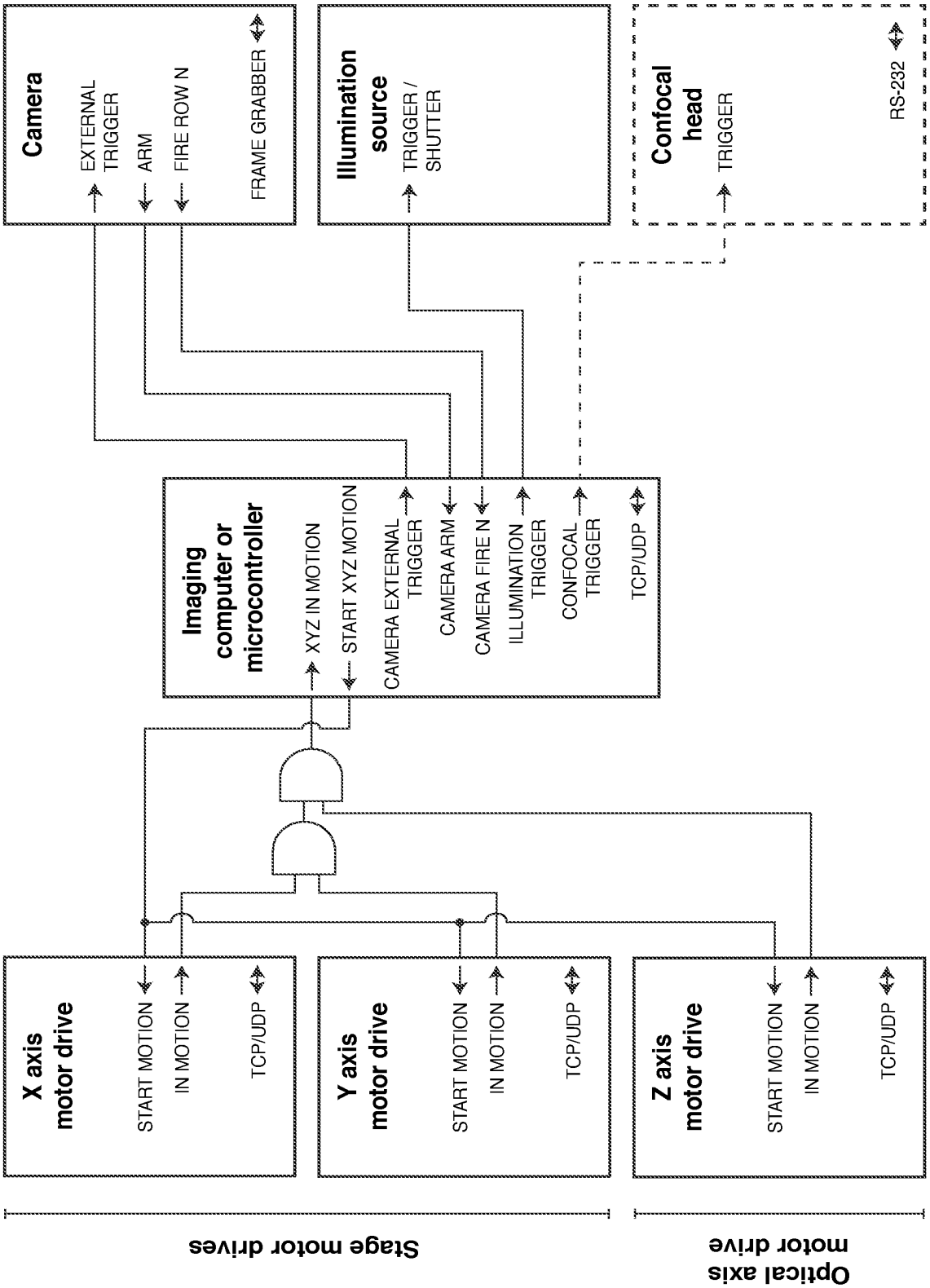

Camera
EXTERNAL TRIGGER
ARM
FIRE ROW N
FRAME GRABBER
Illumination source
TRIGGER / SHUTTER
Confocal head
TRIGGER
RS-232
Imaging computer or microcontroller
XYZ IN MOTION
START XYZ MOTION
CAMERA EXTERNAL TRIGGER
CAMERA ARM
CAMERA FIRE N
ILLUMINATION TRIGGER
CONFOCAL TRIGGER
TCP/UDP
X axis motor drive
START MOTION
IN MOTION
TCP/UDP
Y axis motor drive
START MOTION
IN MOTION
TCP/UDP
Z axis motor drive
START MOTION
IN MOTION
TCP/UDP
Stage motor drives
Optical axis motor drive

FIG. 8

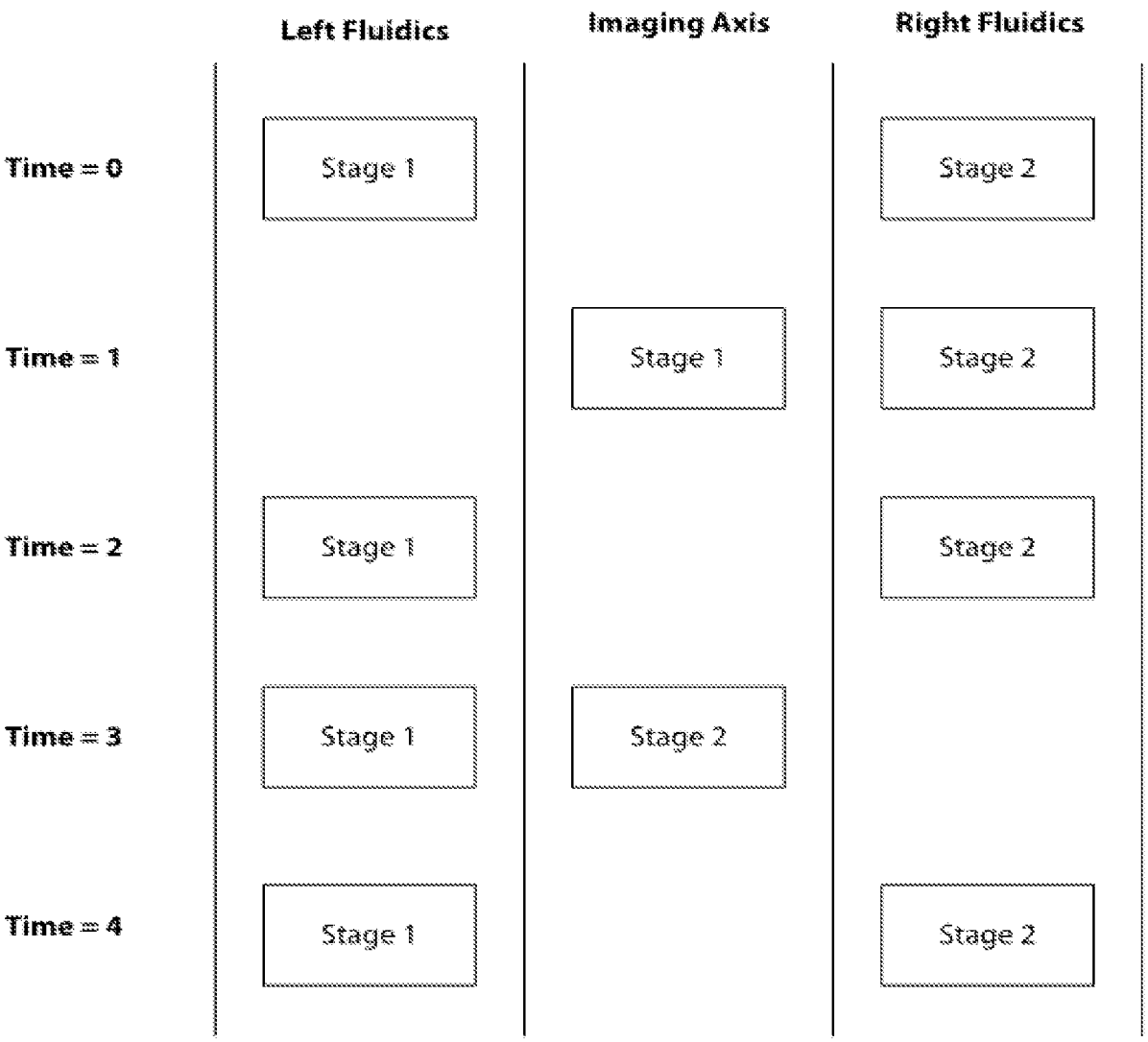

Left Fluidics
Imaging Axis
Right Fluidics
Time = 0
Stage 1
Stage 2
Time = 1
Stage 1
Stage 2
Time = 2
Stage 1
Stage 2
Time = 3
Stage 1
Stage 2
Time = 4
Stage 1
Stage 2

FIG. 9

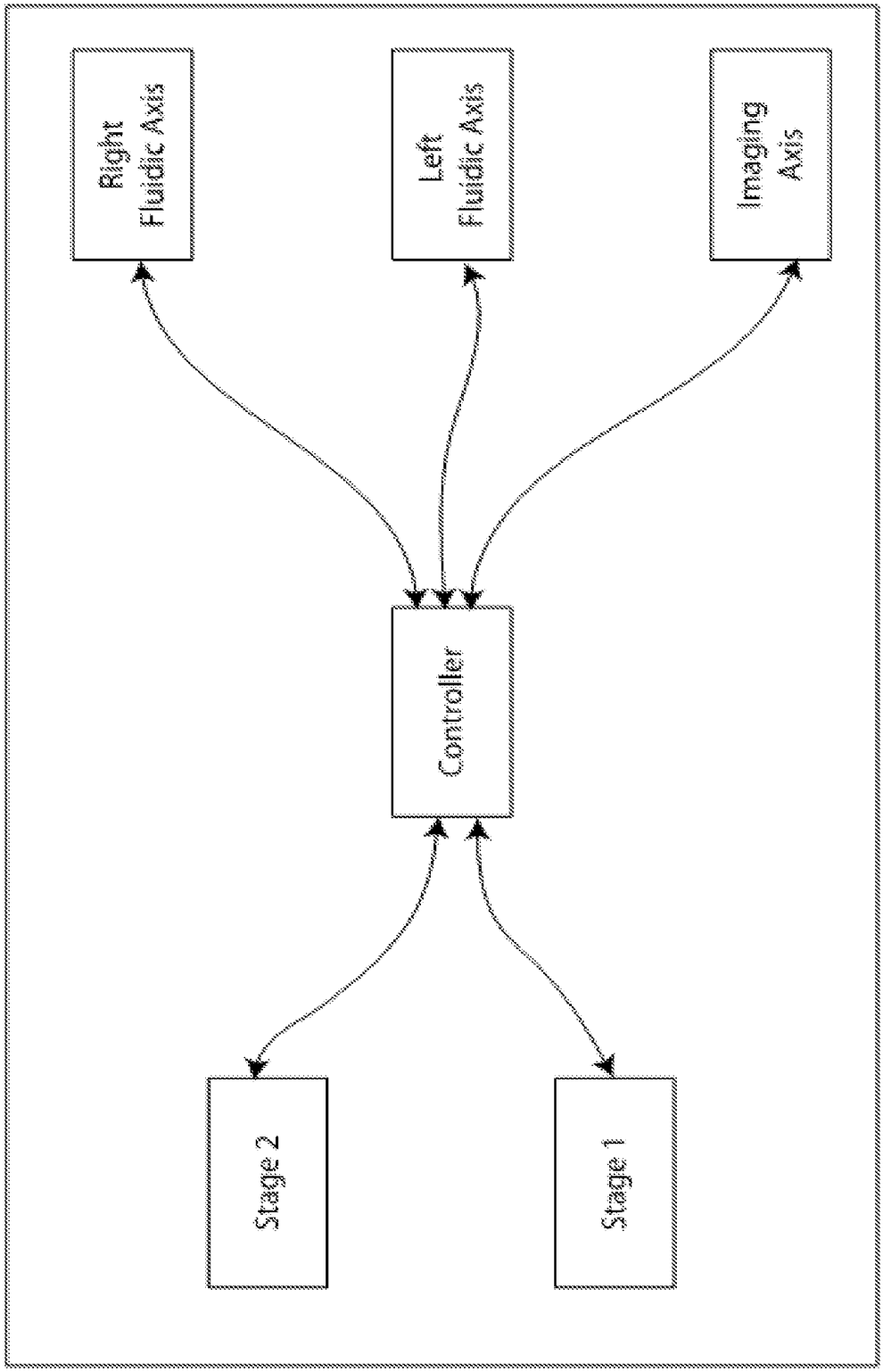
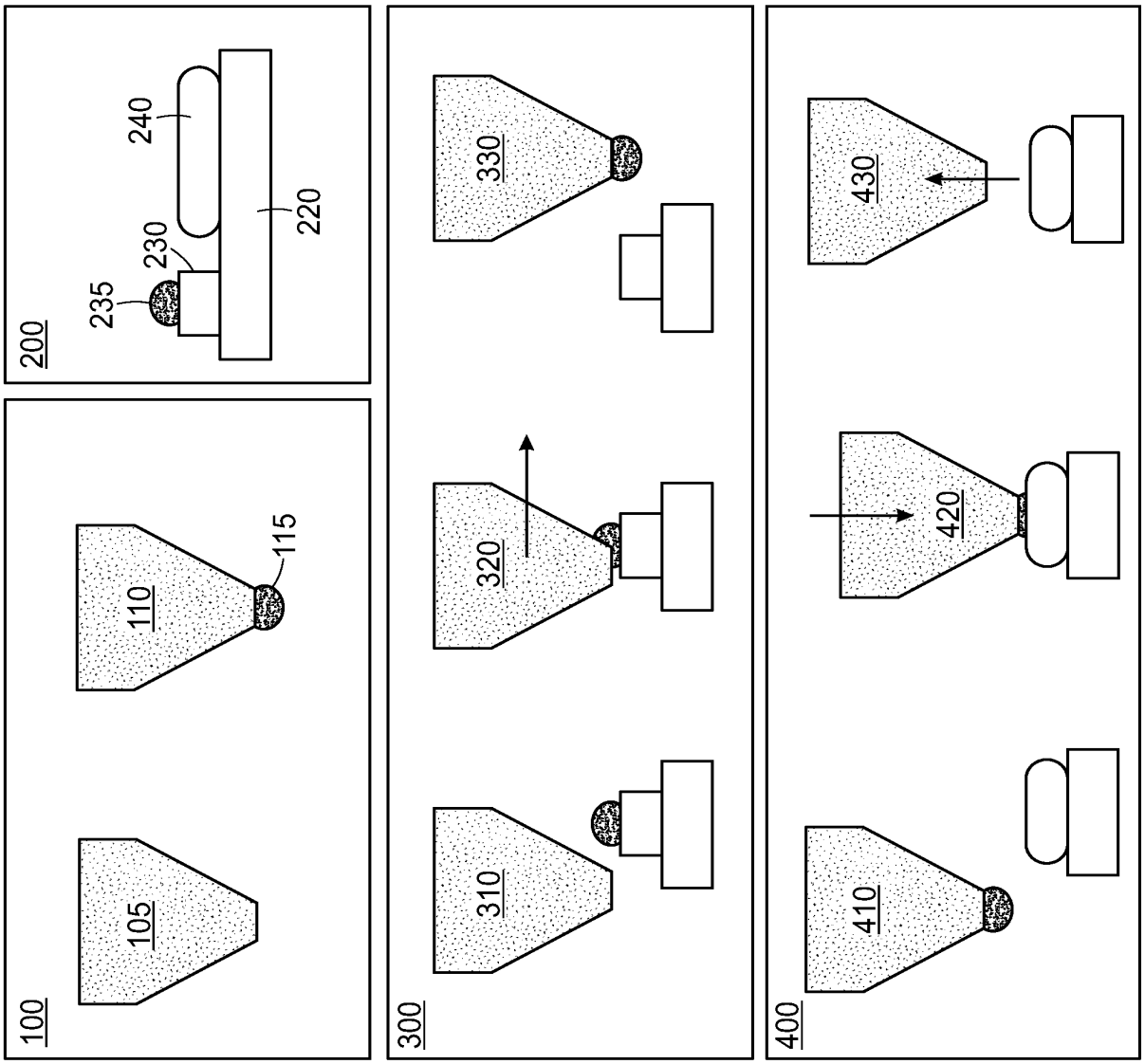

Right Fluidic Axis
Left Fluidic Axis
Imaging Axis
Controller
Stage 2
Stage 1

FIG. 10


100
105
110
115
200
220
230
235
240
300
310
320
330
400
410
420
430

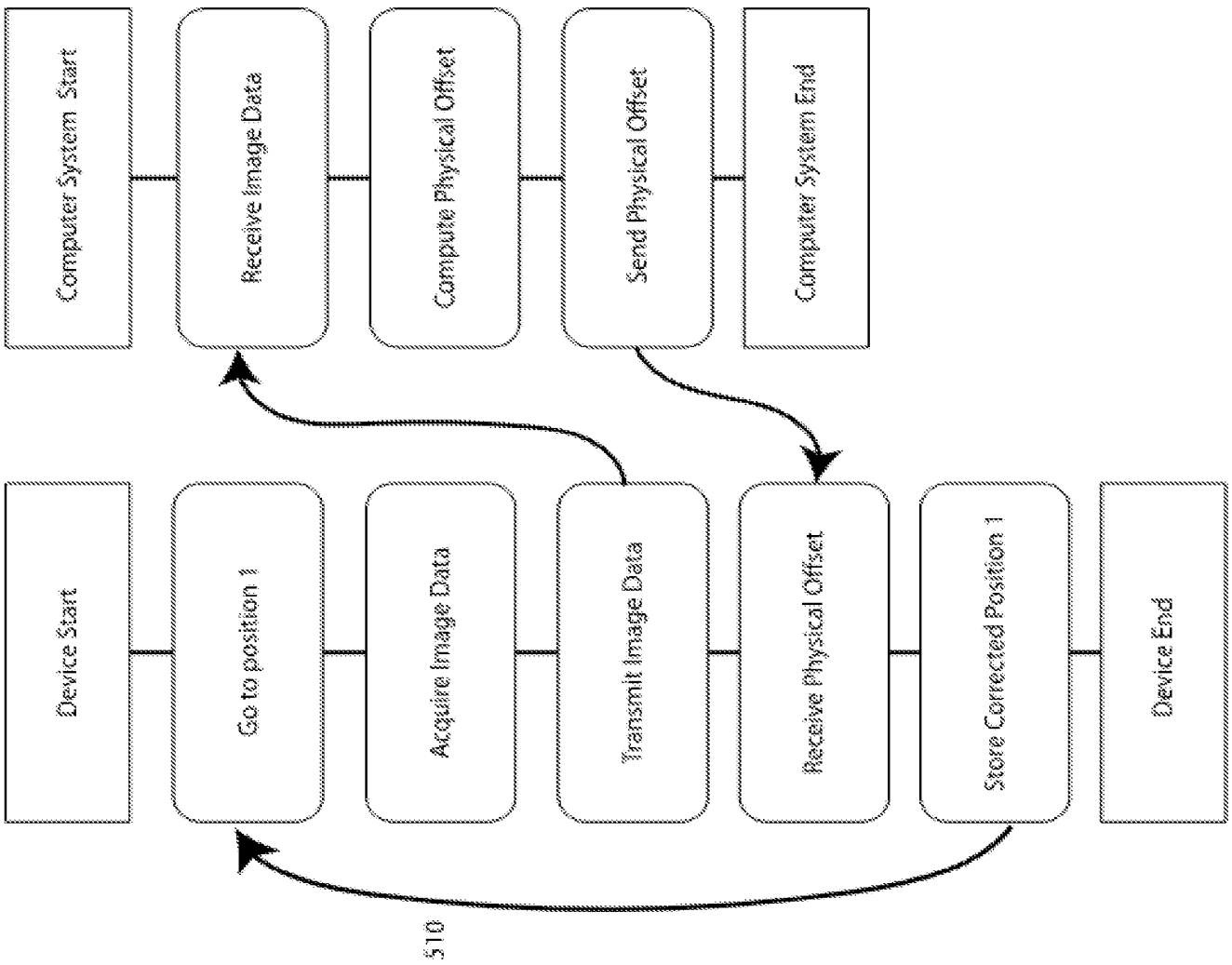

Computer System Start
Receive Image Data
Compute Physical Offset
Send Physical Offset
Computer System End
Device Start
Go to position 1
Acquire Image Data
Transmit Image Data
Receive Physical Offset
Store Corrected Position 1
Device End
510

FIG. 11

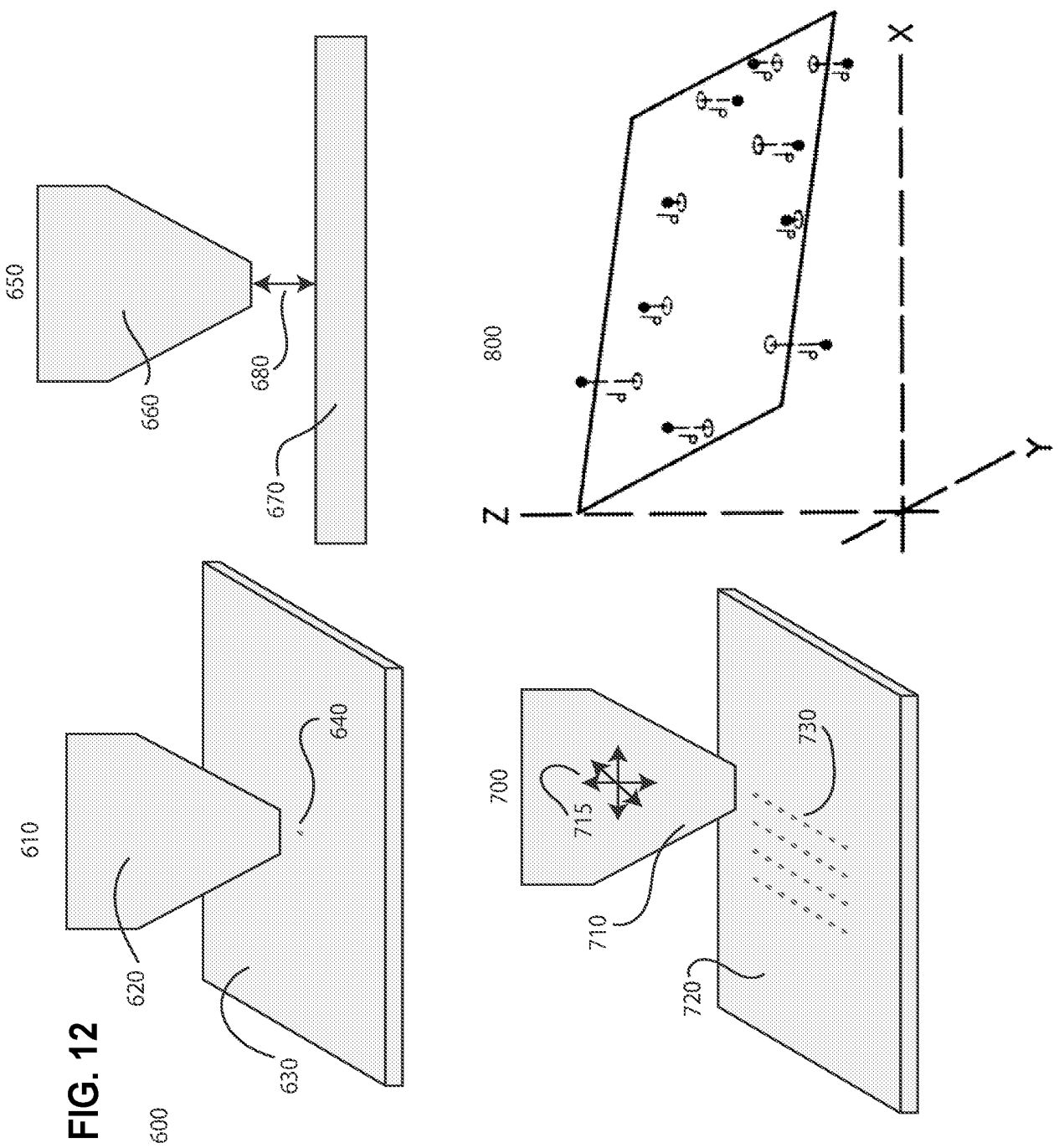


FIG. 12

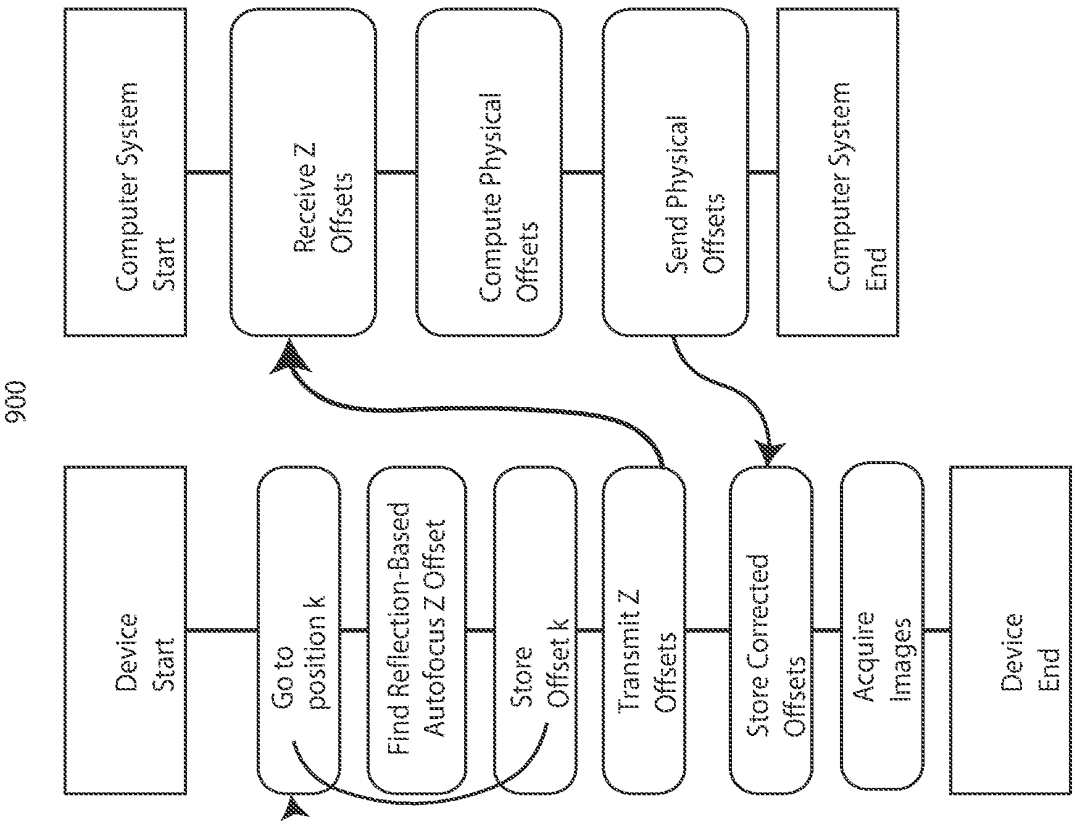

900
Computer System Start
Receive Z Offsets
Compute Physical Offsets
Send Physical Offsets
Computer System End
Device Start
Go to position k
Find Reflection-Based Autofocus Z Offset
Store Offset k
Transmit Z Offsets
Store Corrected Offsets
Acquire Images
Device End

FIG. 12 (Cont.)

PATENT
ATTORNEY DOCKET NO. 010498.01437\US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Evan R. Daugharthy, et al.	)	Examiner:
	)	TBD
Serial No.: TBD	)	
	)	Art Unit: TBD
Filed: Herewith	)	
	)	Conf. No.: TBD
Title: Method and Apparatus for Volumetric Imaging	)	

Commissioner of Patents
P. O. Box 1450
Alexandria, VA 22313-1450

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Applicants submit herewith two of forms PTO/SB/08, identifying information for consideration by the Examiner. All references cited herein were previously cited in parent U.S. Application No. 16/386,337. A copy of the previously cited references is not submitted with this filing. In addition, copies of those of the listed references that are U.S. patents or published applications are not enclosed.

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Respectfully submitted,

Date: August 5, 2021

/John P. Iwanicki/

John P. Iwanicki, Reg. No. 34,628
BANNER & WITCOFF, LTD.
28 State Street, Suite 1800
Boston, MA 02109
Telephone: (617) 720-9640 (direct dial)

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Filer:	John P. Iwanicki/Jennifer Hazzard
Attorney Docket Number:	010498.01437\US

Filed as Large Entity

Filing Fees for Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	320	320
UTILITY SEARCH FEE	1111	1	700	700
UTILITY EXAMINATION FEE	1311	1	800	800
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4200	4200
Pages:				
Claims:				
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**6160**

Electronic Acknowledgement Receipt

EFS ID:	43443163
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22910
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	06-AUG-2021
Filing Date:	
Time Stamp:	09:39:16
Application Type:	Utility under 35 USC 111(a)

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37 CFR 1.21 (Miscellaneous fees and charges)

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	Application_Data_Sheet_Fillable_PDF_21388695.pdf	1256168 831fdae292325af726db2a62092e3b8a69ed153f	no	10
Warnings:					
Information:					
2	TrackOne Request	Certification_and_Request_for_Prioritized_Examination_21392135.pdf	33853 4a4992d256a370012b7152aa71c1b7e1ed4ed180	no	1
Warnings:					
Information:					
3	Transmittal of New Application	Utility_Patent_Application_Transmittal_21392136.pdf	32663 2999b13d8e5ce1115aafaf716eda47eeda4c7b87	no	1
Warnings:					
Information:					
4	Power of Attorney	POA_or_Authorization_of_Agent_21392137.pdf	16214 0ca31d7ae6805aeac292d807fc98a314a1d32cc4	no	1
Warnings:					
Information:					
5	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_1_of_5_21389679.pdf	1044329 dcfc48398dbfb7dc09d6f4e82c89ab6c918133aec	no	26
Warnings:					
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6	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_2_of_5_21389680.pdf	1039512 5714b7a337f003c7a1025a54b3983bdde0c09b44	no	10
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9	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_5_of_5_21389683.pdf	1036585 83045b5c783250b48ace8e505136183a420670a0	no	6
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10	Power of Attorney	POA_21386974.PDF	1129609 6bad5e4703bd741ff3226effe81607a2fd4c7ba2	no	1
Warnings:					
Information:					
11	Oath or Declaration filed	Executed_Declarations_21385726.PDF	1166013 788c19fc742136ec5f4a53b4331eaf4e7ea073bd	no	5
Warnings:					

Information:					
12	Drawings-only black and white line drawings	Drawing_Sheets_21385724.PDF	584721 66a4918baee049046fe2d895418a31165e0ea89c	no	13
Warnings:					
Information:					
13	Transmittal Letter	IDS_Cover_Sheet_21392142.pdf	16277 ef0654fb511759e99bc3dcbc403c22c5f2ad984f	no	1
Warnings:					
Information:					
14	Specification	Continuation_Application_21392143.pdf	190945 7754d6a79e8cac4f19bf5052c707dea2fa0c8cee	no	62
Warnings:					
Information:					
15	Fee Worksheet (SB06)	fee-info.pdf	51312 c39620ba8331dc5ae493c571f7d41c5b28abc978	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	9675533		

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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	010498.01437\US
	Application Number	
Title of Invention	Method and Apparatus for Volumetric Imaging	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

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☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Evan	R.	Daugharthy	

Residence Information (Select One) ● US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Cambridge	MA	US

Mailing Address of Inventor:

Address 1	5 Cowperthwaite Street, Apt. 326
Address 2	
City	Cambridge
State/Province	MA
Postal Code	02138
Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Richard	C.	Terry	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Carlisle	MA	US

Mailing Address of Inventor:

Address 1	100 Nathane Lane
Address 2	
City	Carlisle
State/Province	MA
Postal Code	01741
Country	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Je-Hyuk		Lee	

Residence Information (Select One) ◉ US Residency Non US Residency Active US Military Service

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	010498.01437\US
	Application Number	
Title of Invention	Method and Apparatus for Volumetric Imaging	

City	Allston	State/Province	MA	Country of Residence	US

Mailing Address of Inventor:

Address 1	59 Brainerd Road, Apt. 513		
Address 2			
City	Allston	State/Province	MA
Postal Code	02134	Country	US

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	George	M.	Church	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Brookline	State/Province	MA	Country of Residence	US

Mailing Address of Inventor:

Address 1	218 Kent Street		
Address 2			
City	Brookline	State/Province	MA
Postal Code	02446	Country	US

Inventor 5 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Benjamin	W.	Pruitt	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Cambridge	State/Province	MA	Country of Residence	US

Mailing Address of Inventor:

Address 1	42 Lopez Street		
Address 2			
City	Cambridge	State/Province	MA
Postal Code	02139	Country	US

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Correspondence Information:

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	010498.01437\US
	Application Number	
Title of Invention	Method and Apparatus for Volumetric Imaging	

Customer Number	22907		
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Application Information:

Title of the Invention	Method and Apparatus for Volumetric Imaging		
Attorney Docket Number	010498.01437\US	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	13	**Suggested Figure for Publication (if any)**	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	● Customer Number	US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
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Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending			Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)	
	Continuation of	16/386337	2019-04-17	
Prior Application Status	Patented			Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
16/386337	Continuation of	15/969118	2018-05-02	10266888	2019-04-23
Prior Application Status				Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)	
15/969118	Continuation of	PCT/US16/60279	2016-11-03	
Prior Application Status	Expired			Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)	
PCT/US16/60279	Claims benefit of provisional	62/250182	2015-11-03	

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This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

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Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

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Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

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Title of Invention	Method and Apparatus for Volumetric Imaging	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

● Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.	Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

Organization Name	President and Fellows of Harvard College		
Mailing Address Information For Applicant:			
Address 1	17 Quincy Street		
Address 2			
City	Cambridge	**State/Province**	MA
Country	US	Postal Code	02138
Phone Number	(617) 495-3067	Fax Number	(617) 495-9568
Email Address	patents@harvard.edu		

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

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	Application Number	
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Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

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Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

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See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/John P. Iwanicki/			Date (YYYY-MM-DD)	2021-08-05
First Name	John	Last Name	Iwanicki	Registration Number	34,628

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This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

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METHOD AND APPARATUS FOR VOLUMETRIC IMAGING OF A THREE-DIMENSIONAL NUCLEIC ACID CONTAINING MATRIX

RELATED APPLICATION DATA

This application is a continuation application which claims priority to U.S. Patent Application No. 16/386,337, filed on April 17, 2019, which is a continuation application which claims priority to U.S. Patent Application No. 15/969,118, filed on May 2, 2018, which is a continuation of PCT application no. PCT/US2016/060279, designating the United States and filed November 3, 2016; which claims the benefit U.S. Provisional Patent Application No. 62/250,182 filed on November 3, 2015 each of which is hereby incorporated herein by reference in their entirety for all purposes.

STATEMENT OF GOVERNMENT INTERESTS

This invention was made with Government support under grant number P50 HG005550 awarded by National Institutes of Health, RC2HL102815 awarded by National Institutes of Health, MH098977 awarded by National Institutes of Health, GM080177 awarded by National Institutes of Health and DGE1144152 awarded by National Science Foundation. The Government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to a method and apparatus for volumetric imaging of a three-dimensional matrix of nucleic acids where the nucleic acids have been amplified, detected and sequenced within the matrix.

BACKGROUND OF THE INVENTION

Since many gene products such as RNA and proteins are enriched in regions where they function, their location provides an important clue to their function. This property has been used for in situ fluorescent hybridization, immunohistochemistry and tissue-specific reporter assays in numerous areas of biological research. Most optical sequencing methods either utilize a two-dimensional solid substrate or microwells/microchambers to immobilize sequencing templates during the sequencing process in order to maintain spatial invariability for optical detection, and therefore enable reconstruction of a single nucleic acid template sequence. In both cases, optical detection of signal occurs in at most two two-dimensional planes.

SUMMARY

Embodiments of the present invention are directed to volumetric imaging of nucleic acids within a three-dimensional matrix, such as within a fixed biological specimen, and an apparatus for volumetric imaging of nucleic acids within a three-dimensional matrix, such as within a fixed biological specimen. Volumetric imaging detects fluorescence- or optically-encoded signals in three dimensions. According to one aspect, the three-dimensional positioning of a molecule within the three dimensional matrix is determined. According to certain aspects, methods are provided for imaging an arbitrary volume in a method of volumetric detection, imaging, and reconstruction. Exemplary volumetric detection methods include both methods for volumetric imaging of optical sections that utilize optical sectioning (e.g. two-dimensional acquisition of images throughout a volume), as well as those that do not utilize optical sectioning (e.g. digital holography and physical sectioning).

Aspects of the present disclosure may include in situ nucleic acid sequencing methods using a three-dimensional matrix for immobilization of nucleic acid sequencing templates during sequencing, maintaining the spatial relationships in three dimensions between sequencing templates, enabling detection and reconstruction of both sequences and three-dimensional positional information. Exemplary methods described herein are also directed to reconstruct three-dimensional biological features, such as proteins and cell membranes. Exemplary volumetric imaging approaches include those described herein and those known in the art which measure light signals, systematically or otherwise, in three-dimensional space. Accordingly, aspects of the present disclosure include a method and apparatus for volumetric imaging of in situ sequenced nucleic acids. Aspects of the present disclosure further include an automated method and apparatus for volumetric imaging of in situ sequenced nucleic acids including an in situ sequencing method or apparatus, a fluidics method or reagent delivery method or apparatus to regulate flow of reagents and deliver reagents and a volumetric imaging method or apparatus for imaging and/or detecting light signals from the in situ sequenced nucleic acids or other molecules or structures of interest. According to certain aspects, the methods and apparatus described herein are not limited to nucleic acids. Any molecule or structure within a three dimensional matrix that can be detected, for example, using detectable moieties, such as fluorescent moieties and other detectable moieties known to those of skill in the art can be the subject of the volumetric imaging methods described herein. Such molecules or structures can include DNA, RNA, proteins, biomolecules, cellular structures and the like.

Exemplary methods of making a three dimensional matrix of nucleic acid sequences, amplifying such nucleic acid sequences, sequencing such nucleic acid sequences and imaging such nucleic acid sequences are provided in PCT US2014/18580 hereby incorporated by reference in its

entirety. Such methods include making a three dimensional matrix including nucleic acids covalently bound into a matrix or into or to a matrix material. The nucleic acids may be co-polymerized with the matrix material or cross-linked to the matrix material or both. According to one aspect, a plurality of nucleic acid sequences of certain length, such as DNA or RNA sequences are part of a three-dimensional copolymer. According to one aspect, nucleic acids such as DNA or RNA sequences of given length are covalently attached to a matrix material to preserve their spatial orientation in the x, y and z axes within the matrix. It is to be understood that the three dimensional matrix may include a matrix material and that the term copolymer, matrix and matrix material may be used interchangeably. Useful methods also include immobilizing naturally occurring nucleic acids within their native environment, such as within a cell or within a tissue sample. The three dimensional nucleic acid matrix can be generated in situ in a cell or tissue sample to preserve the naturally occurring nucleic acid sequence diversity (such as DNA and RNA) and spatial orientation in cells, tissues or any other complex biomaterial. According to this aspect, the location of nucleic acids and their relative position is identified as a three dimensional structure, such as within subcellular compartments, within cells, within tissues, as three dimensional nucleic acid assemblies, as three dimensional nucleic acid material, etc. The nucleic acids can be amplified and sequenced, if desired, in situ thereby providing positional information of the nucleic acids within the cell or tissue.

According to a related aspect, nucleic acids of interest or other molecules of interest, whether naturally occurring or synthetic, can be present within a three dimensional matrix material and covalently attached to the three dimensional matrix material such that the relative position of each nucleic acid is fixed, i.e. immobilized, within the three dimensional matrix material. In this manner, a three-dimensional matrix of covalently bound nucleic acids of any desired sequence is

provided. Each nucleic acid has its own three dimensional coordinates within the matrix material and each nucleic acid represents information. According to one aspect, individual nucleic acids, such as DNA or RNA can be amplified and sequenced in situ, i.e., within the matrix.

According to a further aspect, the nucleic acids can be amplified to produce amplicons within the three dimensional matrix material. The amplicons can then be covalently attached to the matrix, for example, by copolymerization or cross-linking. This results in a structurally stable and chemically stable three dimensional matrix of nucleic acids. According to this aspect, the three dimensional matrix of nucleic acids allows for prolonged information storage and read-out cycles. The nucleic acid / amplicon matrix allows for high throughput sequencing of a wide ranging array of biological and non-biological samples in three dimensions.

According to certain aspects, a three dimensional nucleic acid matrix is provided where a plurality of nucleic acid molecules, such as DNA or RNA, amplicons or nucleic acid structural units are immobilized, such as by covalent bonding to the matrix, in a three dimensional space relative to one another. In this context, the nucleic acid molecules are rigidly fixed to the extent that they maintain their coordinate position within the matrix. It is to be understood that even though a nucleic acid molecule may be covalently attached to the three dimensional matrix material, the nucleic acid molecule itself may be capable of movement though bound to the matrix, such as for example, when a nucleic acid sequence is bound to the matrix at a single location on the nucleic acid.

According to one aspect, the three dimensional matrix including nucleic acids is porous. According to one aspect, the three dimensional matrix including nucleic acids is porous to the

extent that reagents typically used in amplification methods can diffuse or otherwise move through the matrix to contact nucleic acids and thereby amplify nucleic acids under suitable conditions. Porosity can result from polymerization and/or crosslinking of molecules used to make the matrix material. The diffusion property within the gel matrix is largely a function of the pore size. The molecular sieve size is chosen to allow for rapid diffusion of enzymes, oligonucleotides, formamide and other buffers used for amplification and sequencing (>50-nm). The molecular sieve size is also chosen so that large DNA or RNA amplicons do not readily diffuse within the matrix (<500-nm). The porosity is controlled by changing the cross-linking density, the chain lengths and the percentage of co-polymerized branching monomers according to methods known to those of skill in the art.

According to one aspect, the three dimensional matrix material is chemically inert and thermally stable to allow for various reaction conditions and reaction temperatures. According to this aspect, the three dimensional matrix material is chemically inert and thermally stable to conditions used in amplification and sequencing methods known to those of skill in the art.

According to one aspect, the three dimensional matrix material is optically transparent. According to one aspect, the three dimensional matrix material is optically transparent to allow for three dimensional imaging techniques known to those of skill in the art.

According to one aspect, the nucleic acids are amplified to an extent to produce sufficient levels of amplicons for three dimensional imaging. For example, the nucleic acids are amplified and include a label sufficient for a high level of fluorescence compatible with three dimensional imaging.

According to one aspect, the material used to form the matrix is compatible with a wide range of biological and non-biological specimens in situ so as to avoid extracting the nucleic acid molecules away from their native environment.

According to one aspect, the matrix material may be a semi-solid medium that can be made from polyacrylamide, cellulose, alginate, polyamide, cross-linked agarose, cross-linked dextran or cross-linked polyethylene glycol. In certain aspects, the semi-solid medium has x, y and z axes, and the nucleic acids are present randomly or non-randomly within the three dimensional matrix.

In certain aspects, the semi-solid medium can be attached to a solid support such as a microscope slide or a flow cell. The solid support can be attached to the bottom surface of the semi-solid medium.

According to one aspect, an automated method and device is provided for introducing reagents into the matrix, such as for example, by using fluidics or microfluidics devices including one or more reservoirs which reagents are stored, channels or conduits to direct the reagents to the matrix and one or more pumps to force or draw the reagents from the reservoirs through the channels or conducts and to the matrix. The automated method and device may be controlled by a microprocessor and software.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other features and advantages of the present invention will be more fully understood from the following detailed description of illustrative embodiments taken in conjunction with the accompanying drawings.

Fig. 1 depicts in schematic the process of creating a matrix of nucleic acids within cells in situ, followed by amplifying the nucleic acids, such as DNA or RNA, in situ, co-polymerizing the amplicons in situ, covalently attaching the amplicons to the matrix material, interrogating the amplicons and imaging the amplicons along with a reconstructed 3D cell image with DNA/RNA amplicons on the order of 10-7m.

Fig. 2 is a perspective view in schematic of components of an automated sequencing and three-dimensional volumetric imaging device.

Fig. 3 is a front view in schematic of components of an automated sequencing and three-dimensional volumetric imaging device.

Fig. 4 is a perspective view in schematic of components of an automated sequencing and three-dimensional volumetric imaging device.

Fig. 5 is a perspective view in schematic of a stage with sample holders of an automated sequencing and three-dimensional volumetric imaging device.

Fig. 6 is an exemplary schematic timing diagram useful with embodiments described herein.

Fig. 7 is an exemplary schematic block diagram illustrating aspects of an exemplary TTL communication structure.

Fig. 8 depicts an exemplary timing and resource usage timing diagram for sequencing device fluidics, imaging, and dual stage subsystems.

Fig. 9 depicts an exemplary schematic block diagram illustrating the controller organization for the dual stages, fluidic, and imaging subsystems.

Fig. 10 depicts exemplary device systems for managing the state of and interfacing with an objective lens.

Fig. 11 depicts a flow chart for repeatable XYZ positioning of the sample relative to the imaging axis over time.

Fig. 12 depicts an exemplary system for mapping a surface of a sample holder comprising a solid substrate.

DETAILED DESCRIPTION

The present invention provides a method and apparatus for analysis of molecules present within a three-dimensional matrix, such as a plurality of nucleic acids within a three-dimensional matrix. According to one aspect, an automated sequencing and three-dimensional imaging device (i.e., volumetric imaging device) is provided that can measure, resolve and optionally localizing light signals in three-dimensional space to measure fluorescently- or optically-encoded nucleic-acid sequencing within a three-dimensional matrix.

According to one aspect, the nucleic acids have been amplified and sequenced using optical sequencing methods known to those of skill in the art so that the nucleic acid can be optically detected. According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus, apparatus for fluid exchange, apparatus for temperature control, and apparatus for computational analysis. The automated sequencing and three-dimensional imaging device uses biochemical methods to execute fluorescence- or optically-encoded nucleic acid sequencing, acquisition of image data, and optionally, data processing.

According to one aspect, a plurality of nucleic acids within a three-dimensional matrix that have been amplified and sequenced using optical sequencing methods known to those of skill in the art so that the nucleic acid can be optically detected is volumetrically imaged. According to this aspect, during detection of fluorescence- or optically-encoded nucleic acid sequencing chemistry, light emanates from a nucleic acid template molecule or multimolecular amplicon in three dimensions upon excitation. The three-dimensional distribution of emission light, called a point spread function, is created by an optical system when imaging a point source. In traditional wide-field microscopy systems, as the focal plane distance increases from the point source in Z, the image becomes less point-like. The total integrated intensity at each out-of-focus plane is the same as that at the focal plane, but in practice the intensity level drops below the sensitivity of the detector after a certain distance. The out-of-focus light is also dispersed, enabling resolution of objects in another focal plane whose intensity is greater than the out-of-focus background. The size and shape of the point spread function is determined by the optical system, especially the numerical aperture of the objective. Although wide-field microscopy can be used to image a volume in this way, additional methods achieve greater axial resolution and signal-to-noise.

Exemplary volumetric imaging approaches used in the automated sequencing and three-dimensional imaging device described herein are broadly divided into two categories: structured illumination and emission manipulation. Additional approaches include computational reconstruction and measurement of angular components of emission light. These approaches provide a systematic measurement of the intensity and wavelength of light in three-dimensional space.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using 3D Structured Illumination (3DSIM). In 3DSIM, spatially patterned light is used for excitation, and fringes in the Moiré pattern generated by interference of the illumination pattern and the sample, are used to reconstruct the source of light in three dimensions. Multiple spatial patterns are used to excite the same physical region in order to illuminate the whole field. Digital processing or analog methods are used to reconstruct the final image. See York, Andrew G., et al. "Instant super-resolution imaging in live cells and embryos via analog image processing." *Nature methods* 10.11 (2013): 1122-1126 and Gustafsson, Mats GL, et al. "Three-dimensional resolution doubling in wide-field fluorescence microscopy by structured illumination." *Biophysical journal* 94.12 (2008): 4957-4970 each of which are hereby incorporated by reference in their entireties.

Two-photon, or multi-photon, microscopic modalities are useful structured illumination microscopy methods. See Denk W., Strickler J., Webb W. (1990). "Two-photon laser scanning fluorescence microscopy". *Science* **248** (4951): 73–6 hereby incorporated by reference in its entirety. Two-photon microscopy is a type of microscopy that enables imaging deep within a sample by using two photons per excitation event. These systems typically use long-wavelength light for excitation, which penetrates more effectively into tissue due to reduced scattering. The use of two-photon excitation also reduces background signal as single-photon absorption provides insufficient energy to excite emission by the fluorophore. Two-photon microscopy can also utilize larger or more efficient optical and sensor configurations to detect the emission, as the localization of excitation over time is known to the imaging system during scanning. Other benefits to this modality include reduced photodamage to the sample.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using a planar illumination method such as selective planar illumination microscopy (SPIM) or light sheet microscopy (LSM). In SPIM, optical detecting moieties are excited selectively in each plane in a third dimension while a two-dimensional image is acquired in the plane orthogonal to the illumination axis, providing effective separation over imaging time/frames between objects distributed in the third dimension. See Huisken, Jan, et al. "Optical sectioning deep inside live embryos by selective plane illumination microscopy." *Science* 305.5686 (2004): 1007-1009 hereby incorporated by reference in its entirety. Systematic sequential imaging of planes provides for volumetric measurement. The planar illumination light may be generated using any number of approaches such as Gaussian and Bessel beam shaping.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using emission manipulation, such as confocal microscopy where one or more pinholes are positioned at the confocal plane of the lens, blocking out-of-focus light from reaching the detector. The focal plane of the lens is systematically shifted through the third dimension, enabling volumetric imaging. See Wilson, Tony. "Confocal microscopy." *Academic Press: London, etc* 426 (1990): 1-64 hereby incorporated by reference in its entirety.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a confocal imaging or microscopy modality. According to a certain aspect, the confocal imaging or microscopy modality is a scanning laser confocal modality. According to another aspect, the confocal modality is a spinning disk confocal modality. The

spinning disk may be a Nipkow disk. According to another aspect, the confocal microscopy modality is a parallel beam scanning laser modality, wherein two or more pinholes are scanned across the sample, such as by using a mirror galvanometer. The confocal modality may comprise one or more microlens arrays, such as for focusing excitation light onto the pinhole array.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using a parallel confocal method including aperture correlation. See Wilson, Tony, et al. "Confocal microscopy by aperture correlation." *Optics letters* 21.23 (1996): 1879-1881 hereby incorporated by reference in its entirety.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using a microlens array for volumetric imaging, known as light field microscopy. See, Broxton et al. (2013) "Wave Optics Theory and 3-D Deconvolution for the Light Field Microscope" *Stanford Computer Graphics Laboratory Technical Report 2013-1* hereby incorporated by reference in its entirety. According to this aspect, a microlens array between the main lens and the detector pass light, which would otherwise focus at an intermediate plane, onto the light field for detection. 3D reconstruction algorithms are applied to generate a volumetric image.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using a method of volumetric reconstruction from slices. According to this aspect of imaging a volume of arbitrary dimension, a specimen may be sectioned into segments

of arbitrary dimension for the purpose of imaging. The original volume is reconstructed using information about the relative position of each segment imaged with respect to the original volume. This process is typically referred to as "acquiring serial sections," and is used due to limitations in depth of scanning of volumetric imaging modalities, as well as by limitations of manipulating and labeling thick samples, where diffusion may limit penetration of reagents deep into specimens. In extraordinary cases, the sections may be thinner than the diffraction limit of light, enabling resolution in the axis of sectioning beyond what is achievable by the diffraction limit of light. According to the 3D volumetric reconstruction from serial sections method described herein, the sample is sectioned either before or after creation of the 3D sequencing library, but specifically in such a way that the spatial relationship between sections is preserved. For example, each section is placed in a separate well of a flowcell and given a unique identification. During sectioning, the samples are attached onto a solid support substrate, particularly by creation of covalent cross-links between the sample matrix and the solid substrate or by creation of a new encapsulating structural matrix. According to one aspect, the section is transferred onto functionalized glass and covalent chemical cross-links are formed between the glass and the 3D matrix of the sample. According to one aspect, the section is transferred onto functionalized glass and a new supporting matrix, which is covalently linked to the glass surface, is formed to encapsulate the sample and provide structural support. For example, a 4% 1:19 acrylamide:bis gel is formed around and through a section of a biological sample as the primary 3D matrix for FISSEQ; or a pre-existing FISSEQ gel (including those formed using a polyacrylamide matrix as the primary 3D matrix) is sectioned and encased in a secondary 3D stabilizing matrix.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using deconvolution microscopy including computational algorithmic methods of processing digital image data so as to remove the effect of the optical components of the microscope. See Biggs, David SC. "3D deconvolution microscopy." *Current Protocols in Cytometry* (2010): 12-19 hereby incorporated by reference in its entirety. Because optical microscopy detects point sources of light as a point spread function that exists in three dimensions, deconvolution methods described herein reassign out of focus light to the point source, often using models or measurements of the point spread function of the optical system. This can serve to effectively increase resolution in three dimensions.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using aberration-corrected multifocus microscopy to simultaneously capture images from multiple sample planes, using Diffractive Fourier optics to create an instant array of focal of 2D wide-field images, recorded simultaneously in one or more camera frames. See Abrahamsson, Sara, et al. "Fast multicolor 3D imaging using aberration-corrected multifocus microscopy." *Nature methods* 10.1 (2013): 60-63 hereby incorporated by reference in its entirety. As with microlens array microscopy, the entire imaging volume is recorded without mechanical movement of parts.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which includes a three-dimensional imaging apparatus where the three-dimensional matrix is imaged using digital holographic microscopy. According to this aspect, digital

holographic microscopy does not record a projected image, but rather records the light wave front information as a hologram. See Manoharan "Digital Holographic Microscopy for 3D Imaging of Complex Fluids and Biological Systems" hereby incorporated by reference in its entirety. The amplitude and phase of light is measured using digital sensors. The hologram contains all information needed for reconstruction of the volume. If the object wave front is measured from multiple angles, all optical characteristics of the object may be fully characterized. Because there is no image-forming lens, reconstruction algorithms that model the optical system will reconstruct the volume without aberration.

According to one aspect, the automated sequencing and three-dimensional imaging device described herein can carry out one or more volumetric imaging methods, such as by including one or more volumetric imaging apparatuses to carry out one or more volumetric imaging methods. A combination of volumetric imaging methods as described herein may be used to further enhance resolution, imaging speed, light efficiency, or gain additional benefits. For example, SIM and confocal microscopy principles have been combined into multifocal SIM (mSIM). See York, Andrew G., et al. "Resolution doubling in live, multicellular organisms via multifocal structured illumination microscopy." *Nature methods* 9.7 (2012): 749-754 hereby incorporated by reference in its entirety. Also, volumetric reconstruction of slices may be combined with other methods, such as confocal microscopy, since confocal microscopy has a depth limit of hundreds of microns, while it is desirable to sequence within three-dimensional matrices of arbitrary dimensions.

According to one aspect, the automated sequencing and three-dimensional imaging device described herein comprises an imaging modality with one image sensor. According to another

aspect, the automated sequencing and three-dimensional imaging device described herein comprises an imaging modality with two or more image sensors. According to one aspect, the automated sequencing and three-dimensional imaging device described herein comprises an imaging modality with four image sensors. According to one aspect, the image sensor is a photon multiplier tube (PMT). According to another aspect, the image sensor is a charge-coupled device (CCD). According to a separate aspect, the image sensor is a Complementary metal–oxide–semiconductor (CMOS). According to one aspect, the image sensor is cooled by an integrated air or liquid cooling apparatus, such as for the purpose of reducing electrical noise or to stabilize the thermal operating conditions of the sensor. Further according to this aspect, a liquid cooling apparatus may provide for cooling with reduced vibration relative to active air cooling, such as by a fan. The cooling apparatus or unit may use a heat sink in conjunction with a fan to dissipate heat produced during temperature changes. The heating or cooling apparatus or unit may use a radiator and liquid cooling / circulating system to dissipate heat produced during temperature changes. The heating or cooling apparatus or unit may use temperature sensors or thermistors to provide temperature feedback to a control system, which may be a microcontroller or built-to-task electronic circuit.

According to one aspect, the automated sequencing and three-dimensional imaging device described herein comprises a color multiplexer for detection of one or more distinct colors of light. The light emitted from one or more fluorescent emitters is detected by a detector. In certain aspects, the detector is configured to detect photons of light with certain wavelengths. According to another aspect, the emission light is filtered such that only photons of certain wavelengths are detected by a photon detector. Further according to another aspect, the device contains one or more emission filters, excitation filters, and/or dichroics for directing certain wavelengths of

light within the optical system. Further according to one aspect, the device contains one or more acousto-optical tunable filter(s) (AOTF) for directing certain wavelengths of light to a certain detector or detectors. According to one aspect, colors of light signals are detected in serial. According to another aspect, two or more distinguishable colors of light are detected in parallel by one or more sensors.

According to one aspect, the automated sequencing and three-dimensional imaging device described herein is used to detect two or more colors of light in serial or parallel. According to one aspect, the colors of light being detected are spaced along the electromagnetic spectrum to facilitate discrimination between the colors. According to certain aspects, the fluorescence signals are spaced along the electromagnetic spectrum by emission wavelength to facilitate specific detection of certain fluorescent moieties. According to certain aspects, the fluorescence signals are spaced along the electromagnetic spectrum by excitation wavelength to facilitate specific excitation of certain fluorescent moieties. According to one exemplary aspect, the colors of emission light are distributed around about 510 nm, 570 nm, 620 nm, and/or 680 nm. According to another exemplary aspect, the colors of excitation light are distributed around about 480 nm, 530 nm, 590 nm, and/or 640 nm.

According to one aspect, the automated sequencing and three-dimensional imaging device described herein comprises one or more sources of light. According to one aspect, the light source is used for the purpose of exciting fluorescence emission by the sample. According to another aspect, the light source is used for the purpose of detecting absorbance, Raman scattering, or other modalities of interaction between the light and the sample. According to a certain aspect, the light source is comprised of one or more light emitting diodes (LED).

According to another aspect, the light source is comprised of one or more lasers. According to another aspect, the light source is comprised of one or more lamps, such as a mercury or metal halide lamp. According to a certain aspect, the light source is coupled to the device by free space optics, wherein the light is propagated through gas or vacuum from the source to the optical system of the device. According to another aspect, the light source is coupled to the device by a fiber optic or liquid light guide. According to an exemplary aspect, the light is transmitted in its entirety or in part along a fiber optic with a square core. Further according to this aspect, the square core is paired with a square aperture or a square field stop. Further according to this aspect, the dimensions of the square core and/or square aperture may be designed to match the dimensions of the image sensor within the optical system. According to certain aspects, multiple fibers are fused as a mechanism of combining multiple colors of light into a single fiber. According to certain aspects, multiple fibers are optically combined as a mechanism of combining multiple colors of light into a single fiber. According to certain aspects, the excitation light is passed through a shutter to control the propagation of the light into or through the optical system. According to one aspect, an acousto-optical tunable filter (AOTF) is used to control the propagation of the light into or through the optical system. According to certain aspects, the propagation of the excitation light into or through the optical system is mechanically, electrically, or electromechanically coupled with a controller system for the purpose of synchronizing one or more events within the device.

According to one aspect, the sample to be analyzed by the automated sequencing and three-dimensional imaging device described herein is a three dimensional matrix including a plurality of nucleic acids bound thereto. According to one aspect, the matrix is a three dimensional nucleic acid-containing polymer. The nucleic acids may be naturally occurring nucleic acids or non-

naturally occurring nucleic acids, such as nucleic acids that have been made using synthetic methods. The nucleic acids in the three dimensional matrix may be ordered or unordered. The nucleic acids in the three dimensional matrix may be present in their natural spatial relationship within a cell, tissue or organism. The nucleic acids in the three dimensional matrix may be present in rows and columns within the three dimensional matrix such as with a regular or repeating array.

According to one aspect, the nucleic acids are modified to incorporate a functional moiety for attachment to the matrix. The functional moiety can be covalently cross-linked, copolymerize with or otherwise non-covalently bound to the matrix. The functional moiety can react with a cross-linker. The functional moiety can be part of a ligand-ligand binding pair. dNTP or dUTP can be modified with the functional group, so that the function moiety is introduced into the DNA during amplification. A suitable exemplary functional moiety includes an amine, acrydite, alkyne, biotin, azide, and thiol. In the case of crosslinking, the functional moiety is cross-linked to modified dNTP or dUTP or both. Suitable exemplary cross-linker reactive groups include imidoester (DMP), succinimide ester (NHS), maleimide (Sulfo-SMCC), carbodiimide (DCC, EDC) and phenyl azide. Cross-linkers within the scope of the present disclosure may include a spacer moiety. Such spacer moieties may be functionalized. Such spacer moieties may be chemically stable. Such spacer moieties may be of sufficient length to allow amplification of the nucleic acid bound to the matrix. Suitable exemplary spacer moieties include polyethylene glycol, carbon spacers, photo-cleavable spacers and other spacers known to those of skill in the art and the like.

According to one aspect, a matrix-forming material is contacted to a plurality of nucleic acids spatially arrange in three-dimensions relative to one another.

Matrix forming materials include polyacrylamide, cellulose, alginate, polyamide, cross-linked agarose, cross-linked dextran or cross-linked polyethylene glycol. The matrix forming materials can form a matrix by polymerization and/or crosslinking of the matrix forming materials using methods specific for the matrix forming materials and methods, reagents and conditions known to those of skill in the art.

According to one aspect, a matrix-forming material can be introduced into a cell. The cells are fixed with formaldehyde and then immersed in ethanol to disrupt the lipid membrane. The matrix forming reagents are added to the sample and are allowed to permeate throughout the cell. A polymerization inducing catalyst, UV or functional cross-linkers are then added to allow the formation of a gel matrix. The un-incorporated material is washed out and any remaining functionally reactive group is quenched. Exemplary cells include any cell, human or otherwise, including diseased cells or healthy cells. Certain cells include human cells, non-human cells, human stem cells, mouse stem cells, primary cell lines, immortalized cell lines, primary and immortalized fibroblasts, HeLa cells and neurons.

According to one aspect, a matrix-forming material can be used to encapsulate a biological sample, such as a tissue sample. The formalin-fixed embedded tissues on glass slides are incubated with xylene and washed using ethanol to remove the embedding wax. They are then treated with Proteinase K to permeabilized the tissue. A polymerization inducing catalyst, UV or functional cross-linkers are then added to allow the formation of a gel matrix. The un-incorporated material is washed out and any remaining functionally reactive group is quenched.

Exemplary tissue samples include any tissue samples of interest whether human or non-human. Such tissue samples include those from skin tissue, muscle tissue, bone tissue, organ tissue and the like. Exemplary tissues include human and mouse brain tissue sections, embryo sections, tissue array sections, and whole insect and worm embryos.

The matrix-forming material forms a three dimensional matrix including the plurality of nucleic acids. According to one aspect, the matrix-forming material forms a three dimensional matrix including the plurality of nucleic acids while maintaining the spatial relationship of the nucleic acids. In this aspect, the plurality of nucleic acids is immobilized within the matrix material. The plurality of nucleic acids may be immobilized within the matrix material by co-polymerization of the nucleic acids with the matrix-forming material. The plurality of nucleic acids may also be immobilized within the matrix material by crosslinking of the nucleic acids to the matrix material or otherwise cross-linking with the matrix-forming material. The plurality of nucleic acids may also be immobilized within the matrix by covalent attachment or through ligand-protein interaction to the matrix.

According to one aspect, the matrix is porous thereby allowing the introduction of reagents into the matrix at the site of a nucleic acid for amplification of the nucleic acid. A porous matrix may be made according to methods known to those of skill in the art. In one example, a polyacrylamide gel matrix is co-polymerized with acrydite-modified streptavidin monomers and biotinylated DNA molecules, using a suitable acrylamide:bis-acrylamide ratio to control the cross-linking density. Additional control over the molecular sieve size and density is achieved by adding additional cross-linkers such as functionalized polyethylene glycols. According to one aspect, the nucleic acids, which may represent bits of information, are readily accessed by

oligonucleotides, such as labeled oligonucleotide probes, primers, enzymes and other reagents with rapid kinetics.

According to one aspect, the matrix is sufficiently optically transparent or otherwise has optical properties suitable for standard Next Generation sequencing chemistries and deep three dimensional imaging for high throughput information readout. The Next Generation sequencing chemistries that utilize fluorescence imaging include ABI SoLiD (Life Technologies), in which a sequencing primer on a template is ligated to a library of fluorescently labeled oligonucleotides with a cleavable terminator. After ligation, the beads are then imaged using four color channels (FITC, Cy3, Texas Red and Cy5). The terminator is then cleaved off leaving a free-end to engage in the next ligation-extension cycle. After all dinucleotide combinations have been determined, the images are mapped to the color code space to determine the specific base calls per template. The workflow is achieved using an automated fluidics and imaging device (i.e. SoLiD 5500 W Genome Analyzer, ABI Life Technologies). Another sequencing platform uses sequencing by synthesis, in which a pool of single nucleotide with a cleavable terminator is incorporated using DNA polymerase. After imaging, the terminator is cleaved and the cycle is repeated. The fluorescence images are then analyzed to call bases for each DNA amplicons within the flow cell (HiSeq, Illumia).

According to certain aspects, the plurality of nucleic acids may be amplified to produce amplicons by methods known to those of skill in the art. The amplicons may be immobilized within the matrix generally at the location of the nucleic acid being amplified, thereby creating a localized colony of amplicons. The amplicons may be immobilized within the matrix by steric factors. The amplicons may also be immobilized within the matrix by covalent or noncovalent

bonding. In this manner, the amplicons may be considered to be attached to the matrix. By being immobilized to the matrix, such as by covalent bonding or crosslinking, the size and spatial relationship of the original amplicons is maintained. By being immobilized to the matrix, such as by covalent bonding or crosslinking, the amplicons are resistant to movement or unraveling under mechanical stress.

According to one aspect, the amplicons, such as DNA amplicons, are then copolymerized and/or covalently attached to the surrounding matrix thereby preserving their spatial relationship and any information inherent thereto. For example, if the amplicons are those generated from DNA or RNA within a cell embedded in the matrix, the amplicons can also be functionalized to form covalent attachment to the matrix preserving their spatial information within the cell thereby providing a subcellular localization distribution pattern.

As used herein, the term "attach" refers to both covalent interactions and noncovalent interactions. A covalent interaction is a chemical linkage between two atoms or radicals formed by the sharing of a pair of electrons (i.e., a single bond), two pairs of electrons (i.e., a double bond) or three pairs of electrons (i.e., a triple bond). Covalent interactions are also known in the art as electron pair interactions or electron pair bonds. Noncovalent interactions include, but are not limited to, van der Waals interactions, hydrogen bonds, weak chemical bonds (i.e., via short-range noncovalent forces), hydrophobic interactions, ionic bonds and the like. A review of noncovalent interactions can be found in Alberts et al., in Molecular Biology of the Cell, 3d edition, Garland Publishing, 1994, incorporated herein by reference in its entirety for all purposes.

As used herein, the term "nucleic acid" includes the term "oligonucleotide" or "polynucleotide" which includes a plurality of nucleotides. The term "nucleic acid" is intended to include naturally occurring nucleic acids and synthetic nucleic acids. The term "nucleic acid" is intended to include single stranded nucleic acids and double stranded nucleic acids. The term "nucleic acid" is intended to include DNA and RNA, whether single stranded or double stranded. Nucleotides of the present invention will typically be the naturally-occurring nucleotides such as nucleotides derived from adenosine, guanosine, uridine, cytidine and thymidine. When oligonucleotides are referred to as "double-stranded," it is understood by those of skill in the art that a pair of oligonucleotides exists in a hydrogen-bonded, helical array typically associated with, for example, DNA. In addition to the 100% complementary form of double-stranded oligonucleotides, the term "double-stranded" as used herein is also meant to include those form which include such structural features as bulges and loops (see Stryer, Biochemistry, Third Ed. (1988), incorporated herein by reference in its entirety for all purposes). As used herein, the term "polynucleotide" refers to a strand of nucleic acids that can be a variety of different sizes. Polynucleotides may be the same size as an oligonucleotide, or may be two-times, three-times, four-times, five-times, ten-times, or greater than the size of an oligonucleotide.

Oligonucleotides and/or polynucleotides may be isolated from natural sources or purchased from commercial sources. Oligonucleotide and/or polynucleotide sequences may be prepared by any suitable method, e.g., the phosphoramidite method described by Beaucage and Carruthers ((1981) Tetrahedron Lett. 22: 1859) or the triester method according to Matteucci et al. (1981) J. Am. Chem. Soc. 103:3185), both incorporated herein by reference in their entirety for all purposes, or by other chemical methods using either a commercial automated oligonucleotide synthesizer or high-throughput, high-density array methods described herein and known in the

art (see U.S. Patent Nos. 5,602,244, 5,574,146, 5,554,744, 5,428,148, 5,264,566, 5,141,813, 5,959,463, 4,861,571 and 4,659,774, incorporated herein by reference in its entirety for all purposes). Pre-synthesized oligonucleotides may also be obtained commercially from a variety of vendors.

In certain embodiments of the invention oligonucleotides and/or polynucleotides may be prepared using a variety of microarray technologies known in the art. Pre-synthesized oligonucleotide and/or polynucleotide sequences may be attached to a support or synthesized in situ using light-directed methods, flow channel and spotting methods, inkjet methods, pin-based methods and bead-based methods set forth in the following references: McGall et al. (1996) Proc. Natl. Acad. Sci. U.S.A. 93:13555; Synthetic DNA Arrays In Genetic Engineering, Vol. 20:111, Plenum Press (1998); Duggan et al. (1999) Nat. Genet. S21:10; Microarrays: Making Them and Using Them In Microarray Bioinformatics, Cambridge University Press, 2003; U.S. Patent Application Publication Nos. 2003/0068633 and 2002/0081582; U.S. Patent Nos. 6,833,450, 6,830,890, 6,824,866, 6,800,439, 6,375,903 and 5,700,637; and PCT Application Nos. WO 04/031399, WO 04/031351, WO 04/029586, WO 03/100012, WO 03/066212, WO 03/065038, WO 03/064699, WO 03/064027, WO 03/064026, WO 03/046223, WO 03/040410 and WO 02/24597; incorporated herein by reference in their entirety for all purposes.

Nucleic acids may be obtained from libraries, e.g., genomic libraries, cDNA libraries and the like. Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) Proc. Natl. Acad. Sci. USA 90:6909; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al. (1994) J. Med. Chem. 37:2678; Cho et al. (1993) Science 261:1303; Carrell et al. (1994) Angew. Chem. Int. Ed. Engl. 33:2059; Carell et

al. (1994) Angew. Chem. Int. Ed. Engl. 33:2061; and in Gallop et al. (1994) J. Med. Chem. 37:1233, incorporated herein by reference in their entirety for all purposes.

In certain embodiments, nucleic acids are those found naturally in a biological sample, such as a cell or tissue.

In still other aspects, a matrix is used in conjunction with a solid support. For example the matrix can be polymerized in such a way that one surface of the matrix is attached to a solid support (e.g., a glass surface), while the other surface of the matrix is exposed or sandwiched between two solid supports. According to one aspect, the matrix can be contained within a container.

Solid supports of the invention may be fashioned into a variety of shapes. In certain embodiments, the solid support is substantially planar. Examples of solid supports include plates such as slides, microtitre plates, flow cells, coverslips, microchips, and the like, containers such as microfuge tubes, test tubes and the like, tubing, sheets, pads, films and the like. Additionally, the solid supports may be, for example, biological, nonbiological, organic, inorganic, or a combination thereof.

Embodiments of the present invention are further directed to the amplification of nucleic acid sequences within the matrix, i.e. in situ, within the matrix. Methods of amplifying nucleic acids include rolling circle amplification in situ. In certain aspects, methods of amplifying nucleic acids involves the use of PCR, such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91:360-364; incorporated herein by reference in their

entirety for all purposes). Alternative amplification methods include: self-sustained sequence replication (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87:1874, incorporated herein by reference in its entirety for all purposes), transcriptional amplification system (Kwoh et al. (1989) Proc. Natl. Acad. Sci. US. 86:1173, incorporated herein by reference in its entirety for all purposes), Q-Beta Replicase (Lizardi et al. (1988) BioTechnology 6:1197, incorporated herein by reference in its entirety for all purposes), recursive PCR (Jaffe et al. (2000) J. Biol. Chem. 275:2619; and Williams et al. (2002) J. Biol. Chem. 277:7790; incorporated herein by reference in their entirety for all purposes) or any other nucleic acid amplification method using techniques well known to those of skill in the art. A variety of amplification methods are described in U.S. Patent Nos. 6,391,544, 6,365,375, 6,294,323, 6,261,797, 6,124,090 and 5,612,199, incorporated herein by reference in their entirety for all purposes. Embodiments of the present invention are directed to methods of amplifying nucleic acids in situ within the matrix by contacting the nucleic acids within the matrix with reagents, such as primers and nucleotides, and under suitable reaction conditions sufficient to amplify the nucleic acids. According to one aspect, the matrix is porous to allow migration of reagents into the matrix to contact the nucleic acids.

In accordance with certain examples, methods of sequencing a nucleic acid in situ within a matrix are provided. General sequencing methods known in the art, such as sequencing by extension with reversible terminators, fluorescent in situ sequencing (FISSEQ), pyrosequencing, massively parallel signature sequencing (MPSS) and the like (described in Shendure et al. (2004) Nat. Rev. 5:335, incorporated herein by reference in its entirety), are suitable for use with the matrix in which the nucleic acids are present. Reversible termination methods use step-wise sequencing-by-synthesis biochemistry that coupled with reversible termination and removable fluorescence (Shendure et al. supra and U.S. Patent Nos. 5,750,341 and 6,306,597, incorporated

herein by reference. FISSEQ is a method whereby DNA is extended by adding a single type of fluorescently-labelled nucleotide triphosphate to the reaction, washing away unincorporated nucleotide, detecting incorporation of the nucleotide by measuring fluorescence, and repeating the cycle. At each cycle, the fluorescence from previous cycles is bleached or digitally subtracted or the fluorophore is cleaved from the nucleotide and washed away. FISSEQ is described further in Mitra et al. (2003) Anal. Biochem. 320:55, incorporated herein by reference in its entirety for all purposes. Pyrosequencing is a method in which the pyrophosphate (PPi) released during each nucleotide incorporation event (i.e., when a nucleotide is added to a growing polynucleotide sequence). The PPi released in the DNA polymerase-catalyzed reaction is detected by ATP sulfurylase and luciferase in a coupled reaction which can be visibly detected. The added nucleotides are continuously degraded by a nucleotide-degrading enzyme. After the first added nucleotide has been degraded, the next nucleotide can be added. As this procedure is repeated, longer stretches of the template sequence are deduced. Pyrosequencing is described further in Ronaghi et al. (1998) Science 281:363, incorporated herein by reference in its entirety for all purposes. MPSS utilizes ligation-based DNA sequencing simultaneously on microbeads. A mixture of labelled adaptors comprising all possible overhangs is annealed to a target sequence of four nucleotides. The label is detected upon successful ligation of an adaptor. A restriction enzyme is then used to cleave the DNA template to expose the next four bases. MPSS is described further in Brenner et al. (2000) Nat. Biotech. 18:630, incorporated herein by reference in its entirety for all purposes.

According to certain aspects, the nucleic acids within the matrix can be interrogated using methods known to those of skill in the art including fluorescently labeled oligonucleotide/DNA/RNA hybridization, primer extension with labeled ddNTP, sequencing by

ligation and sequencing by synthesis. Ligated circular padlock probes described in Larsson, et al., (2004), Nat. Methods 1:227-232 can be used to detect multiple sequence targets in parallel, followed by either sequencing-by-ligation, -synthesis or –hybridization of the barcode sequences in the padlock probe to identify individual targets.

Fig. 1 depicts in schematic the process of creating a matrix of nucleic acids within cells in situ, followed by amplifying the nucleic acids, such as DNA or RNA, in situ, co-polymerizing the amplicons in situ, covalently attaching the amplicons to the matrix material, interrogating the amplicons and imaging the amplicons along with a reconstructed 3D cell image with DNA/RNA amplicons on the order of 10-7m. According to certain aspects, FISSEQ methods and materials useful in the practice of the methods described herein are provided in Lee et al., Nature Protocols, vol. 10, No. 3 (2015) pp. 442-458, Lee et al., Science 343, 1360-1363 (2014) and Supplementary Materials published 27 February 2014 on Science Express DOI: 10.1126/scienmce.1250212 each of which are hereby incorporated by reference in its entirety.

The automated sequencing and three-dimensional imaging device may be used for detecting optical signals distributed in three dimensions. The automated sequencing and three-dimensional imaging device may be used for detecting optical signals by fluorescence microscopy. According to certain aspects, the fluorescence is generated by fluorescent dyes or fluorophores, such as cyanine. According to certain aspects, the fluorescence is generated by quantum dots or other types of nanoscale semiconductors. According to certain aspects, the fluorescence is generated by fluorescent proteins, such as GFP. The automated sequencing and three-dimensional imaging device may be used for detecting optical signals of autofluorescence, chemiluminescence, or non-fluorescent optical signals such as light absorption properties of a sample (e.g., color) or

light scattering properties of a sample, e.g. Raman spectroscopy and CARS, coherent anti-Stokes Raman spectroscopy.

According to one aspect, an automated sequencing and three-dimensional imaging device is provided which uses volumetric three dimensional imaging modalities to image a three dimensional nucleic acid matrix. The optical configuration may be upright, inverted, side-view, dual-view, multi-view, or have any other orientation to enable measurement of light signals in three dimensions. The device includes hardware and software functionally assembled to enact a protocol of chemical manipulation (i.e., nucleic acid sequencing) and imaging of the three dimensional nucleic acid containing matrix which is contained within a suitable vessel or stage. The device may be referred to as a fluidic sequencing microscope to the extent that it includes hardware and software to automate sequencing and hardware and software for volumetric imaging.

The three-dimensional sequencing substrate may be contained within a sample holder such as an enclosed flow cell, or be fully or partially exposed in an open well format. An enclosed flowcell may be taken to mean any solid or semi-solid substrate with an optically clear region for imaging and a channel in which laminar flow may be established for the purposes of liquid exchange. The flowcell may have one more more inlets or outlets or ports for the purposes of liquid exchange. These physical interfaces may be statically or dynamically coupled to a fluidics system and may include wells or other reservoirs to store excess or reserve fluidics to facilitate dispensing or extraction regimes. The sample holder may be designed with additional physical features intended to interface or cooperate with fluidics systems, optical systems, or physical retention systems.

The device may include a stage for retaining the sample holder. The stage may be positioned using high-precision motion control systems, such as linear servo motors with optical encoders. Optical encoder systems may be furnished in either absolute or relative formats. In the case where the motor controller reads one or more relative encoders, homing routines may be implemented relative to limit sensors and/or physical limits to provide repeatable axis positioning. Software limits may be dynamically imposed to prevent samples or sample holders (i.e., flowcells) from colliding or interfering with other physical aspects of the device. Motor drivers responsible for the motor control may interface, over a standard communications protocol (e.g., RS-323, TCP/IP, UDP, etc), with other aspects of device software and hardware for the purposes of multi-axis, coordinated routines.

The stage may include physical mechanisms for positioning and retaining sample holders, such as slots with retention mechanisms (e.g., leaf springs or magnets) that reproducibly position sample holders / samples for interaction with fluidics and/or optical systems. The stage may include a physical means of coupling fluidic apparatuses to sample holders and/or samples. Stage components may be selected to minimize the tendency for thermal changes to introduce physical deformations or translations to the sample or sample holder. Stage components may be selected to minimize chemical reactivity with fluidics used during chemistry protocols.

According to certain aspects, the device may include a mechanism for sample holder tracking, such as by reference IDs, barcodes, RFID tags, or inclusion of other trackable labels in the sample holder. The trackable label may be automatically detected by the device, such as by detection of RFID or optical sensor for detecting a barcode, or the trackable label may be input into a computer software system by a user. One or more types of sample holders may vary in

physical organization of the samples, reagent input/output, imaging interface, and other aspects of the sample holder. The sample holder tracking may further comprise a mechanism for indicating to the device controller software the configuration of the sample holder, such as by referencing an index in a database of sample holder configurations. According to other aspects, the device may contain a mechanism for automatically configuring the sample holder configuration, such as by use of RADAR/LIDAR, including RADAR/LIDAR on-a-chip systems, or by machine vision, wherein physical aspects of the sample holder are automatically detected by the system, parsed, and used to configure the device interface with the sample holder. According to other aspects, the device may utilize reference diagrams or fixtures for the purpose of configuring the device interface with the sample holder, such as a fixture for determining a physical offset for a feature of the sample holder.

According to certain aspects, the device also comprises a software interface, such as a command line interface (CLI) or graphical user interface (GUI) for configuring a sample holder definition or otherwise configuring the interface between the device and the sample holder. The device may automatically scan the imaging region of a sample holder for the purpose of generating an overview scan of the image area within the sample holder. According to one aspect, the overview scan is presented to the user via a GUI for the purpose of selecting regions of the sample for sequencing and/or imaging. According to another aspect, the device contains software programs for automatically determining the appropriate regions of the sample for sequencing and/or imaging, such as by calculation of a metric such as fluorescence intensity or entropy. The device may further comprise a computer vision system or machine learning mechanism for feature recognition of the sample holder or components thereof.

The stage may include a heating or cooling apparatus where the stage may be heated or cooled (such as through thermoelectric cooling using Peltier elements), such as according to a programmed time and temperature, such as is useful with thermo-cycling for applications such as nucleic acid hybridization, amplification and sequencing. The heating or cooling apparatus or heating or cooling unit may be capable of rapid temperature cycling. The heating or cooling apparatus or unit may use a heat sink in conjunction with a fan to dissipate heat produced during temperature changes. The heating or cooling apparatus or unit may use a radiator and liquid cooling / circulating system to dissipate heat produced during temperature changes. The heating or cooling apparatus or unit may use temperature sensors or thermistors as a means of providing temperature feedback to a control system, which may be a microcontroller or other electronic circuit.

The device may include a fluidics dispenser or fluidics unit where programmed volumes of liquid reagents are dispensed to sample holders on the stage, such as to wells or flow cells on the stage, and to the three-dimensional nucleic acid containing matrix. The fluidics dispenser or unit may include temperature control (such as through thermoelectric cooling using Peltier elements) to store reagents at a consistent temperature, and may implement either liquid or water cooling as a means of heat dissipation. A pump, such as a syringe pump, may be used to deliver fluid reagents through the fluidics dispenser. According to certain aspects, the fluidics and pressure-based fluidic dispense may be driven by the pressure differential between the inside and outside of a liquid container. For example, a pressurized bottle may be connected to an electrically actuated valve, as by a piece of tubing, such that when the valve is opened, fluid is driven through the tubing and through the valve onto a sample in an open well or into a closed flowcell. In certain aspects, two or more reagents may be dispensed to a sample holder from the same

valve. These reagents may be selected upstream of the valve by means of another valve (e.g., a rotary valve) or other physical means. In other aspects, valves may be addressable by two selectable lines, such that one line may deliver water or another liquid meant to flush or otherwise clean the valve in between dispenses. A reservoir or absorbent material may be statically or dynamically positioned beneath the valve to collect rinse solution or reagent during priming and cleaning operations.

In certain implementations, the exchange of liquids in a closed flowcell may take place in an entirely closed-loop system, where tubing is connected to the flowcell in a fixed manner and a system of valves and pumps (or other pressure generating devices) controls the movement and selection of reagents delivered to the flowcell. Closed flowcells may be designed to allow for non-contact reagent dispensing into wells external to the sample channel. Such wells may be designed to hold a volume appropriate to partially or fully displace the volume of the sample-containing channel. The movement of dispensed reagent into the flowcell may then be driven by positive pressure exerted on the wells, or by negative pressure exerted on outlets at the opposite end of the sample channel. The interface between the suction-generating device and the flowcell may be transient or fixed, and may be created or adjusted by motion axes such as those driven by stepper motors, servo motors or solenoids. The interface may be further defined by the presence of suction cup devices designed to create and maintain a consistent seal while minimally perturbing the physical positioning of the sample.

In other aspects, sample holders with open well(s) may be designed with features to accommodate reagent dispensing and extraction to minimize sample perturbations, such as by including channels, slots, or other physical areas designed to sequester higher levels of liquid

flow from the sample. The open well(s) may additionally have chamfered or otherwise tapered sides to accommodate the specific shape of optical system interfaces, such as by matching the side profile of an optical objective.

According to a certain implementation of the pressure-driven fluidics block, multiple distinct tubes of reagent sit inside a pressurized container, each connected to its own valve, such that when any one or more valves are actuated fluid is driven from the tube through the valve onto the sample. The valves may be of one or more general types, including but not limited to solenoid valves, rotary valves and microfluidic valves. The valves may be select to maximize precision and/or minimize dead volume. According to certain aspects, the valve bodies may be designed in such a way as to place the actuating mechanism as close to the orifice as possible, to minimize problems associated with reagent drying and/or clogging. The valves may have upstream filters, screens, or other means of preventing non-liquid materials from clogging or otherwise interfering with the valves' operation. The amount of reagent dispensed using such a pressure-driven system is determined based on the valve internal diameter, liquid viscosity, pressure differential, and time of valve actuation, and can be determined empirically and used to configure a software controller such that a desired volume is converted into a length of time (given a particular hardware configuration with particular liquid viscosity, valve internal diameter, and pressurization) during which the valve is opened or actuated. According to certain aspects, the reagent containers may be individually or collectively pressurized. The pressurization medium is ideally an inert gas such as argon. The fluidic systems may combine pump-driven and pressure-driven fluidic components. The device may include a mixer for mixing programmed volumes of liquid reagents. The aspiration system described herein is used to remove fluid from a flow cell by applying a suction force to the liquid using a pump or

vacuum source. Pressure-based dispense may also be used to exchange fluids during sequencing by introducing a new reagent under pressure as above to displace the existing fluid through a channel. Pressure based dispense may be used alongside aspiration to exchange liquid in a flow cell.

According to one aspect, the device may contain a reservoir for containing bulk reagents, or reagents with larger volume. According to one such aspect, the bulk reagent reservoir may contain one or more pressurized containers within an enclosing container. According to another such aspect, the bulk reagent reservoir may contain one or more containers within a pressurized enclosing container. According to one aspect, the bulk reagent reservoir is temperature controlled, such as through thermoelectric cooling using Peltier elements, for the purpose of extending the life of and activity of one or more reagents. The heating or cooling apparatus or unit may use a heat sink in conjunction with a fan to dissipate heat produced during temperature changes. The heating or cooling apparatus or unit may use a radiator and liquid cooling / circulating system to dissipate heat produced during temperature changes. The heating or cooling apparatus or unit may use temperature sensors or thermistors as a means of providing temperature feedback to a control system, which may be a microcontroller or other electronic circuit. The bulk reagent reservoirs may be connectorized, such as by split septum, enabling facile exchange of individual reservoirs. Other connector types include, but are not limited to, Luer locks, ¼ 28, MINSTACK (LEE Co).

The device may include a mechanism of extracting liquid from a flow cell. According to one aspect, a vacuum is generated by a pump, such as a main pump or syringe pump, which is used to aspirate liquid contained in a flow cell, such as an open well or partially enclosed flow cell.

According to one aspect, a motorized sipper tube is used to contact the sample holder or liquid therein for the purpose of extracting liquid. Further according to this aspect, the motorized sipper tube may comprise a pneumatic, servo, or stepper motor, a positional encoder, and electromechanical controller system. The device may contain one or more waste liquid repositories, into which waste or extracted liquid is directed for the purpose of temporary storage. Waste liquid may be directed by means of pumps and valves, such as a rotary valve. The waste liquid repository may comprise a feedback system for automatically notifying a user upon reaching a certain level. The waste liquid may be directed to one or more repositories based on the nature of the reagent, to prevent undesired chemical reactions, or to facilitate downstream disposal according to applicable procedures, laws, or regulations.

The device may include a mechanism of covering one or more flow cells, wells, sample holders and/or stages for the purpose of preventing accumulation of airborne particulates in the sample and/or preventing evaporation of liquid reagents. According to one aspect, the device contains a motorized flowcell cover that uses one or more motion axes to position an airtight cover over one or more flow cells, wells, sample holders and/or stages. According to another aspect, the stage contains a dynamically extendable cover. According to another aspect, the device contains a static cover capable of interfacing with the motorized stage for the purpose of enclosing the wells and/or sample holders.

The device may include an optical assembly including one or more optical axes. The device may include one or more detectors, such as large area detectors, for volumetric imaging of the three dimensional nucleic acid containing matrix. The detectors may be cameras, and in particular, cameras with physical attributes tailored to high-speed, low-noise scientific imaging, such as an

sCMOS camera. In one such embodiment, a reflection based autofocus system provides closed loop control of the optical axis in order to attain and/or maintain sample focus. In another configuration, a microcontroller, FPGA, or other computing device may provide software focus and positioning feedback using one or more image analysis algorithms such as that described in Mario, A. Bueno, Josue Alvarez-Borrego, and L. Acho. "Autofocus algorithm using one-dimensional Fourier transform and Pearson correlation." *5th Iberoamerican Meeting on Optics and 8th Latin American Meeting on Optics, Lasers, and Their Applications*. International Society for Optics and Photonics, 2004 hereby incorporated by reference in its entirety. Such automated sample positioning may include coordination of one or more motion axes in conjunction with the imaging system. The sample positioning may account for physical shifts in sample position over the course of imaging and may be tolerant of shifts that are greater than the field of view captured in a single image frame. The device may implement a means of mapping a planar surface, rendering the need for autofocusing in real time unnecessary. Such a system may include using a reflection- or software-based autofocus system to sample three or more points at the sample surface and then fitting those points to the equation for a plane or the surface geometry of the solid substrate. The fitting process may involve excluding one or more points based on autofocus signal data, its residual as a result of regression analysis, or other factors indicating its fitness as a data point. The fitting process may include allowances for surface variance consistent with the sample mounting medium, such that the final surface map may include local deviations from a perfectly flat plane to reflect variations in the actual substrate surface. As described in Figure 12, according to one aspect the device comprises a system for mapping the surface of a sample holder with respect to one or more motion axes for the purpose of reproducible positioning of the sample relative to the imaging system, comprising the steps of using: 1) an

autofocus system, such as a laser-reflection autofocus mechanism, to acquire one or more distance measurements between the optical system and the sample, and 2) computing one or more offsets relative to the positional encoder(s) along the one or more motion axes, which are used during image acquisition.

The device may use image-based software programs for determining, adjusting, correcting, and/or tracking the position of the sample. According to one aspect, image data is computationally registered to a reference for the purpose of calculating a positional shift along one or more dimensions. Further according to this aspect, a Fourier transform (FT), such as the discrete Fourier transform (DFT) or fast Fourier transform (FFT) is used to compute a shift between two or more images or image volumes along one or more dimensions. According to one aspect, sub-pixel shifts are calculated, such as by using the upscaled DFT. According to one aspect, translational shifts are calculated along one or more dimensions. According to another aspect, rotational shifts are calculated along one or more dimensions. According to one aspect, features or fiducial markers contained within the sample holder, flowcell, well, or sample, are used for the purpose of aiding positional tracking using image analysis. Features or fiducial markers include features manufactured into or added onto the sample holder, flowcell, or well, such as engraved features, laser-engraved features, printed features, deposited features, microcontact printed features, beads, and other types of patterns in one or more dimensions. According to one aspect, fiducial markers are embedded into the sample, such as into the 3D hydrogel. According to one aspect, the fiducial markers are microscopy beads, which may be fluorescent or autofluorescent. According to one aspect, features are an aspect of one surface of the solid substrate forming part of the sample holder. According to a certain aspect, the features are an aspect of a different surface of the sample holder than the surface containing the sample.

In one example, the flowcell is understood to be a glass slide containing two or more open wells on the top surface, with beads serving as fiducial markers deposited on either the top or bottom surface of the glass slide.

A microcontroller system coordinates motion systems in XYZ axes along with the illumination/excitation light source and camera sensor such that the motion systems position the sample relative to the optical system, then image data is acquired at that position with global shutter capture or rolling shutter or "all lines firing" rolling shutter capture using synchronized illumination. In other cases, the motion along one or more axes are synchronized with the capture of lines along the camera sensor such that the sample does not need to come to a resting position relative to the optical system. Software and hardware handshaking protocols are implemented over low-latency communication protocols (e.g., digital TTL, I2C, RS-232, UDP, TCP/IP) to coordinate between the subsystems, e.g. initiating axis motion or triggering a camera exposure.

The device may include one or more objective lenses for imaging. In a certain aspect, the device may include one objective lens. In another aspect, the device may contain two objective lenses. In another aspect, the device may contain three or more objective lenses. The objective lenses may be water immersion lenses, oil immersion lenses, water dipping lenses, air lenses, lenses with a refractive index matching another imaging medium, or lenses with an adjustable refractive index. In a certain aspect the device contains a single water dipping objective lens, which provides for higher image quality by eliminating the refractive index mismatch occurring at the interface between two media with distinct refractive indexes, such as an air-water interface or water-glass interface.

In aspects comprising one or more objective lenses with refractive index not matched to air, the objective lens must interface with an imaging media, such as water, oil, or other imaging buffer. In these aspects, the device may comprise a mechanism of wetting the objective lenses or otherwise creating an interface between the objective lens and the imaging medium. Certain mechanisms of lens wetting include dipping the lens into an imaging medium or otherwise dispensing an imaging medium onto the lens, such as by a syringe, needle valve, or other mechanisms known to those familiar with the art of dispensing a liquid reagent. In a certain aspect, the device dispenses a certain amount of liquid into a well for the purpose of creating an incident angle between the objective lens and the liquid interface for deposition of liquid onto the objective lens without forming bubbles. In another aspect, the device dispenses a certain amount of liquid onto the objective, such as by using a syringe or needle valve, without forming bubbles, as by controlling the speed and angle of incidence between the liquid droplet and the objective lens.

During imaging in a liquid imaging medium, bubbles may form either on the objective lens, within the sample, or between the objective lens and the sample; including in devices imaging within an open flowcell and through a glass interface present in a closed flowcell. The device may contain a mechanism of detecting bubbles formed on the objective lens, or bubbles present between the objective lens and the sample. Mechanisms of bubble detection include detection via scattering of light; by image analysis, e.g., by measurement of the point spread function of the optical system, which is perturbed by bubbles; by external machine vision, such as by a camera or other imaging system observing the lens, connected to a computer system with software programmed to detect bubbles on the lens. The device further may contain a mechanism for eliminating bubbles formed on the objective lens. According to one aspect, the device comprises

a mechanism for contacting the objective lens with an aspirating needle, which removes any liquid present on the objective lens. According to another aspect, the device comprises a mechanism for contacting the objective lens with an absorbent material, which absorbs any liquid present on the objective lens. The device may contain a mechanism for drying or removing liquid from the objective lens. The device may execute a software and hardware routine for removing and replacing a liquid imaging medium from the lens upon detection of a bubble. The device may contain a mechanism for alerting a user upon detection of bubbles on the objective lens, within the sample, or between the objective lens and the sample.

During operation of the device, the objective lens may accumulate dirt, dust, deposited salts or other reagent solutes, or other types of materials which interfere with imaging. The device may contain a mechanism for detecting such interference, via scattering of light or other properties of the interaction between light and the interfering material; by image analysis, e.g., by measurement of the point spread function of the optical system, which is perturbed by the presence of interfering materials; by external machine vision, such as by a camera or other imaging system observing the lens, connected to a computer system with software programmed to detect interfering materials on the lens. The device may further contain a mechanism for cleaning the objective lens. According to one aspect, the device may include a lens cleaning reagent, which is dispensed onto the lens for the purpose of cleaning the lens, such as by a syringe or needle valve, or by dipping the objective lens into a cleaning reagent dispensed into a well or onto a non-abrasive material, which is made to contact the objective lens. The device may contain a mechanism for alerting a user upon detection of an interfering material on the objective lens, within the sample, or between the objective lens and the sample.

The device comprises one or more optical light paths. The device may contain optics for the purpose of correcting refractive index mismatches between certain components of the optical system and the sample or imaging medium. The device may contain optics for the purpose of correcting other types of optical distortion within the optical system, such as spherical or chromatic aberration. The device may contain a mechanism of detecting optical distortion, such as by using an image sensor combined with software to detect changes in a point-spread function of the optical system or other property of the optical system. According to one aspect, the device comprises one or more beam characterizing cameras. According to another aspect, prior to, during, or after operation of the device, the device may contain an automated or manual routine for measuring the point spread function or other optical property of the system and alerting a user or engaging a mechanical, electromechanical, or optical system for correcting the optical distortion. According to a certain aspect, the device contains an adaptive optical system (AO), which is used to improve the performance of optical systems by reducing the effect of wavefront distortions. Adaptive optics can correct deformations of an incoming wavefront by deforming a mirror in order to compensate for the distortion. The adaptive optics system may comprise a deformable mirror, image sensor, and hardware and software feedback systems. According to one aspect, the adaptive optic system contains a wavefront sensor, such as the Shack-Hartmann wavefront sensor. The adaptive optical system and other corrective optical systems may be open loop, where errors are measured before they have been corrected by the corrector. The adaptive optical system and other corrective optical systems may be closed loop, where the errors are measured after they have been corrected by the corrector. Adaptive optics may be used to improve the image quality within a 3D sample by correcting for optical aberrations within the sample.

The device may include one or more electromechanical, electronic, or fully computerized systems for the purposes of controlling and coordinating the timing of fluidic, optical, and motion-related events. Device subsystems such as motor controllers, temperature controllers, pneumatics controller, valve controllers, cameras, optical tuning or gating systems, sensors, and other electronic systems may leverage a variety of communication protocols for the purposes of such coordination. Communication protocols may be selected on the basis of latency, interoperability, electromechanical constraints or other application-focused considerations. Subsystems may conform to consistent or well-defined application program interfaces (APIs) such that they may be individually addressed and/or operated from generic computers or human machine interfaces.

Timing of optical systems such as cameras, confocal optical systems, illumination devices, AOTFs, mechanical shutters, etc, and single- or multi-axis motion control systems may be coordinated by microcontrollers, motor controllers, electronic circuits, and/or computerized systems. Optical sensor exposure timing may be optimized such that motion control movements overlap with non-measurement sensor events such as pixel readin / readout or background measurements. Optical illumination timing (e.g., laser illumination gated) may be implemented such that it is tied to specific optical sensor events (e.g., readin / readout) so as to minimize sample exposure to excitation light. Single- or multi-axis motion control for the purposes of optical imaging may be further optimized to account for the sensor exposure regimen (e.g., rolling shutter exposure) for continuous motion applications. Under certain specialized imaging regimes, e.g., time delay integration (TDI), it is possible to execute continuing axis motion during the acquisition of imaging data.

In certain aspects, the dimension order of multi-axis motion control (when coupled with optical imaging) may be selected so as to minimize frame-to-frame move times. For example, it is often the case the vertical axis (i.e., Z or optical axis) move times are the fastest, so it is desirable to perform three-dimensional imaging in a series of "Z stacks" in which frames are acquired while the vertical axis is driven up or down. These Z stacks are performed repeatedly across an X/Y plane or plane-like surface in order to acquire a fully three-dimensional volume of image data.

In some cases, it may be desirable to image a three-dimensional volume that is not cuboid in nature. In these cases, a three-dimensional coordinate system based on physical or engineering units may be employed to dictate arbitrary three-dimensional imaging positions that are then disseminated to the participating motion control and imaging systems. Employing such a system allows for imaging that is constrained exclusively to the region of a three-dimensional matrix in which sample voxels of interest exist.

It may be desirable to image the volume with a particular spatial sampling frequency. In certain aspects, the sampling frequency of imaging along one or more axes is determined relative to the Nyquist frequency of the optical system. In a certain implementation, image data acquired using a 40X 1.0 NA objective is sampled in the axial (Z) axis at approximately 500 nanometer intervals. In another aspect, the sampling frequency of imaging along one or more axes is oversampled, such as by acquiring image data or sampling the same area of the sample volume twice. Further according to this aspect, oversampling the volume may facilitate computational volumetric reconstruction, such as by providing redundant image data in neighboring image frames for the purpose of volumetric image stitching. In a certain implementation, image data

may be acquired with 10% overlaps along one or more axes, with 20% overlaps along one or more axes, or with 30% or more overlapping pixel data along one or more axes.

The device may include more than one stage, fluidics unit, and/or optical system such that each sample holder and/or sample may be addressed by multiple systems. Under such a regime access to certain hardware resources must be shared and therefore a physical resource allocation and scheduling system is necessary. Such a scheduling system may be passive or active, and may include predictive modeling as a means of anticipating and scheduling hardware resource access based on future fluidic and optical imaging needs on a per-sample basis. Further aspects of the scheduling system may allow for external events, such as those performed by a user or an external computer system to interrupt or otherwise modify the activities of the fluidic imaging system. In one such aspect, a physical media storage system may become full and no longer able to store acquired images. This event could trigger a cessation of imaging activities on the device pending external user intervention. In another aspect, a user may want to add a sample in a sample holder to a slot on a device stage. The user may be able to, through an HMI, graphical user interface, or using a physical button, temporarily pause or otherwise modify the operational state of the device to allow for the addition of the sample holder.

The device may be operated and otherwise programmed through a command line interface, graphical user interface, or application programming interface (API) exposed through local software libraries over a network connection. The user may be able to perform activities including but not limited to: physical configuration of the device, testing and calibration tasks, sample holder and sample definition generation and modification, fluidic manipulations and routine specification, optical measurement and routine specification, sequencing protocol

development, as well as other tasks related to general fluidics, optical imaging, and motion control. The device may include hardware or software that enables real time analysis of imaging data, such as FPGAs or user-space software running on GPUs that allows for activities such as three-dimensional image alignment, objective finding, signal processing or measurement, and other image analysis tasks as required by sequencing and optical sample analysis. The device may include hardware and software designed to store image data locally. The device may be part of a network of devices and services designed to collect, analyze, store, and visualize three-dimensional imaging and sequencing data. This network may include physically co-located hardware as well as cloud computing faculties. In certain embodiments it may implement open standards to interface with third party hardware and software.

It is to be understood that the device includes software to automate the procedures of amplification and sequencing as described herein. It is to be further understood that the hardware components described herein are commercially available in whole or in part. According to one aspect, a commercially available sequencing apparatus called the "I15 Pollinator" manufactured by Danaher Corporation may be used for the sequencing aspects described herein and may be modified to include the hardware for volumetric imaging described herein.

This invention is further illustrated by the following examples, which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference in their entirety for all purposes.

EXAMPLE I

Operative Components of an Automated Sequencing and Three-Dimensional Imaging Device

Fig. 2 is a perspective view in schematic of operative components of an automated sequencing and three-dimensional imaging device of the present disclosure. It is to be understood that further components may be added to the device including housing elements. As shown in Fig. 2, the automated sequencing and three-dimensional imaging device includes a laser confocal head enclosure including a pinhole array and camera detectors for receiving light from the sample. Such a hardware arrangement allows for volumetric imaging of a three dimensional nucleic acid containing matrix. The device further includes an optical path with reflection-based autofocus to the laser confocal head enclosure. The device further includes a temperature controlled fluidics head on a pressurized fluidics block which serves to provide one or more reagents to the sample. Operatively connected bulk fluidics valves and an aspiration tube for removing liquid from the sample are provided. A sample stage with temperature control and XY motion system is provided and is shown with a glass slide sample holder. A track for stage motion with linear encoders is operatively connected to the stage. An enclosure door for an oxygen-free (argon) environment is provided with the rest of enclosure not shown. The device rests on an anti-vibration foundation.

Fig. 3 is a front view in schematic of operative components of an automated sequencing and three-dimensional imaging device of the present disclosure. The device incudes an optical path including laser reflection-based autofocus and a laser confocal head. A bulk reagent valve array for bulk reagent dispense is shown (pressurized bulk reagent storage not shown). A Peltier element for reagent block temperature control and heat sink (shown) or liquid cooling is shown connected to a fluidics block for pressure-based fluidics dispense. A dipping aspiration tube for removing liquid from the flow cell is shown. Electronics are provided for valve actuation and fluidics block temperature and pressure control and feedback. A valve array is shown connected

to the fluidics block. A stage for holding samples, with temperature control by Peltier element and heat sink (shown) or liquid cooling is provided. Electronics for stage motion and temperature control and feedback are provided. Track for stage motion with linear encoders for positional feedback is provided. An optical axis with microscope objective is shown. A dipping objective is shown for open flowcell. An enclosure door for oxygen-free (argon) environment is provided (rest of enclosure not shown). The device includes a vibration control rigid foundation. As can be seen in Fig. 3, the device for volumetric imaging of a 3D sequencing matrix utilizes two independent stages and two independent fluidics systems along with a single optical axis for image acquisition. Image acquisition is in practice the temporally limiting step. No matter how long the biochemistry and fluidics for sequencing take per base, this is a fixed time per base (e.g. 2 hours), but the imaging time is proportional to the size of the volume being imaged. By using two stages, each operating independently and with independent fluidics, one stage can be doing biochemistry/fluidics while the other stage is imaging. Therefore the system is always effectively waiting to image and the fixed biochemistry time is effectively zero as the system is never waiting on fluidics to complete in order to proceed to the next step, but is always waiting on imaging to complete. Scan time may be an issue for 3D optical scanning vs. 2D optical scanning, as 2D scan time increases to the second power with scanning area, but in 3D scan time increases to the third power with scanning volume. The configuration of two independent stages and two independent fluidics systems along with a single optical axis for image acquisition is also cost effective, as the stage with motion/temperature control stage and fluidics subsystems are relatively cheap compared to the optical subsystem, which requires costly optics, camera detectors, data acquisition electronics (e.g. frame grabber hardware to capture image data from the cameras), and laser sources for fluorescent excitation.

Fig. 4 is a perspective view of the device of the present disclosure. Laser scanning confocal head and camera detectors are provided. An optical path light bend into the confocal head is provided. A microscope objective is shown, e.g. a dipping immersion objective for perfectly matched refractive index with the sample for high fidelity imaging. A mirror for capturing feedback light signal of reflection-based autofocus system is provided. An open flow cell is shown inserted into the stage. A Z-axis linear motor with linear encoder feedback for Z motion and Z scanning of objective focal plane through a sample is provided. Located behind the Z-axis linear motor is the reflection-based autofocus system (not visible). A pressurized and temperature controlled fluidic block is shown. A valve for fluidic dispense is provided. Tubes holding reagents for dispense are shown inside the pressurized fluidics block.

Fig. 5 is a perspective view of a stage according to the present disclosure. A heat sink for the Peltier cooler for stage temperature control is provided. A flow cell foundation is shown as a standard 1x3 inch 1mm thick glass microscopy slide onto which the 3D sequencing library matrix is attached. The 3D sequencing library matrix attached inside the well. A fluidic well component of the open-format flowcell is shown as a 2-well format. An open-format flowcell avoids problems with bubbles and laminar flow over irregular 3D matrix that would cause uneven diffusion of reagents into and out of the 3D sequencing library matrix. A spring-loaded registration point for reproducible slide positioning is provided. The flow cell contacts a thermo-cycling stage top. A rail system for reproducible slide positioning is provided that provides 2D directional force onto the slide (downward and along one of the long edges of the slide) and secures the sample. A registration point for reproducible slide positioning is provided. In one implementation, the flow cell consists of a series of open-chambers or wells. Reagents are dispensed into the wells from the top by the fluidic system and removed by an aspiration tube.

Open well format is preferable for 3D matrix samples as formats enclosed on top and bottom may suffer from issues related to fluid dynamics such as uneven laminar flow over an irregularly-shaped 3D matrix, and introduction or trapping of bubbles by vortexes created in the dynamic liquid field. Open well format is amenable to upright (from the top) microscopy modalities by one or more optical axes where the objective physically dips into an imaging medium such as an imaging buffer, minimizing or eliminating refractive index mismatches between the optical system and the sample (e.g. air/glass interface).

Fig. 6 is a schematic timing diagram illustrating an example of coordination between optical imaging, illumination, motion control, and acquisition systems for the purposes of achieving optimal imaging frame rates.

Fig. 7 is a schematic block diagram illustrating an example TTL communication structure for the purposes of optimal real time coordination of optical imaging, illumination, motion control, and acquisition systems.

Fig. 8 depicts the timing and resource usage timing diagram for sequencing device fluidics, imaging, and dual stage subsystems. This figure depicts a device with two independent motion stages, which house the sample holders, two intendent fluidic systems, denoted "Left" and "Right", and a single imaging axis. A controller manages the motion stages such that at Time=0, both stages are engaged in fluidic routines. At Time=1, image data is acquired from the samples on Stage 1 while Stage 2 is engaged in fluidic routines. At Time=2, image data acquisition from Stage 1 has completed, and fluidic routines for Stage 2 have completed. At Time=3, image data is acquired from the samples on Stage 2, while Stage 1 is engaged in fluidic routines. At

Time=4, image data acquisition from Stage 2 has completed, and fluidic routines for Stage 1 have completed.

Fig. 9 depicts a schematic block diagram illustrating the controller organization for the dual stages, fluidic, and imaging subsystems. A controller synchronizes event timing, such as that depicted in Figure 8.

Fig. 10 depicts device systems for managing the state of and interfacing with an objective lens. Subpanel 100 is directed to depiction of a dry objective (105) and a wet objective (110) comprising the objective lens with a droplet of imaging medium (115). Imaging medium include aqueous imaging buffers, such as water and anti-fade reagents, as well as oil and other types of imaging liquids. Subpanel 200 depicts a configuration of an apparatus for objective wetting and drying, such as an apparatus for removing a bubble from an objective lens or for cleaning an objective lens; a carrier is depicted (220), which may be a fixture of the device or a consumable insert, which further comprises a modality (230) for depositing a reagent (235) onto the objective lens, such as a well or reagent dispensing apparatus, and also further comprises a modality (240) for removing liquid from an objective lens, such as a non-abrasive absorbent material or aspirating port. Subpanel 300 depicts a routine for depositing liquid onto an objective lens, comprising the steps of forming a liquid interface on the liquid wetting modality (310), contacting the objective lens with the liquid interface (320), as by translating the objective lens or objective wetting modality, such that the objective is wetted (330). Subpanel 400 depicts a routine for removing liquid from an objective lens (410), comprising the steps of translating the objective lens or drying modality such that the drying modality contacts the liquid forming an

interface (420) such that the liquid is absorbed or otherwise aspirated by the drying modality, resulting in an objective lens without a liquid droplet (430).

Fig. 11 depicts a flow chart for repeatable XYZ positioning of the sample relative to the imaging axis over time. In the case where the solid substrate may shift over time, this system for determining shifts of a sample holder can increase the accuracy of XYZ positional repeatability over time between the sample and the imaging system. At the initialization of imaging, the device moves Position 1 under the imaging axis and acquires initial image data, which is transmitted to a computer system. The Computer system computes a physical offset relative to a reference image dataset, which is communicated to the Sequencing Device and stored. As depicted in Loop 510, the sequencing device may repeat these steps until the physical offset received by the computer system is lower than a threshold amount, e.g., until the computer system confirms the physical positional information stored by the device corresponding to Position 1 is within a given physical shift relative to a reference positional state.

Fig. 12 depicts a system for mapping a surface of a sample holder comprising a solid substrate. In the case where the solid substrate may shift over time and a particular autofocus system may have a certain failure rate, this system for mapping the surface of a sample holder can increase the accuracy of Z positional repeatability over time between the sample and the imaging system, such as by discarding outliers in the Z offsets as determined by the autofocus system. This system may also increase image acquisition speed, as pre-computing physical offsets for the motion system may be significantly faster than allowing the autofocus to determine the physical offset in real time at each potential imaging position. Panel 600, which includes Subpanels 610, 650, and 700, depicts a diagram of the device state. In Subpanel 610, an objective lens (620) is

positioned relative to a solid substrate (630), such as glass, silicon, metal, plastic, or another solid material, which is understood to immobilize a mounted sample such as a FISSEQ hydrogel. This depiction includes a laser-based autofocus device, which shines a laser (640) onto the solid substrate in order to determine the distance between the objective and the solid substrate, such as by analyzing the reflection of the laser light from the surface. Subpanel 650 depicts a side view of subpanel 610, wherein the distance 680 between the objective and the solid substrate corresponds to a known value, such as the focal length of the objective, when the objective lens is determined to be focused on the solid surface. Subpanel 700 depicts scanning the objective (710) in one, two, or three axes (715 depicts three possible motion axes) over the solid substrate (720), such that an Z autofocus offset is measured at each scan position (730). Subpanel 900 depicts a flow chart for the behavior of the device and a computer system during surface scanning, wherein the objective lens is positioned at position k, and a Z offset is determined and stored, as in an array. Upon sampling a number of positions, the Z offsets are communicated to a computer system, which computes physical offsets, such as by fitting the autofocus offsets to a plane. The physical offsets are communicated back to the sequencing device, which proceeds to acquire 3D imaging data with accurate Z physical offsets. Subpanel 800 depicts a diagram of autofocus Z offsets being fit to a plane for the purpose of determining accurate physical Z offsets.

The practice of the methods disclosed herein may employ conventional biology methods, software, computers and computer systems. Accordingly, the methods described herein may be computer implemented methods in whole or in part. Computer software utilized in the methods of the present disclosure include computer readable medium having computer-executable instructions for performing logic steps of the method of the invention. Suitable computer

readable medium include, but are not limited to, a floppy disk, CD-ROM/DVD/DVD-ROM, hard-disk drive, flash memory, ROM/RAM, magnetic tapes, and others that may be developed. The computer executable instructions may be written in a suitable computer language or combination of several computer languages. The methods described herein may also make use of various commercially available computers and computer program products and software for a variety of purposes including obtaining and processing light intensity into data values, automation of reagent delivery, automation of reaction conditions such as thermo-cycling, automation of movement of a stage including a sample holder and a sample and processing and storage of light intensity data and other methods and aspects described herein.

Aspects of the present disclosure are directed to a method of analyzing a plurality of nucleic acids within a three dimensional polymerized matrix including amplifying the plurality of nucleic acids to produce amplicons within the matrix, covalently bonding the amplicons to the matrix, sequencing the plurality of amplicons using an optical sequencing method where the plurality of amplicons are labeled with a detectable label, and volumetrically imaging the plurality of amplicons to produce three dimensional imaging data of the plurality of amplicons wherein light intensity data is processed into a three-dimensional volumetric image. According to one aspect, the plurality of nucleic acids is contained within a biological sample and the matrix-forming material is introduced into the biological sample. According to one aspect, the plurality of nucleic acids is contained within a cell and the matrix-forming material is introduced into the cell. According to one aspect, the plurality of nucleic acids is contained within a tissue sample and the matrix-forming material is introduced into the tissue sample. According to one aspect, the three dimensional imaging data identifies the relative position of the plurality of amplicons within the cell. According to one aspect, the plurality of amplicons is sequenced

using fluorescence in situ sequencing. According to one aspect, the plurality of nucleic acids are volumetrically imaged using one or more of 3D structured illumination, selective planar illumination microscopy, light sheet microscopy, emission manipulation, volumetric imaging using pinhole confocal microscopy, volumetric imaging using aperture correlation confocal microscopy, volumetric imaging using volumetric reconstruction from slices, volumetric imaging using deconvolution microscopy, volumetric imaging using aberration-corrected multifocus microscopy, volumetric imaging using digital holographic microscopy.

Aspects of the present disclosure are directed to an automated sequencing and volumetric imaging device including a multi axis stage or positioning system including a sample holder for a three dimensional nucleic acid containing matrix, a heating or cooling apparatus operationally connected to the stage, whereby the heating or cooling apparatus is programmable for time and temperature useful with thermo-cycling for amplification and sequencing, a fluidics dispenser positioned to dispense one or more reagents into the sample holder wherein the fluidics dispenser is in fluid communication with one or more reservoirs for containing one or more reagents, whereby the fluidics dispenser is programmable for dispensing programmed volumes of liquid reagents to the sample holder, a pump operationally connected to the fluidics dispenser whereby the pump forces or withdraws one or more regents from the one or more reservoirs through the fluidics dispenser, an optical assembly including one or more optical axis, one or more detectors positioned in light receiving communication with the sample holder, whereby the one or more detectors receives light intensity signals which processed into a three-dimensional volumetric image of the nucleic acid sample, and one or more microprocessors with software for automating and controlling introduction of reagents into the sample holder, thermocycling of the sample holder, and image detection and acquisition.

REFERNCES

Each reference is incorporated herein by reference in its entirety for all purposes.

Drmanac, R., Sparks, A.B., Callow, M.J., Halpern, A.L., Burns, N.L., Kermani, B.G., Carnevali, P., Nazarenko, I., Nilsen, G.B., Yeung, G., et al. (2010). Human genome sequencing using unchained base reads on self-assembling DNA nanoarrays. Science 327, 78-81.

Islam, S., Kjallquist, U., Moliner, A., Zajac, P., Fan, J.B., Lonnerberg, P., and Linnarsson, S. (2011). Characterization of the single-cell transcriptional landscape by highly multiplex RNA-seq. Genome Res 21, 1160-1167.

Larsson, C., Grundberg, I., Söderberg, O., and Nilsson, M. (2010). In situ detection and genotyping of individual mRNA molecules. Nature methods 7, 395-397.

Larsson, C., Koch, J., Nygren, A., Janssen, G., Raap, A.K., Landegren, U., and Nilsson, M. (2004). In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes. Nature methods 1, 227-232.

Shendure, J., Porreca, G.J., Reppas, N.B., Lin, X., McCutcheon, J.P., Rosenbaum, A.M., Wang, M.D., Zhang, K., Mitra, R.D., and Church, G.M. (2005). Accurate multiplex polony sequencing of an evolved bacterial genome. Science 309, 1728-1732.

What is claimed is:

1. A method of analyzing a plurality of nucleic acids within a three dimensional polymerized matrix comprising

amplifying the plurality of nucleic acids to produce amplicons within the matrix,

covalently bonding the amplicons to the matrix,

sequencing the plurality of amplicons using an optical sequencing method where the plurality of amplicons are labeled with a detectable label, and

volumetrically imaging the plurality of amplicons to produce three dimensional imaging data of the plurality of amplicons wherein light intensity data is processed into a three-dimensional volumetric image.

2. The method of claim 1 wherein the plurality of nucleic acids is contained within a biological sample and the matrix-forming material is introduced into the biological sample.

3. The method of claim 1 wherein the plurality of nucleic acids is contained within a cell and the matrix-forming material is introduced into the cell.

4. The method of claim 1 wherein the plurality of nucleic acids is contained within a tissue sample and the matrix-forming material is introduced into the tissue sample.

5. The method of claim 1 wherein the three dimensional imaging data identifies the relative position of the plurality of amplicons within the cell.

6. The method of claim 1 wherein the plurality of amplicons are sequenced using fluorescence in situ sequencing.

7. The method of claim 1 wherein the plurality of nucleic acids are volumetrically imaged using one or more of 3D structured illumination, selective planar illumination microscopy, light sheet microscopy, emission manipulation, volumetric imaging using pinhole confocal microscopy, volumetric imaging using aperture correlation confocal microscopy, volumetric imaging using volumetric reconstruction from slices, volumetric imaging using deconvolution microscopy, volumetric imaging using aberration-corrected multifocus microscopy, volumetric imaging using digital holographic microscopy.

8. An automated sequencing and volumetric imaging device comprising

a multi axis stage or positioning system including a sample holder for a three dimensional nucleic acid containing matrix,

a heating or cooling apparatus operationally connected to the stage, whereby the heating or cooling apparatus is programmable for time and temperature useful with thermo-cycling for amplification and sequencing,

a fluidics dispenser positioned to dispense one or more reagents into the sample holder wherein the fluidics dispenser is in fluid communication with one or more reservoirs for containing one or more reagents, whereby the fluidics dispenser is programmable for dispensing programmed volumes of liquid reagents to the sample holder,

a pump operationally connected to the fluidics dispenser whereby the pump forces or withdraws one or more regents from the one or more reservoirs through the fluidics dispenser,

an optical assembly including one or more optical axis,

one or more detectors positioned in light receiving communication with the sample holder, whereby the one or more detectors receives light intensity signals which are processed into a three-dimensional volumetric image of the nucleic acid sample,

one or more microprocessors with software for automating and controlling introduction of reagents into the sample holder, thermocycling of the sample holder, and image detection and acquisition.

ABSTRACT

Methods of volumetric imaging of a three-dimensional matrix of nucleic acids within a cell is provided. An automated apparatus for sequencing and volumetric imaging of a three-dimensional matrix of nucleic acids is provided.

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Evan R. Daugharthy	Nonprovisional Application Number (if known):	Not Yet Assigned
Title of Invention:	Method and Apparatus for Volumetric Imaging		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.
2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.
3. The applicable box is checked below:

I. [x] **Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)**

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. [ ] **Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)**

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature	/John P. Iwanicki/	Date	August 5, 2021
Name (Print/Typed)	John P. Iwanicki	Practitioner Registration Number	34,628

***Note**: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

[ ] *Total of 1 forms are submitted.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
17/395,534	08/06/2021	1636	1820	010498.01437\US	8	2

CONFIRMATION NO. 5147

FILING RECEIPT

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051



Date Mailed: 08/20/2021

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

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Inventor(s)

Evan R. Daugharthy, Cambridge, MA;
Richard C. Terry, Carlisle, MA;
Je-Hyuk Lee, Allston, MA;
George M. Church, Brookline, MA;
Benjamin W. Pruitt, Cambridge, MA;

Applicant(s)

President and Fellows of Harvard College, Cambridge, MA;

Power of Attorney: The patent practitioners associated with Customer Number 22910

Domestic Priority data as claimed by applicant

This application is a CON of 16/386,337 04/17/2019
which is a CON of 15/969,118 05/02/2018 PAT 10266888
which is a CON of PCT/US16/60279 11/03/2016
which claims benefit of 62/250,182 11/03/2015

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

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If Required, Foreign Filing License Granted: 08/18/2021
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/395,534**
Projected Publication Date: 11/25/2021
Non-Publication Request: No
Early Publication Request: No
Title

Method and Apparatus for Volumetric Imaging

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 17/395,534

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	700
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	8 minus 20=	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*				x 480 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1820

APPLICATION AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
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If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	1	Tillberg et al., "Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies," Nat Biotechnol., Vol. 34, No. 9, pp. 987-992 (2016).	

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-09-20
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	43816613
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	21-SEP-2021
Filing Date:	06-AUG-2021
Time Stamp:	09:32:08
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	Tillberg_et_al_21446027.PDF	1850984 44405a3a31af985325b7a3daaf54006a5846b61c	no	21
Warnings:					

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21479760.pdf	1034253 bdbe5192198 4f8953305a27f3908999300b5accf	no	4

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	H04-268359	JP	A	1992-09-24	Taiho Pharmaceutical Co Ltd		X

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

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	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

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If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

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☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-10-04
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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Electronic Acknowledgement Receipt

EFS ID:	43940253
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	04-OCT-2021
Filing Date:	06-AUG-2021
Time Stamp:	15:17:04
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	JPH04268359A_21505335.pdf	536983 1893cc81a83941a5d89032ad30a4cfa878373e7c	no	7
Warnings:					

Information:					
2	Foreign Reference	Translation_of_JPH04268359A_21505346.pdf	298987 612f9d7ace01fda26e716492fcb524dc0204727e	no	8
Warnings:					
Information:					
3	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21505673.pdf	1034159 caabeaeb613855d7171955f8b3abd7b42443fa70	no	4
Warnings:					
Information:					
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New Applications Under 35 U.S.C. 111

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National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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Espacenet

Bibliographic data: JPH04268359 (A) — 1992-09-24

STIMULUS-RESPONSIVE HYDROGEL AND PRODUCTION THEREOF

Inventor(s): YONESE MASAKATSU; KISHIMOTO HIROSHI; BABA KAZUHIKO ± (YONESE MASAKATSU, ; KISHIMOTO HIROSHI, ; BABA KAZUHIKO)

Applicant(s): TAIHO PHARMACEUTICAL CO LTD ± (TAIHO YAKUHIN KOGYO KK)

Classification: **- international:** ***C08F6/00; C08J3/00; C08L29/02; C08L29/04;*** (IPC1-7): C08F6/00; C08J3/00; C08L29/04

- cooperative:

Application number: JP19910050804 19910222

Priority number(s): JP19910050804 19910222

Abstract of JPH04268359 (A)

PURPOSE:To produce a functional hydrogel which is based on polyvinyl alcohol and practically applicable to the medical and pharmaceutical fields.
CONSTITUTION:An aq. soln. of polyvinyl alcohol having a degree of saponification of 97mol% or higher and an average degree of polymn. of 1000 or higher is freeze-dried to produce a high-strength transparent hydrogel, which is dipped in an aq. alkaline soln. of boric acid or a borate in a concn. of 0.001 mol/l or higher with a pH of 7 or higher for 30min or longer to give the title gel.

(19)日本国特許庁（ＪＰ）　(12) **公開特許公報**（Ａ）　(11)特許出願公開番号

特開平4－268359

(43)公開日　平成４年(1992)９月24日

(51)Int.Cl.[5]	識別記号	庁内整理番号	ＦＩ	技術表示箇所
Ｃ０８Ｌ　29/04	ＬＧＷ	6904－４Ｊ		
Ｃ０８Ｆ　6/00	ＭＦＵ	8016－４Ｊ		
Ｃ０８Ｊ　3/00		7918－４Ｆ		
// Ｃ０８Ｌ　29:04		6904－４Ｊ		

審査請求　未請求　請求項の数８(全　６　頁)

(21)出願番号　特願平3－50804

(22)出願日　平成３年(1991)２月22日

(71)出願人　000207827
大鵬薬品工業株式会社
東京都千代田区神田錦町１－27

(72)発明者　米勢　政勝
愛知県名古屋市名東区平和ケ丘１－135
光ケ丘ハイツＣ－102

(72)発明者　岸本　博
愛知県名古屋市緑区篠の風２－208

(72)発明者　馬場　一彦
徳島県徳島市住吉４－３－26　第一福岡ハイツ201

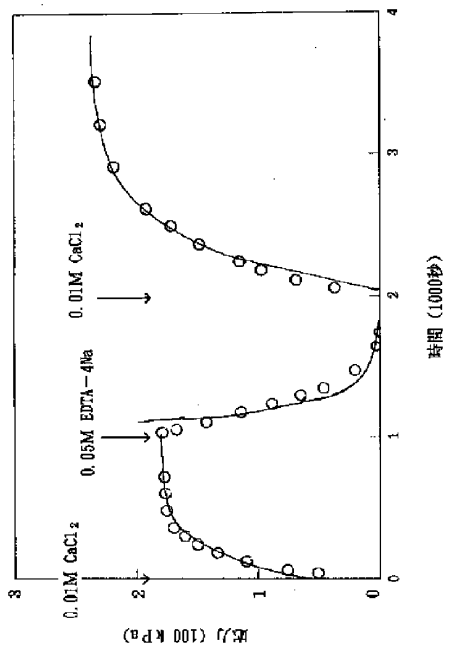
(74)代理人　弁理士　田村　巌

(54)【発明の名称】　刺激応答性ヒドロゲル及びその製造方法

(57)【要約】　(修正有)

【目的】　ポリビニルアルコールを用いた医療、医薬分野へ実用可能な機能性のヒドロゲル及びその製造方法を提供することにある。

【構成】　けん化度97モル％以上、平均重合度1000以上のポリビニルアルコール水溶液を用いて凍結乾燥法により高強度で透明なヒドロゲルを得、このゲルを0.001モル／１以上の濃度のホウ酸あるいはホウ酸塩のｐＨ７以上のアルカリ性水溶液中に30分以上浸漬して得られる刺激応答性ヒドロゲル及びその製造方法。



1

【特許請求の範囲】

【請求項１】　ポリビニルアルコールより作製した刺激応答性ヒドロゲル。

【請求項２】　単糖類及び二糖類の濃度変化に対して応答する請求項１の刺激応答性ヒドロゲル。

【請求項３】　二価金属イオンの濃度変化に対して応答する請求項１の刺激応答性ヒドロゲル。

【請求項４】　ｐＨ変化に対して応答する請求項１の刺激応答性ヒドロゲル。

【請求項５】　温度変化に対して応答する請求項１の刺激応答性ヒドロゲル。

【請求項６】　電場の印加に対して応答する請求項１の刺激応答性ヒドロゲル。

【請求項７】　けん化度97モル％以上、平均重合度1000以上のポリビニルアルコール水溶液を用いて凍結乾燥法により高強度で透明なヒドロゲルを得、このゲルを0.001モル／１以上の濃度のホウ酸あるいはホウ酸塩のｐＨ７以上のアルカリ性水溶液中に30分以上浸漬することを特徴とする刺激応答性ヒドロゲルの製造方法。

【請求項８】　凍結乾燥法が、ポリビニルアルコールの水溶液を、任意の形状の容器または成型用鋳型へ注入後、これを非凍結下で乾燥させ、しかる後、この乾燥体を一定圧の水蒸気中に放置して一定量の水分を吸着させた後、速やかに－20℃以下で凍結し、凍結状態のまま乾燥して得られた試料を必要に応じ水中に浸漬することによりヒドロゲルを得る方法である請求項７の刺激応答性ヒドロゲルの製造方法。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明はポリビニルアルコールを用いて、グルコース等の単糖類及び二糖類又は二価金属イオンの濃度変化、ｐＨ、温度及び電場に応答して大きな体積変化及び強度変化を起こす全く新しい高強度な刺激応答性ヒドロゲル及びその製造方法に関する。

【０００２】

【従来の技術】ポリビニルアルコールゲルは現在最も多く研究されているヒドロゲルの１つであり、優れた力学的特性、保水性を有し、しかも安価で毒性も極めて低いことから医療、医薬分野においても種々の応用が試みられている。しかし、その試みは大きく分けて２つのカテゴリーに限定され、１つはゲル中に薬物、酵素及び微生物等を包埋した物質担体及び支持体としての利用であり、もう１つは人工関節、人工角膜等の生体組織の代替としての利用である。しかし、これらは単にポリビニルアルコールゲルの持つ物質保持能、高含水性及び弾力性を利用しているに過ぎなかつた。

【０００３】又、近年、荷電基を有する高分子（高分子電解質）を用いて、外部のｐＨ、温度及び溶媒組成等の変化に応答して体積変化（膨潤収縮）するゲルが調製されており、これらは機能性ゲルと総称されている。機能

2

性ゲルはゲルの応用分野に全く新しい道を開いたが、その殆どは医療面への使用実績がなく毒性も未確認の高分子電解質を用いたものであり、医療、医薬分野への応用には程遠い段階である。更に糖の濃度変化に応答して膨潤収縮するヒドロゲルは、従来の高分子電解質を用いたゲルにおいても報告例はない。

【０００４】

【発明が解決しようとする課題】本発明の目的は、ポリビニルアルコールを用いた医療、医薬分野へ実用可能な機能性ヒドロゲル及びその製造方法を提供することにある。

【０００５】

【課題を解決するための手段】本発明はポリビニルアルコールより作製した刺激応答性ヒドロゲルに係る。

【０００６】本発明者らは、上記問題点を解決するため鋭意研究した結果、独自の製法により作製した高強度で透明なポリビニルアルコールヒドロゲルをホウ酸及びホウ酸塩で処理することにより、グルコース等の単糖類及び二糖類又は二価金属イオンの濃度変化、ｐＨ、温度及び電場等の種々の変化に応答して大きな膨潤収縮を示す新しい機能性ヒドロゲルを見出し本発明を完成した。本発明の刺激応答性ヒドロゲルは以下の様な機能を有し、医療、医薬の分野で非常に有用である。

【０００７】（１）ゲルをホウ酸塩の水溶液中に入れ、溶液中のグルコース濃度を増加減少させる。この時、グルコース濃度を増大させるとゲルは収縮し、濃度を低下させるとゲルは膨潤する。この収縮膨潤の変化量はグルコースの濃度に比例して増加することから、本ヒドロゲルは溶液中のグルコース濃度を決定できるグルコースセンサーとして利用できる。感度は良好であり、血糖値等の低濃度のグルコース測定にも応用可能である。現在一般的に使用されているグルコースセンサーは酵素を用いた複雑かつ高価なものであり、酵素の失活により使用不能となる。本製法で得られるヒドロゲルをグルコースセンサーとして使用すれば、安価でかつ簡便なグルコース濃度の決定が可能であり、また長期間変質が起こらないので繰り返し使用できる。

【０００８】一方、溶液中にカルシウムイオン等の二価金属イオンが一定濃度以上存在する時は、グルコース濃度の増大に伴いゲルは膨潤し、濃度の低下に伴いゲルは収縮する。この性質を利用して新しい糖尿病治療システムが設計可能である。例えば、ゲル中にインシュリンを封入しておき、ゲルを生体内に投与するかあるいは体外に血液を循環させてゲルを接触させる。血糖値が上がるとゲルが膨潤してゲルの網目構造が広がり、その結果封入されていたインシュリンが放出され、血糖値の増加を抑制するというシステムが考えられる。グルコース応答性薬物徐放システムは数多い糖尿病患者からも待ち望まれているものである。

【０００９】（２）ゲルをカルシウム、バリウム等の二

価金属イオンの水溶液に入れると急速に収縮し、体積が初めの30％程度にまで減少する。収縮したゲルにエチレンジアミン四酢酸四ナトリウム等のイオンキレート化剤を添加するとゲルは速やかに膨潤し、完全にもとの体積となる。次に二価金属イオンを加えると再び収縮が起こり、完全に可逆的な体積変化（膨潤収縮）を起こすことが可能である。又、ゲルの両端を固定しておくと収縮の際に非常に強い張力を発生する。すなわち化学的な刺激を物理的な仕事に変換する機能を持つたヒドロゲルであると言える。この機能を利用して、例えば人工筋肉等への応用が可能である。

【００１０】（３）ゲルのｐＨを６～12の間で変化させると、ゲルは酸性側に行くほど収縮し、アルカリ性側に行くほど膨潤する。この体積変化は可逆的であり、変化量は30％以上に及ぶ。これを利用してｐＨ感受性の薬物放出システムに応用可能である。

【００１１】（４）ゲルの温度を増加させると収縮が起こり、温度を元に戻すと膨潤する。これは可逆的な変化であり、この性質を利用して温度感受性の薬物放出システム、例えば体温の変化に対応して薬物の供給を制御するシステムに応用可能である。

【００１２】（５）ゲルを浸漬した水溶液に電場をかけることにより、ゲルに膨潤収縮や屈曲運動をさせることができ、電気刺激による薬物放出システムや人工筋肉等への応用が可能である。

【００１３】以上述べたゲルの体積変化や張力変化の大きさや速度は用いるポリビニルアルコールの種類、ゲルの形状（薄膜状、棒状等）、ホウ酸塩等の濃度等によつて制御することができる。

【００１４】又、本発明は、上記刺激応答性ヒドロゲルの製造方法を提供するものである。即ち、本発明のヒドロゲルは、けん化度97モル％以上、平均重合度1000以上のポリビニルアルコール水溶液を用いて凍結乾燥法により高強度で透明なヒドロゲルを得、このゲルを0.001モル／１以上の濃度のホウ酸あるいはホウ酸塩のｐＨ７以上のアルカリ性水溶液中に30分以上浸漬して得ることができる。

【００１５】本発明の製造方法は大きく分けて２つの工程からなり、第１工程は凍結乾燥法により高強度で透明なゲルを作製する工程である。即ち、けん化度97モル％以上、平均重合度1000以上のポリビニルアルコールの水溶液を、任意の形状の容器または成型用鋳型へ注入後、これを非凍結下で乾燥させる。このときの含水率は約５～10％である。しかる後、この乾燥体を一定圧の水蒸気中に放置して一定量の水分を吸着させる。この操作により含水率は20～40％となる。この試料を速やかに－20℃以下で凍結し、凍結乾燥を行い、得られた試料を必要に応じ水中に浸漬することによりヒドロゲルとする工程である。この高強度で透明なゲルはポリビニルアルコール水溶液の乾燥、水蒸気中での水分吸着及び凍結乾燥という３つの過程を経てはじめて得られるものであり、後記実施例で示すごとくこのうち１つの過程が欠けても同様の性質を有するヒドロゲルは得られない。又、ポリビニルアルコール水溶液を乾燥濃縮して含水率を20～40％として凍結乾燥を行つても、形状を維持し得ない程度の弱いゲルが得られるだけである。第１工程で得られるゲルの含水率はポリビニルアルコールの種類、水蒸気圧によつて変化し、約40～90％の範囲となる。

【００１６】第２工程は、第１工程で得られたゲルを0.001モル／１以上の濃度のホウ酸あるいはホウ酸塩のｐＨ７以上のアルカリ性水溶液中に30分以上浸漬する工程であり、これによりゲル内にホウ酸イオンによる架橋が形成され、ゲルに荷電基が付与されることとなる。

【００１７】本発明に用いるポリビニルアルコールのけん化度は97モル％以上、好ましくは98モル％以上を要する。また平均重合度は1000以上であることが必要であり、これよりもけん化度及び重合度の低いポリビニルアルコールを用いても得られるゲルの強度は低く、目的は達成されない。

【００１８】ポリビニルアルコールの水溶液は、その濃度が高くなりすぎると溶液中から気泡を除去することが困難となるため、その濃度は約40％程度以下の方が好ましい。

【００１９】ポリビニルアルコール水溶液を乾燥する際の温度は水溶液が凍結せず、かつポリビニルアルコール自身が融解しない温度範囲とする必要があり、－５～60℃の範囲が好ましい。

【００２０】水蒸気中でゲルに少量の水分を含有させる過程も同様の温度範囲で行う必要がある。この際の水蒸気圧が凍結前のゲルの含水量を決定し、最終的に得られるゲルの強度、透明度に影響する。例えば30～80℃の飽和水蒸気圧中に置くことにより、0.04～0.3atmの水蒸気圧とすることができる。又、温度を一定にして水蒸気圧を調整することもできる。

【００２１】凍結時の温度もゲルの強度に影響し、高強度のゲルを得るためには－20℃以下、好ましくは－40℃以下が必要であり、－６℃～－20℃ではゲルの強度が低下する。

【００２２】ホウ酸塩としてはホウ砂（四ホウ酸ナトリウム十水和物）、メタホウ酸ナトリウム、過ホウ酸ナトリウム等を使用することができる。

【００２３】ホウ酸塩水溶液の濃度、ｐＨ及び温度はゲルの刺激応答性及びゲルの強度に大きく影響する。濃度は例えばホウ酸を用いる場合、約0.01モル／１以上、ホウ砂を用いる場合は約0.001モル／１以上とする必要があり、これ以下では十分な機能を発揮しない。またｐＨはホウ酸が解離するアルカリ性領域とする必要があり、約７以上、好ましくは９以上に調整する。温度は50℃以上ではホウ酸塩による架橋が解離するため、約50℃以下とする必要がある。本発明の刺激応答性ヒドロゲルの機

5

能は、高強度のポリビニルアルコールゲルを作製しかつそれにホウ酸塩による架橋を施すという２つの工程の相乗効果により初めて発揮されるものであり、従来のポリビニルアルコールの水溶液にホウ酸塩を添加して得られるゲルは非常に脆弱であり、その機能性を調べる以前のものである。又、ポリビニルアルコールの水溶液を繰り返し凍結解凍する従来の方法で調製したゲルを本製法の第１工程で得られるゲルに代用することもできるが、強度が低く不透明であるためその用途は大きく限定される。

【００２４】

【実施例】参考例１

6

＊市販ポリビニルアルコール（けん化度98.8モル％、平均重合度2000）の水溶液（４ｗ／ｖ％）をペトリ皿に流延し、30℃の乾燥空気中で乾燥させた後、30～70℃の飽和水蒸気圧中に24時間放置し、得られた試料を－40℃で12時間凍結させ、凍結乾燥を行つた。これを蒸留水中で膨潤させて膜状のヒドロゲルを得た。得られたヒドロゲルはいずれも強靭で透明な膜であつた。このゲルについて破断伸び及び引張強度試験の測定を行つた。測定結果を表１に示す。

【００２５】

【表１】

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飽和水蒸気温度（℃）	破断伸び（％）	引張強度（ＭＰａ）
３０	４７５	3.25
４０	４１４	4.94
５０	３７７	2.59
６０	５１４	9.03
７０	４９２	6.87

【００２６】実施例１

参考例１で得たヒドロゲル膜を0.03モル／１のホウ砂水溶液に24時間浸漬し架橋処理を行つた。得られたゲルはいずれも透明であつた。得られたゲルの含水率及び10％※の引張り歪みを加えた時の弾性率を表２に示す。

【００２７】

【表２】

飽和水蒸気温度（℃）	含水率（％）	弾性率（ＭＰａ）
３０	８０.２	０.５０
４０	７８.８	０.６８
６０	７７.８	０.８５
７０	７５.７	１.１５

【００２８】比較例１

ポリビニルアルコール（けん化度98.8モル％、平均重合度2000）の水溶液（10w／v％）をペトリ更に流延し、その上に0.03モル／１のホウ砂水溶液を加えてゲル化させた。得られたゲルは粘着性のゼリー状であり、極めて強度の低いものであつた。

【００２９】比較例２

市販ポリビニルアルコール（けん化度98.8モル％、平均重合度2000）の水溶液（10w／v％）を調製し、－50℃での凍結と７℃での解凍を５回繰り返した後、水中で膨潤させることにより、ヒドロゲルを作製した。このヒドロゲルを0.03モル／１のホウ砂水溶液に24時間浸漬し架橋処理を行つた。得られたゲルは不透明であり、このゲルに引張り歪みを加えたときの弾性率は0.09ＭＰａであり、実施例１で得たヒドロゲルと比較してはるかに強度が低かつた。

【００３０】実施例２

実施例１で得た飽和水蒸気温度40℃のヒドロゲルにカルシウムイオンを添加した時の応力、体積及び含水率の変化を調べた。応力測定は0.03モル／１のホウ砂水溶液にてゲルに10％の引張り歪みを加え、応力が一定に達した後、外部溶液をホウ砂（0.03モル／１）／塩化カルシウム（0.01モル／１）混合水溶液に交換し、それに伴うゲル膜の応力変化を測定した。更にその後外部溶液をホウ砂（0.03モル／１）／エチレジアミン四酢酸四ナトリウム（0.05モル／１）混合水溶液に交換した。応力変化を図１に示す。

【００３１】カルシウムイオンの添加と同時に急激な応力の増加が見られ、約10分で一定値に達した。次に外部溶液をエチレンジアミン四酢酸四ナトリウムに交換することにより応力は速やかに減少し、約10分でカルシウムイオン添加前の応力値にまで回復した。再度カルシウムイオンを添加することにより応力は増加し、以後も可逆的な応力変化が起こつた。カルシウムイオン添加時の応

7

力は初めの約４倍に増加した。カルシウムイオン添加による応力の増加量は約0.2ＭＰａであり、非常に大きな張力が発生した。

【００３２】ゲルの体積変化を図２に示す。体積変化はカルシウムイオンの添加と同時に急速なゲルの収縮が起こり、20分後には元の体積の半分以下となつた。その後エチレンジアミン四酢酸四ナトリウムに交換することに＊

8

＊よるゲルの体積は完全に回復した。

【００３３】ゲルの含水率を表３に示す。カルシウムイオンの添加により含水率は20％以上減少した。またエチレンジアミン四酢酸四ナトリウムへの交換により含水率は回復した。

【００３４】

【表３】

外部溶液	含水率（％）
ホウ砂	７３．９
ホウ砂／塩化カルシウム	５０．２
ホウ砂／ＥＤＴＡ－４Ｎａ	７７．８

【００３５】実施例３

実施例１で得た飽和水蒸気温度40℃のヒドロゲルに0.03モル／１のホウ砂水溶液中にて10％の引張り歪みを加え、溶液中のグルコース濃度を０～125ｍＭの間で増加減少させ、ゲルの応力変化を測定した。応力変化を図３に示す。グルコース濃度を増大させると応力は増加し、濃度を低下させると応力は減少した。応力変化量とグルコース濃度の関係は比例関係にあつた。従つて本ヒドロゲルはグルコースセンサーとして利用できることが示された。

【００３６】実施例４

実施例１で得た飽和水蒸気温度40℃のヒドロゲルに0.03モル／１のホウ砂水溶液中にて10％の引張り歪みを加え、溶液のｐＨを６～12の間で変化させ、ゲルの応力変化、体積変化を25℃にて測定した。ｐＨを12から６に低下させることにより、ゲルの応力は３倍以上に増大した。再びｐＨを12に戻すと応力は減少し、元の値となり、可逆的な応力変化を示した。一方、体積変化はｐＨを12から６に低下させることにより、体積は半分以下に収縮した。再びｐＨを12に戻すと膨潤して元の体積となり、可逆的なｐＨ応答性を示した。

【００３７】実施例５

実施例１で得た飽和水蒸気温度40℃のヒドロゲルを0.03モル／１のホウ砂水溶液に浸漬して液温を室温（約22℃）から50℃に上げるとゲルは収縮し、約17％体積が減少した。室温に戻すとゲルは膨潤し、体積は回復した。

【００３８】実施例６

実施例１で得た飽和水蒸気温度40℃のヒドロゲル膜を短冊状に切つて0.03モル／１のホウ砂水溶液の入つたペトリ皿の中に置きその片端を固定した。二枚の白金極板をゲル膜をはさんで平行に置き、極板間に40Ｖの電圧を加えた。電圧の印加と同時にゲル膜は正極側に大きく屈曲した。

【００３９】

【発明の効果】本発明においてはグルコース等の単糖類及び二糖類又は二価金属イオンの濃度変化、ｐＨ、温度及び電場等の種々の変化に応答して大きな膨潤収縮を示す新しい機能性ヒドロゲルを得ることができる。

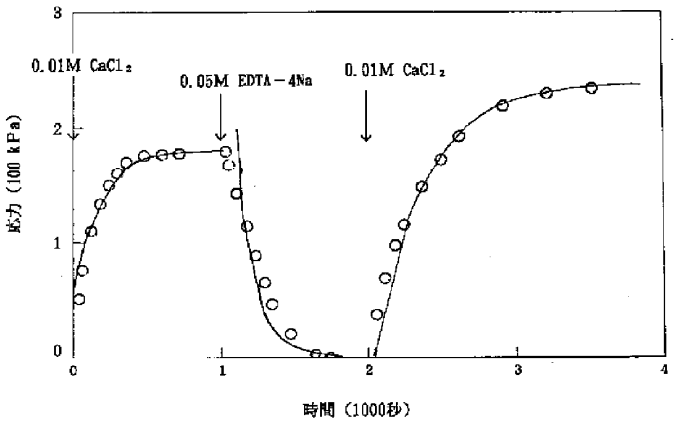
【図面の簡単な説明】

【図１】本発明のヒドロゲルの二価金属イオンに対する応力変化を示す応答曲線である。

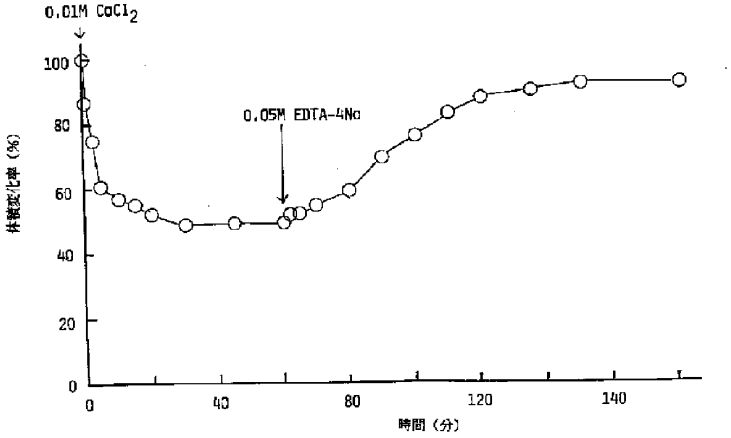
【図２】本発明のヒドロゲルの二価金属イオンに対する体積変化を示す応答曲線である。

【図３】本発明のヒドロゲルのグルコースに対する応力変化を示す応答曲線である。

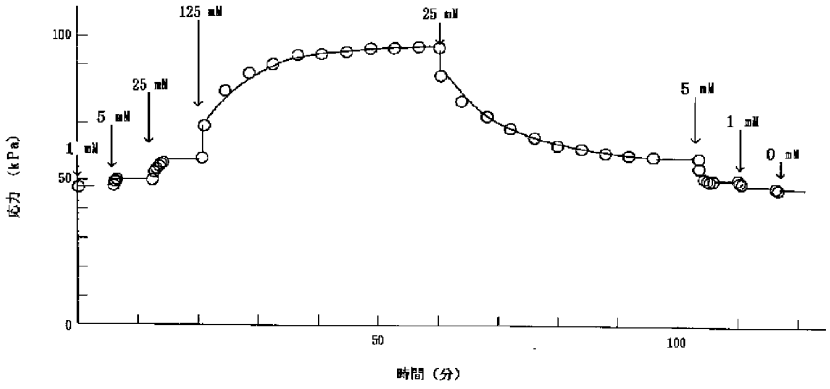
【図１】


0.01M CaCl₂
0.05M EDTA－4Na
0.01M CaCl₂
応力（100 kPa）
時間（1000秒）

【図２】


0.01M CaCl2
0.05M EDTA-4Na
体積変化率（%）
時間（分）

【図３】


125 mM
25 mM
25 mM
5 mM
1 mM
5 mM
1 mM
0 mM
応力（kPa）
時間（分）

Espacenet

Bibliographic data: JPH04268359 (A) — 1992-09-24

STIMULUS-RESPONSIVE HYDROGEL AND PRODUCTION THEREOF

Inventor(s): YONESE MASAKATSU; KISHIMOTO HIROSHI; BABA KAZUHIKO ± (YONESE MASAKATSU, ; KISHIMOTO HIROSHI, ; BABA KAZUHIKO)

Applicant(s): TAIHO PHARMACEUTICAL CO LTD ± (TAIHO YAKUHIN KOGYO KK)

Classification: **- international:** ***C08F6/00; C08J3/00; C08L29/02; C08L29/04;*** (IPC1-7): C08F6/00; C08J3/00; C08L29/04

- cooperative:

Application number: JP19910050804 19910222

Priority number(s): JP19910050804 19910222

Abstract of JPH04268359 (A)

PURPOSE:To produce a functional hydrogel which is based on polyvinyl alcohol and practically applicable to the medical and pharmaceutical fields.
CONSTITUTION:An aq. soln. of polyvinyl alcohol having a degree of saponification of 97mol% or higher and an average degree of polymn. of 1000 or higher is freeze-dried to produce a high-strength transparent hydrogel, which is dipped in an aq. alkaline soln. of boric acid or a borate in a concn. of 0.001 mol/l or higher with a pH of 7 or higher for 30min or longer to give the title gel.

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DESCRIPTION JPH04268359A

[0001]

INDUSTRIAL APPLICABILITY The present invention uses polyvinyl alcohol to make large volume changes and intensities in response to changes in the concentration of monosaccharides such as glucose and disaccharides or divalent metal ions, pH, temperature and electric field. It relates to a completely new high-intensity stimulus-responsive hydrogel that causes change and a method for producing the same.

[0002]

Polyvinyl alcohol gel is one of the most studied hydrogels at present, and has excellent mechanical properties and water retention, is inexpensive, and has extremely low toxicity. Various applications are being attempted in the field as well.

However, the attempt is roughly divided into two categories, one is the use as a substance carrier and a support in which a drug, an enzyme, a microorganism, etc. are embedded in a gel, and the other is an artificial joint or an artificial joint. It is used as a substitute for living tissues such as the cornea. However, these merely utilize the substance retention ability, high water content and elasticity of the polyvinyl alcohol gel.

[0003]

Further, in recent years, a gel having a volume change (swelling / contraction) in response to a change in external pH, temperature, solvent composition, etc. has been prepared using a polymer having a charged group (polyelectrolyte). , These are collectively referred to as functional gels.

Functional gels have opened up a whole new path in the field of gel applications, but most of them use polymer electrolytes that have not been used in the medical field and have not been confirmed to be toxic, and are used in the medical and pharmaceutical fields. Is far from the stage. Furthermore, hydrogels that swell and contract in response to changes in sugar concentration have not been reported even in gels using conventional polyelectrolytes.

[0004]

An object of the present invention is to provide a functional hydrogel practicable in the medical and pharmaceutical fields using polyvinyl alcohol and a method for producing the same.

[0005]

The present invention relates to a stimulus-responsive hydrogel made from polyvinyl alcohol.

[0006]

As a result of diligent research to solve the above problems, the present inventors have treated a high-strength and transparent polyvinyl alcohol hydrogel produced by a unique production method with boric acid and borate to obtain glucose or the like. The present invention has been completed by finding a new functional hydrogel that exhibits large swelling and contraction in response to various changes in the concentration of monosaccharides and disaccharides or divalent metal ions, pH, temperature and electric field.

The stimulus-responsive hydrogel of the present invention has the following functions and is very useful in the fields of medicine and medicine.

[0007]

(1) The gel is placed in an aqueous solution of borate to increase or decrease the glucose concentration in the solution.

At this time, when the glucose concentration is increased, the gel contracts, and when the concentration is decreased, the gel swells.

Since the amount of change in this contraction and swelling increases in proportion to the glucose concentration, this hydrogel can be used as a glucose sensor capable of determining the glucose concentration in the solution.

The sensitivity is good, and it can be applied to the measurement of low-concentration glucose such as blood glucose level. Glucose sensors that are generally used at present are complicated and expensive using enzymes, and become unusable due to enzyme inactivation. If the hydrogel obtained by this production method is used as a glucose sensor, it is possible to determine the glucose concentration inexpensively and easily, and since deterioration does not occur for a long period of time, it can be used repeatedly.

[0008]

On the other hand, when a divalent metal ion such as calcium ion is present in the solution at a certain concentration or more, the gel swells as the glucose concentration increases, and the gel contracts as the concentration decreases.

This property can be used to design a new diabetes treatment system. For example, insulin is encapsulated in a gel, and the gel is administered in vivo or blood is circulated outside the body to bring the gel into contact. When the blood glucose level rises, the gel swells and the network structure of the gel expands, and as a result, the encapsulated insulin is released, and a system that suppresses the increase in the blood glucose level can be considered. The glucose-responsive drug sustained-release system is also awaited by many diabetics.

[0009]

(2) When the gel is put into an aqueous solution of divalent metal ions such as calcium and barium, it shrinks rapidly and the volume is reduced to about 30% of the initial volume.

When an ion chelating agent such as ethylenediaminetetraacetic acid tetrasodium is added to the shrunk gel, the gel quickly swells to the original volume. Next, when divalent metal ions are added, contraction occurs again, and it is possible to cause a completely reversible volume change (swelling contraction). Further, if both ends of the gel are fixed, a very strong tension is generated at the time of contraction. In other words, it can be said that it is a hydrogel that has the function of converting chemical stimuli into physical work. Utilizing this function, it can be applied to, for example, artificial muscles.

[0010]

(3) When the pH of the gel is changed between 6 and 12, the gel contracts toward the acidic side and swells toward the alkaline side.

This volume change is reversible, and the amount of change reaches 30% or more. This can be utilized in pH-sensitive drug release systems.

[0011]

(4) When the temperature of the gel is increased, shrinkage occurs, and when the temperature is returned to the original level, the gel swells.

This is a reversible change, and this property can be applied to temperature-sensitive drug release systems, such as systems that control drug supply in response to changes in body temperature.

[0012]

(5) By applying an electric field to the aqueous solution in which the gel is immersed, the gel can be swelled and contracted and flexed, and can be applied to a drug release system by electrical stimulation, artificial muscles, and the like.

[0013]

The magnitude and rate of the volume change and tension change of the gel described above can be controlled by the type of polyvinyl alcohol used, the shape of the gel (thin film, rod, etc.), the concentration of borate, etc. ..

[0014]

The present invention also provides a method for producing the above-mentioned stimulus-responsive hydrogel.

That is, the hydrogel of the present invention is a high-strength and transparent hydrogel obtained by a freeze-drying method using a polyvinyl alcohol aqueous solution having a saponification degree of 97 mol% or more and an average degree of polymerization of 1000 or more, and this gel is 0.001 mol / l or more. It can be obtained by immersing the borate or borate in an alkaline aqueous solution having a pH of 7 or more for 30 minutes or more

[0015]

The production method of the present invention is roughly divided into two steps, and the first step is a step of producing a high-strength and transparent gel by a freeze-drying method.

That is, an aqueous solution of polyvinyl alcohol having a saponification degree of 97 mol% or more and an average degree of polymerization of 1000 or more is injected into a container having an arbitrary shape or a molding mold, and then dried under non-freezing.

The water content at this time is about 5 to 10%.

After that, the dried product is left in water vapor at a constant pressure to adsorb a certain amount of water. By this operation, the water content becomes 20 to 40%. This is a step of rapidly freezing this sample at −20 ° C. or lower, freeze-drying, and immersing the obtained sample in water as needed to form a hydrogel. This high-strength, transparent gel can only be obtained through the three processes of drying an aqueous polyvinyl alcohol solution, adsorbing water in water vapor, and freeze-drying, and as shown in the examples below, one of these processes is missing. However, a hydrogel having similar properties cannot be obtained. Further, even if the polyvinyl alcohol aqueous solution is dried and concentrated to have a water content of 20 to 40% and freeze-dried, only a weak gel that cannot maintain its shape can be obtained. The water content of the gel obtained in the first step varies depending on the type of polyvinyl alcohol and the water vapor pressure, and ranges from about 40 to 90%.

[0016]

The second step is a step of immersing the gel obtained in the first step in an alkaline aqueous solution of boric acid or borate having a concentration of 0.001 mol / l or more and having a pH of 7 or more for 30 minutes or more. As a result, a crosslink by borate ions is formed in the gel, and a charged group is imparted to the gel.

[0017]

The saponification degree of polyvinyl alcohol used in the present invention is required to be 97 mol% or more, preferably 98 mol% or more.

Further, the average degree of polymerization needs to be 1000 or more, and even if polyvinyl alcohol having a lower saponification degree and a lower degree of polymerization is used, the strength of the obtained gel is low, and the purpose cannot be achieved.

[0018]

If the concentration of the aqueous solution of polyvinyl alcohol becomes too high, it becomes difficult to remove air bubbles from the solution. Therefore, the concentration is preferably about 40% or less.

[0019]

The temperature at which the polyvinyl alcohol aqueous solution is dried needs to be in a temperature range in which the aqueous solution does not freeze and the polyvinyl alcohol itself does not melt, and is preferably in the range of −5 to 60 ° C.

[0020]

The process of adding a small amount of water to the gel in water vapor also needs to be performed in the same temperature range.

The water vapor pressure at this time determines the water content of the gel before freezing, and affects the strength and transparency of the finally obtained gel.

For example, by placing it in a saturated water vapor pressure of 30 to 80 ° C., the water vapor pressure can be 0.04 to 0.3 atm.

It is also possible to adjust the water vapor pressure by keeping the temperature constant.

[0021]

The temperature at the time of freezing also affects the strength of the gel, and in order to obtain a high-strength gel, it is necessary to have a temperature of −20 ° C. or lower, preferably −40 ° C. or lower. The strength decreases.

[0022]

As the borate, borax (sodium tetraborate decahydrate), sodium metaborate, sodium perborate and the like can be used.

[0023]

The concentration, pH and temperature of the borate aqueous solution greatly affect the stimulus responsiveness of the gel and the strength of the gel.

For example, when boric acid is used, the concentration needs to be about 0.01 mol / l or more, and when borax is used, it needs to be about 0.001 mol / l or more, and if it is less than this, a sufficient function is not exhibited.

The pH needs to be in an alkaline region where boric acid dissociates, and is adjusted to about 7 or more, preferably 9 or more.

If the temperature is 50 ° C. or higher, the cross-linking due to borate dissociates, so it is necessary to set the temperature to about 50 ° C. or lower.

The function of the stimulus-responsive hydrogel of the present invention is exhibited for the first time by the synergistic effect of the two steps of producing a high-strength polyvinyl alcohol gel and cross-linking it with borate, and is exhibited for the first time by the conventional polyvinyl alcohol. The gel obtained by adding borate to an aqueous solution is very fragile and has not been investigated for its functionality. Further, the gel prepared by the conventional method of repeatedly freezing and thawing an aqueous solution of polyvinyl alcohol can be substituted for the gel obtained in the first step of this production method, but its use is greatly limited due to its low strength and opacity. NS.

[0024]

Reference Example 1 An aqueous solution (4 w / v%) of commercially available polyvinyl alcohol (saponification degree 98.8 mol%, average degree of polymerization 2000) is poured on a Petri dish and in dry air at 30 ° C. After drying, the sample was allowed to stand in a saturated aqueous steam pressure of 30 to 70 ° C. for 24 hours, and the obtained sample was frozen at −40 ° C. for 12 hours to freeze-dry.

This was swollen in distilled water to obtain a film-like hydrogel. All of the obtained hydrogels were tough and transparent films. The gel was measured for elongation at break and tensile strength test. The measurement results are shown in Table 1.

[0025]

[Table 1]

[0026]

Example 1 The hydrogel membrane obtained in Reference Example 1 was immersed in a 0.03 mol / l borax aqueous solution for 24 hours for cross-linking treatment.

All of the obtained gels were transparent.

Table 2 shows the water content of the obtained gel and the elastic modulus when a tensile strain of 10% is applied.

[0027]

[Table 2]

[0028]

Comparative Example 1 An aqueous solution (10 w / v%) of polyvinyl alcohol (saponification degree 98.8 mol%, average degree of polymerization 2000) was further cast on Petri, and a 0.03 mol / l borax aqueous solution was further cast thereto. Was added and gelled.

The obtained gel was in the form of a sticky jelly and had extremely low strength.

[0029]

Comparative Example 2 An aqueous solution (10 w / v%) of a commercially available polyvinyl alcohol (saponification degree 98.8 mol%, average degree of polymerization 2000) was prepared, frozen at −50 ° C. and thawed at 7 ° C. 5 times. After repeating, a hydrogel was prepared by swelling in water.

This hydrogel was immersed in a 0.03 mol / l borax aqueous solution for 24 hours for cross-linking treatment.

The obtained gel was opaque, and the elastic modulus when tensile strain was applied to this gel was 0.09 MPa, which was much lower in strength than the hydrogel obtained in Example 1.

[0030]

Example 2 Changes in stress, volume and water content when calcium ions were added to the hydrogel having a saturated water vapor temperature of 40 ° C. obtained in Example 1 were investigated.

The stress is measured by applying 10% tensile strain to the gel with a 0.03 mol / l borax aqueous solution, and after the stress reaches a certain level, the external solution is borax (0.03 mol / l) / calcium chloride (0.03 mol / l). It was replaced with a 0.01 mol / l) mixed aqueous solution, and the accompanying stress change in the gel film was measured. After that, the external solution was replaced with a mixed aqueous solution of borax (0.03 mol / l) / tetrasodium ethirediamine tetraacetate (0.05 mol / l). The stress change is shown in FIG.

[0031]

A rapid increase in stress was observed at the same time as the addition of calcium ions, and reached a constant value in about 10 minutes.

Next, the stress was rapidly reduced by exchanging the external solution with tetrasodium ethylenediaminetetraacetate, and recovered to the stress value before the addition of calcium ions in about 10 minutes. The stress increased by adding calcium ions again, and a reversible stress change occurred thereafter. The stress at the time of adding calcium ions increased about 4 times at the beginning. The amount of increase in stress due to the addition of calcium ions was about 0.2 MPa, and a very large tension was generated.

[0032]

The volume change of the gel is shown in FIG

As for the volume change, rapid gel contraction occurred at the same time as the addition of calcium ions, and after 20 minutes, the volume became less than half of the original volume. After that, the volume of the gel was completely restored by exchanging with tetrasodium ethylenediaminetetraacetate.

[0033]

The water content of the gel is shown in Table 3.

The water content was reduced by 20% or more by the addition of calcium ions. The water content was restored by the exchange with ethylenediaminetetraacetic acid tetrasodium.

[0034]

[Table 3]

[0035]

Example 3 A hydrogel having a saturated water vapor temperature of 40 ° C. obtained in Example 1 was subjected to a tensile strain of 10% in a 0.03 mol / l borax aqueous solution to increase the glucose concentration in the solution to 0 to 125 mM. The stress change of the gel was measured by increasing or decreasing between.

The stress change is shown in FIG.

Increasing the glucose concentration increased the stress, and decreasing the concentration decreased the stress. The relationship between the amount of stress change and the glucose concentration was proportional. Therefore, it was shown that this hydrogel can be used as a glucose sensor.

[0036]

Example 4 A 10% tensile strain was applied to the hydrogel obtained in Example 1 at a saturated water vapor temperature of 40 ° C. in a 0.03 mol / l borax aqueous solution, and the pH of the solution was between 6 and 12. The change in stress and volume of the gel were measured at 25 ° C.

By lowering the pH from 12 to 6, the stress on the gel increased more than 3-fold. When the pH was returned to 12, the stress decreased and returned to the original value, showing a reversible stress change. On the other hand, the volume change reduced the pH from 12 to 6 to reduce the volume to less than half. When the pH was returned to 12, it swelled to its original volume and showed reversible pH responsiveness.

[0037]

Example 5 When the hydrogel having a saturated water vapor temperature of 40 ° C. obtained in Example 1 is immersed in a 0.03 mol / l borax aqueous solution and the liquid temperature is raised from room temperature (about 22 ° C.) to 50 ° C., the gel is obtained. Shrank and lost about 17% in volume.

When the temperature was returned to room temperature, the gel swelled and the volume was restored.

[0038]

Example 6 The hydrogel membrane having a saturated water vapor temperature of 40 ° C. obtained in Example 1 was cut into strips and placed in a Petri dish containing a 0.03 mol / l borax aqueous solution, and one end thereof was fixed. ..

Two platinum plates were placed in parallel with a gel film sandwiched between them, and a voltage of 40 V was applied between the plates. At the same time as the voltage was applied, the gel film was greatly bent toward the positive electrode.

[0039]

[Effect of the Invention] In the present invention, a new function showing large swelling and contraction in response to various changes in the concentration of monosaccharides such as glucose and disaccharides or divalent metal ions, pH, temperature and electric field. Sexual hydrogels can be obtained.

[0040]

A brief description of the drawing

[0041]

FIG. 1 is a response curve showing a stress change of the hydrogel of the present invention with respect to divalent metal ions.

[0042]

FIG. 2 is a response curve showing a volume change of the hydrogel of the present invention with respect to a divalent metal ion.

[0043]

FIG. 3 is a response curve showing a stress change of the hydrogel of the present invention with respect to glucose.

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CLAIMS JPH04268359A

1.

A stimulus-responsive hydrogel made from polyvinyl alcohol.

2.

The stimulus-responsive hydrogel according to claim 1, which responds to changes in the concentrations of monosaccharides and disaccharides.

3.

The stimulus-responsive hydrogel according to claim 1, which responds to changes in the concentration of divalent metal ions.

4.

The stimulus-responsive hydrogel of claim 1, which responds to changes in pH.

5.

The stimulus-responsive hydrogel of claim 1, which responds to changes in temperature.

6.

The stimulus-responsive hydrogel of claim 1, which responds to the application of an electric field.

7.

A high-strength and transparent hydrogel was obtained by a freeze-drying method using a polyvinyl alcohol aqueous solution having a saponification degree of 97 mol% or more and an average degree of polymerization of 1000 or more, and this gel was subjected to borate or boric acid having a concentration of 0.001 mol / l or more. A method for producing a stimulus-responsive hydrogel, which comprises immersing the salt in an alkaline aqueous solution having a pH of 7 or more for 30 minutes or more.

8.

In the freeze-drying method, an aqueous solution of polyvinyl alcohol is poured into a container of an arbitrary shape or a molding mold, which is dried under non-freezing, and then the dried product is left in steam at a constant pressure to be constant. The method according to claim 7, which is a method of obtaining a hydrogel by adsorbing an amount of water, promptly freezing at −20 ° C. or lower, drying the sample in a frozen state, and immersing the obtained sample in water as needed. A method for producing a stimulus-responsive hydrogel.

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	1	7941279		2011-05-10	Hwang et al.	
	2	8415102		2013-04-09	Geiss et al.	
	3	8519115		2013-08-27	Webster et al.	
	4	8986926		2015-03-24	Ferree et al.	
	5	9371563		2016-06-21	Geiss et al.	
	6	9714446		2017-07-25	Webster et al.	
	7	9714937		2017-07-25	Dunaway	
	8	7910304		2011-03-22	Drmanac	

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	9	8431691		2013-04-30	McKernan et al.	
	10	6194148		2001-02-27	Hori et al.	

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	WANG et al. "Rapid and Sensitive Detection of Severe Acute Respiratory Syndrome Coronavirus by Rolling Circle Amplification" Journal of Clinical Microbiology, Vol. 43, No. 5, May 2005, pp. 2339-2344	

If you wish to add additional non-patent literature document citation information please click the Add button Add

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (**Not for submission under 37 CFR 1.99)**		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

EXAMINER SIGNATURE			
Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-11-01
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	44170436
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	01-NOV-2021
Filing Date:	06-AUG-2021
Time Stamp:	13:11:43
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	WANG_et_al_J_of_Clinical_Microbiol_2005_21554518.pdf	482270 7d6214019d03e91d718eda72f196284220ea02fe	no	6
Warnings:					

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21554773.pdf	1034689 5b67fb6037c21d8e6051f3975a1fc1d8998ef99a	no	5
Warnings:					
Information:					
			Total Files Size (in bytes):	1516959	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	10030261		2018-07-24	Frisen et al.	US counterpart to JP 2014-513523
	2	7771949		2010-08-10	Kramer	
	3	9017992		2015-04-28	Winther et al.	
	4	9896720		2018-02-20	Raj et al.	
	5	8946389		2015-02-03	Gao et al.	
	6	8658361		2014-02-25	Wu et al.	
	7	10465235		2019-11-05	Gullberg et al.	
	8	8013134		2011-09-06	Fredriksson	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20130323729	A1	2013-12-05	Landegren et al.	

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2014-513523	JP	A	2014-06-05	Spatial Transcriptomics AB		X

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	GORANSSON et al. "A single molecule array for digital targeted molecular analyses" Nucleic Acids Research, 2009, Vol. 37, No. 1, e7, doi:10.1093/nar/gkn921	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-11-04
Name/Print	John P. Iwanicki	Registration Number	34,628

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Electronic Acknowledgement Receipt

EFS ID:	44204654
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	04-NOV-2021
Filing Date:	06-AUG-2021
Time Stamp:	13:59:26
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	JP2014513523A_21559104.pdf	4675047 cc82e8d396e6cc58483a85a2c89e05a35722e0db	no	115

Warnings:

Information:					
2	Foreign Reference	Translation_of_JP2014513523A_21559115.pdf	816109 0e85e658b9f3ac1e188a060cc796d5db2b2eace3	no	117
Warnings:					
Information:					
3	Non Patent Literature	GORANSSON_et_al_Nucleic_Acids_Res_2009_21561615.pdf	1531260 98d754d2863aa0d097e78c1b91541cf7ad8f8a56	no	9
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21563654.pdf	1034703 2aaa8b4a0730ad63a9114225c0011559822cf5d4	no	4
Warnings:					
Information:					
		Total Files Size (in bytes):	8057119		

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<u>New Applications Under 35 U.S.C. 111</u>
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<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Espacenet

Bibliographic data: JP2014513523 (A) — 2014-06-05

METHOD AND PRODUCT FOR LOCALISED OR SPATIAL DETECTION OF NUCLEIC ACID IN A TISSUE SAMPLE

Inventor(s): フライセン， ヨナス, ; スタール， パトリック, ; ルンデベルグ， ヨアキム

Applicant(s): スペイシャル トランスクリプトミクス アーベー SPATIAL TRANSCRIPTOMICS AB

Classification: - international: ***C12N15/09; C12Q1/68; G01N33/53; G01N33/566; G01N37/00***

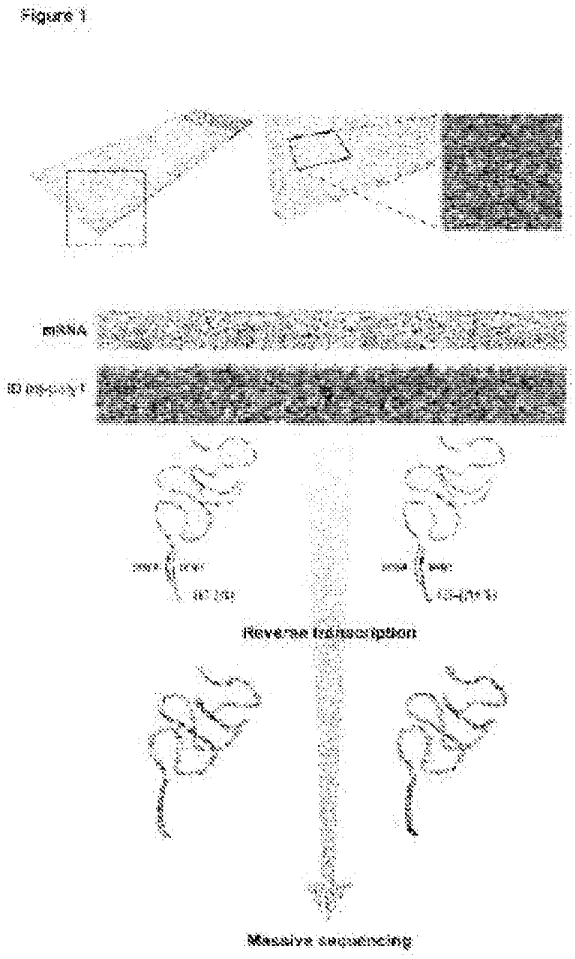
- cooperative: **C12N15/11 (KR); C12Q1/6806 (KR, US); C12Q1/6816 (US); C12Q1/6827 (US); C12Q1/6837 (CN, RU, US); C12Q1/6841 (EP, CN, RU, US); C12Q1/6844 (CN, RU, US); C12Q1/6853 (EP); C12Q1/6876 (US); G16B30/00 (US); G16B50/20 (US); G16B50/30 (US);** C12Y600/00 (US) more

Application number: JP20140504349 20120413
Global Dossier

Priority number(s): GB20110006254 20110413 ; WO2012EP56823 20120413

Also published as: AU2012241730 (A1) AU2012241730 (B2) BR112013026502 (A2) CA2832678 (A1) CA2832678 (C) more

Abstract not available for JP2014513523 (A)
Abstract of corresponding document: WO2012140224 (A1)



The present invention relates to methods and products for the localised or spatial detection of nucleic acid in a tissue sample and in particular to a method for localised detection of nucleic acid in a tissue sample comprising: (a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3': (i) a positional domain that corresponds to the position of the capture probe on the array, and (ii) a capture domain; (b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing nucleic acid of the tissue sample to hybridise to the capture domain in said capture probes; (c) generating DNA molecules from the captured nucleic acid molecules using said capture probes as extension or ligation primers, wherein said extended or ligated

DNA molecules are tagged by virtue of the positional domain; (d) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying said tagged DNA; (e) releasing at least part of the tagged DNA molecules and/or their complements or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof; and (f) directly or indirectly analysing the sequence of the released DNA molecules.

(19)日本国特許庁(JP)　(12)公表特許公報(A)　(11)特許出願公表番号
特表2014-513523
(P2014-513523A)
(43)公表日　平成26年6月5日(2014.6.5)

(51)Int.Cl.		F I			テーマコード（参考）
C12N 15/09	(2006.01)	C12N	15/00	A	4B024
C12Q 1/68	(2006.01)	C12Q	1/68	ZNAA	4B063
G01N 33/53	(2006.01)	C12N	15/00	F	
G01N 37/00	(2006.01)	G01N	33/53	M	
G01N 33/566	(2006.01)	G01N	37/00	102	

審査請求 未請求　予備審査請求 未請求　（全 112 頁）　最終頁に続く

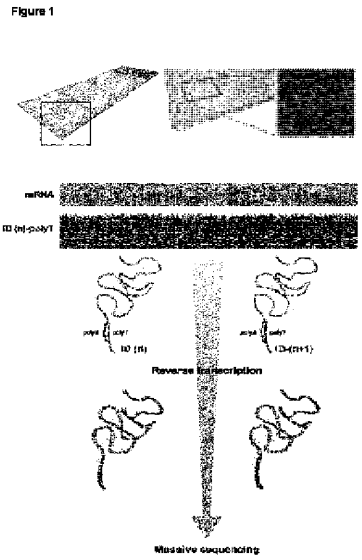
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(71)出願人　513257465
スペイシャル　トランスクリプトミクス　アーベー
ＳＰＡＴＩＡＬ　ＴＲＡＮＳＣＲＩＰＴＯＭＩＣＳ　ＡＢ
スウェーデン，　１１４　２６　ストックホルム，　ブラゲヴァーゲン　５
(74)代理人　100107456
弁理士　池田　成人
(74)代理人　100148596
弁理士　山口　和弘
(74)代理人　100123995
弁理士　野田　雅一

最終頁に続く

(54)【発明の名称】組織試料中の核酸の局在化された検出、又は空間的検出のための方法及び生成物

(57)【要約】
本発明は、組織試料中の核酸の局在化された又は空間的検出のための方法及び生成物に関し、特に組織試料中の核酸の局在化された検出のための方法であって、（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつプローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、（ｉ）アレイ上の捕捉プローブの位置に対応する位置ドメイン、及び（ｉｉ）捕捉ドメインを有する核酸分子を含む、ステップと、（ｂ）アレイ上の捕捉プローブの位置と、組織試料における位置とが関連付けられるようにアレイを組織試料に接触させて、組織試料の核酸を捕捉プローブ中の捕捉ドメインにハイブリダイズさせるステップと、（ｃ）捕捉プローブを伸長又はライゲーションプライマーとして用いて、捕捉された核酸分子か



【特許請求の範囲】
【請求項１】
組織試料中の核酸の局在化された検出のための方法であって、
（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、
前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）前記アレイ上の捕捉プローブの前記位置と、前記組織試料における位置とが関連付けられるように前記アレイを前記組織試料に接触させて、前記組織試料の核酸を前記捕捉プローブ中の前記捕捉ドメインにハイブリダイズさせるステップと、
（ｃ）前記捕捉プローブを伸長又はライゲーションプライマーとして用いて、捕捉された核酸分子からＤＮＡ分子を生成させるステップであって、
前記伸長された又はライゲートされたＤＮＡ分子が前記位置ドメインによりタグ付けされる、ステップと、
（ｄ）場合によってタグ付けされたＤＮＡの相補鎖を生成し、及び／又は場合によってタグ付けされたＤＮＡを増幅するステップと、
（ｅ）前記タグ付けされたＤＮＡ分子及び／又はその相補体若しくはアンプリコンの少なくとも一部を前記アレイの表面から放出するステップであって、
前記一部が前記位置ドメイン又はその相補体を含む、ステップと、
（ｆ）放出されたＤＮＡ分子の配列を直接的に又は間接的に解析するステップと、
を含む方法。
【請求項２】
（ｇ）解析された配列の情報を前記組織試料の画像と関連付けるステップであって、
前記組織試料がステップ（ｃ）の前又は後で撮像される、ステップ、をさらに含む、請求項１に記載の方法。
【請求項３】
（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、
前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブが逆転写酵素（ＲＴ）プライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）前記アレイ上の捕捉プローブの前記位置と、前記組織試料における位置とが関連付けられるように前記アレイを前記組織試料に接触させて、前記組織試料のＲＮＡを前記捕捉プローブ中の前記捕捉ドメインにハイブリダイズさせるステップと、
（ｃ）前記捕捉プローブをＲＴプライマーとして用いて、捕捉されたＲＮＡ分子からｃＤＮＡ分子を生成し、場合によって前記ｃＤＮＡ分子を増幅するステップと、
（ｄ）ｃＤＮＡ分子及び場合によってそのアンプリコンの少なくとも一部を前記アレイの表面から放出するステップであって、
放出された分子が、第一鎖及び／若しくは第二鎖ｃＤＮＡ分子又はそのアンプリコンであり、前記一部が前記位置ドメイン又はその相補体を含むステップと、
（ｅ）放出されたＤＮＡ分子の配列を直接的に又は間接的に解析するステップと、場合に

よって、（ｆ）解析された配列の情報を前記組織試料の画像と関連付けるステップであって、
前記組織試料がステップ（ｃ）の前又は後で撮像される、ステップと、
を含む、
組織試料のトランスクリプトームを決定する、及び／又は解析するための方法である、請求項１に記載の方法。
【請求項４】
前記捕捉プローブがＤＮＡ分子である、請求項１～３のいずれか一項に記載の方法。
【請求項５】
前記捕捉プローブが、前記位置ドメインに対して５’側にあるユニバーサルドメインをさらに含み、
前記ユニバーサルドメインが、
（ｉ）生成されたＤＮＡ分子を増幅するための増幅ドメイン及び／又は
（ｉｉ）生成されたＤＮＡ分子を前記アレイの表面から放出するための切断ドメイン
を含む、請求項１～４のいずれか一項に記載の方法。
【請求項６】
捕捉プローブのそれぞれのスピーシーズの前記位置ドメインが、独自のバーコード配列を含む、請求項１～５のいずれか一項に記載の方法。
【請求項７】
前記捕捉ドメインが、少なくとも１０個のデオキシチミジン残基を含むポリＴ　ＤＮＡオリゴヌクレオチド及び／又はランダム若しくは縮重オリゴヌクレオチド配列を含む、請求項１～６のいずれか一項に記載の方法。
【請求項８】
前記捕捉プローブがその５’端部により前記アレイの表面に直接的に固定化されている、請求項１～７のいずれか一項に記載の方法。
【請求項９】
前記捕捉プローブが、表面プローブへのハイブリダイゼーションにより前記アレイの表面に間接的に固定化されており、
前記捕捉プローブの前記捕捉ドメインが、前記アレイ上に固定化されている表面プローブの５’端部にハイブリダイズすることができる上流配列を含む、請求項１～７のいずれか一項に記載の方法。
【請求項１０】
前記表面プローブがその３’端部により前記アレイの表面に固定化されている、請求項９に記載の方法。
【請求項１１】
前記表面プローブが、
（ｉ）前記捕捉ドメインの少なくとも一部、
（ｉｉ）前記位置ドメイン及び
（ｉｉｉ）前記ユニバーサル増幅ドメインの少なくとも一部
に相補的である配列を含む、請求項９又は１０に記載の方法。
【請求項１２】
前記相補鎖又は前記第二鎖ｃＤＮＡ分子が、前記タグ付けされたＤＮＡ分子又は前記ｃＤＮＡ分子が前記アレイの表面から放出される前に又は後で生成される、請求項１～１１のいずれか一項に記載の方法。
【請求項１３】
鎖置換ポリメラーゼがｃＤＮＡの第二鎖の相補鎖の合成のために使用される、請求項１～１２のいずれか一項に記載の方法。
【請求項１４】
ランダムプライマーが、前記相補鎖又は前記第二鎖ｃＤＮＡの合成のために使用される、請求項１～１３のいずれか一項に記載の方法。

【請求項１５】
前記ランダムプライマーが増幅ドメインを含む、請求項１４に記載の方法。
【請求項１６】
前記ランダムプライマーの前記増幅ドメインが、前記捕捉プローブの前記増幅ドメインとは異なるヌクレオチド配列からなる、請求項１５に記載の方法。
【請求項１７】
アダプターが、タグ付き分子若しくはその相補体又はｃＤＮＡ分子の一方の又は両方の端部にライゲートされている、請求項１～１６のいずれか一項に記載の方法。
【請求項１８】
配列解析に先立ってタグ付き分子又はｃＤＮＡ分子を増幅するステップをさらに含む、請求項１～１７のいずれか一項に記載の方法。
【請求項１９】
前記増幅するステップが、前記アレイの前記基板からのタグ付きＤＮＡ若しくはｃＤＮＡ分子の放出後に実施される、又は前記増幅するステップが前記アレイ上でインサイツで実施される、請求項１８に記載の方法。
【請求項２０】
前記増幅するステップがＰＣＲを含む、請求項１８又は１９に記載の方法。
【請求項２１】
前記分子が、
（ｉ）核酸切断、
（ｉｉ）変性及び／又は
（ｉｉｉ）物理的手段
により前記アレイの表面から放出される、請求項１～２０のいずれか一項に記載の方法。
【請求項２２】
前記分子が、前記捕捉プローブの前記ユニバーサルドメイン又は前記位置ドメインに位置している切断ドメインの酵素的切断により放出される、請求項２１（ｉ）に記載の方法。
【請求項２３】
前記分子が、熱水又はバッファーを前記アレイに適用することにより放出される、請求項２１（ｉｉ）又は（ｉｉｉ）に記載の方法。
【請求項２４】
シーケンシングに先立って放出された分子を精製するステップをさらに含む、請求項１～２３のいずれか一項に記載の方法。
【請求項２５】
前記基板が、ガラス、シリコン、ポリ－Ｌ－リジン被覆物質、ニトロセルロース、ポリスチレン、環状オレフィンコポリマー（ＣＯＣ）、環状オレフィンポリマー（ＣＯＰ）、ポリプロピレン、ポリエチレン及びポリカーボネートからなる群から選択される、請求項１～２４のいずれか一項に記載の方法。
【請求項２６】
前記組織試料が組織切片である、請求項１～２５のいずれか一項に記載の方法。
【請求項２７】
前記組織切片が固定組織、たとえばホルマリン固定パラフィン包埋（ＦＦＰＥ）組織又は急速冷凍組織、を用いて調製される、請求項２６に記載の方法。
【請求項２８】
前記試料をステップ（ｃ）に先立って前記アレイに接触させた後で、前記組織試料を再水和するステップをさらに含む、請求項１～２７のいずれか一項に記載の方法。
【請求項２９】
前記組織試料が植物、動物又は真菌由来である、請求項１～２８のいずれか一項に記載の方法。
【請求項３０】

前記アレイを洗浄して残余組織を取り除くステップであって、前記アレイからの前記分子の放出に先立つステップをさらに含む、請求項１～２９のいずれか一項に記載の方法。
【請求項３１】
前記アレイが、前記アレイ上での前記組織試料の方向付けを可能にする少なくとも１つの位置マーカーを含む、請求項１～３０のいずれか一項に記載の方法。
【請求項３２】
前記アレイが、前記アレイの表面上の別個の位置に少なくとも１０、５０、１００、２００、５００、１０００又は２０００の位置マーカーを含む、請求項３１に記載の方法。
【請求項３３】
前記位置マーカーが、標識された、好ましくは蛍光標識されたマーカー核酸分子にハイブリダイズすることができる、請求項３１又は３２に記載の方法。
【請求項３４】
前記組織試料が、光、明視野、暗視野、位相差、蛍光、反射、干渉若しくは共焦点顕微鏡又はその組み合わせを使用して撮像される、請求項２～３３のいずれか一項に記載の方法。
【請求項３５】
前記組織試料が蛍光顕微鏡を使用して撮像される、請求項３４に記載の方法。
【請求項３６】
配列解析ステップが、シーケンシングステップを含み、好ましくは、シーケンシングステップが可逆的ダイターミネーターに基づくシーケンシング反応を含む、請求項１～３５のいずれか一項に記載の方法。
【請求項３７】
（ｉ）アレイに接触させる組織試料から核酸を捕捉するのに使用するためのアレイ、又は（ｉｉ）組織試料中の核酸の局在化された検出において使用するためのアレイを作る又は作製するための方法であって、
捕捉プローブの複数のスピーシーズをアレイ基板に直接的に又は間接的に固定化するステップを含み、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、方法。
【請求項３８】
捕捉プローブのそれぞれのスピーシーズが前記アレイ上の別個の位置を占める、請求項３７に記載の方法。
【請求項３９】
以下の
（ａ）複数の表面プローブをアレイ基板に直接的又は間接的に固定化するステップであって、前記表面プローブが、
（ｉ）前記捕捉ドメインのオリゴヌクレオチドの一部（前記核酸を捕捉することに関与していない部分）にハイブリダイズすることができるドメイン、
（ｉｉ）相補的位置ドメイン、及び
（ｉｉｉ）相補的ユニバーサルドメインを含む
ステップと、
（ｂ）前記アレイ上に固定化された前記表面プローブに、捕捉ドメインオリゴヌクレオチド及びユニバーサルドメインオリゴヌクレオチドをハイブリダイズさせるステップと、
（ｃ）鋳型重合によりユニバーサルドメインオリゴヌクレオチドを伸長して、前記捕捉プローブの前記位置ドメインを生成するステップと、
（ｄ）前記位置ドメインを前記捕捉ドメインオリゴヌクレオチドにライゲートして、捕捉オリゴヌクレオチドを作製するステップ
のうちのいずれか１つ又は複数をさらに含む、請求項３７又は３８に記載の方法。

【請求項４０】
（ｉ）アレイに接触させる組織試料から核酸を捕捉するのに使用するためのアレイ、又は（ｉｉ）組織試料中の核酸の局在化された検出において使用するためのアレイであって、
捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備え、
前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーションプライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）前記アレイに接触させる前記組織試料の核酸を捕捉するための捕捉ドメイン
を有する核酸分子を含む、
アレイ。
【請求項４１】
前記捕捉プローブが請求項４～９のいずれか一項に定義されるとおりであり、
前記表面プローブが請求項１０又は１１に定義されるとおりであり、
前記基板が請求項２５に定義されるとおりであり、
前記組織試料が請求項２６、２７又は２８のいずれか一項に定義されるとおりであり、
前記アレイが請求項３１～３３のいずれか一項に定義されるとおりであり、
前記位置マーカーが請求項３３に定義されるとおりである、請求項３７～３９のいずれか一項に記載の方法又は請求項４０に記載のアレイ。
【請求項４２】
（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、
前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）前記アレイ上の前記捕捉プローブの前記位置と、前記組織試料中の位置とが関連付けられるように前記アレイを前記組織試料に接触させて、前記組織試料のＤＮＡを前記捕捉プローブの前記捕捉ドメインにハイブリダイズさせるステップと、
（ｃ）前記組織試料中のＤＮＡを断片化するステップであって、
前記断片化が、ステップ（ｂ）において前記アレイを前記組織試料に接触させる前に、間に又は後で実行される、ステップと、
（ｄ）捕捉されたＤＮＡ断片を鋳型として用いてプライマー伸長反応において前記捕捉プローブを伸長させて伸長されたＤＮＡ分子を生成する、又は捕捉されたＤＮＡ断片をライゲーション反応において前記捕捉プローブにライゲートして、ライゲートされたＤＮＡ分子を生成するステップであって、
前記伸長された又はライゲートされたＤＮＡ分子が前記位置ドメインによりタグ付けされる、ステップと、
（ｅ）場合によって、タグ付けされたＤＮＡの相補鎖を生成する及び／又は場合によって、タグ付けされたＤＮＡを増幅するステップと、
（ｆ）前記アレイの表面から前記タグ付けされたＤＮＡ分子並びに／又はその相補体及び／若しくはアンプリコンの少なくとも一部を放出するステップであって、
前記一部が前記位置ドメイン又はその相補体を含む、ステップと、
（ｇ）放出されたＤＮＡ分子の配列を直接的に又は間接的に解析する（たとえば、シー

ケンシングする）ステップと、場合によって
（ｈ）解析された配列の情報を前記組織試料の画像と関連付けるステップであって、
前記組織試料がステップ（ｄ）の前に又は後で撮像される、ステップと、
を含む、
組織試料中のＤＮＡの局在化された検出のための方法である、請求項１又は請求項２に記載の方法。
【請求項４３】
（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、
前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）前記アレイ上の前記捕捉プローブの前記位置と、組織試料中の位置とが関連付けられるように前記アレイを前記組織試料に接触させるステップと、
（ｃ）前記組織試料中のＤＮＡを断片化するステップであって、
前記断片化が、ステップ（ｂ）において前記アレイを前記組織試料に接触させる前に、間に又は後で実行される、ステップと、
（ｄ）前記ＤＮＡ断片に、前記捕捉ドメインにハイブリダイズすることができる結合ドメインを与えるステップと、
（ｅ）前記ＤＮＡ断片を前記捕捉プローブ中の前記捕捉ドメインにハイブリダイズさせるステップと、
（ｆ）捕捉されたＤＮＡ断片を鋳型として用いてプライマー伸長反応において前記捕捉プローブを伸長させて、伸長されたＤＮＡ分子を生成する、又は捕捉されたＤＮＡ断片をライゲーション反応において前記捕捉プローブにライゲートして、ライゲートされたＤＮＡ分子を生成するステップであって、
前記伸長された又はライゲートされたＤＮＡ分子が前記位置ドメインによりタグ付けされる、ステップと、
（ｇ）場合によって、タグ付けされたＤＮＡの相補鎖を生成する及び／又は場合によって、タグ付けされたＤＮＡを増幅するステップと、
（ｈ）前記アレイの表面から前記タグ付けされたＤＮＡ分子並びに／又はその相補体及び／若しくはアンプリコンの少なくとも一部を放出するステップであって、
前記一部が前記位置ドメイン又はその相補体を含む、ステップと、
（ｉ）放出されたＤＮＡ分子の配列を直接的に又は間接的に解析する（たとえば、シーケンシングする）ステップと、場合によって
（ｊ）解析された配列の情報を前記組織試料の画像と関連付けるステップであって、
前記組織試料がステップ（ｆ）の前に又は後で撮像される、ステップと、
を含む、請求項４２に記載の方法。
【発明の詳細な説明】
【発明の詳細な説明】
【０００１】
本発明は一般的には、組織試料中の核酸の局在化された検出、又は空間的検出に関する。核酸はＤＮＡでもＲＮＡであってもよい。したがって、本発明は、組織試料、たとえば個人の細胞における、遺伝子の局在化、分布若しくは発現についての空間情報、又は実際に、任意のゲノム変異（必ずしも遺伝子中ではない）の局在化若しくは分布についての空間的情報を得るために、ＲＮＡ、たとえば、ＲＮＡ転写物又はゲノムＤＮＡを検出する及び／又は解析するための方法を提供する。したがって、本発明は空間ゲノミクス及び空間

トランスクリプトミクスを可能にする。

【０００２】

より詳細には、本発明は、組織試料のトランスクリプトーム又はゲノム、特に包括的トランスクリプトーム又はゲノムを決定する及び／又は解析するための方法に関する。特に、前記方法は、組織試料中のゲノム配列の分布、場所又は発現を解析するための定量的及び／又は定性的方法であって、組織試料内の空間的発現又は分布又は場所パターンが維持される方法に関する。したがって、前記新しい方法は、「空間トランスクリプトミクス」又は「空間ゲノミクス」を実施するためのプロセスであって、使用者が、組織試料中における発現パターン又は発現された遺伝子若しくは存在する遺伝子若しくはゲノム遺伝子座の場所／分布パターンを同時に決定するのを可能にするプロセスを提供する。

【０００３】

本発明は、特に、高処理ＤＮＡシーケンシング技術と結びついたアレイ技術に基づいており、このアレイ技術により、組織試料中の核酸分子（たとえば、ＲＮＡ又はＤＮＡ分子）、特にｍＲＮＡ又はＤＮＡを捕捉し位置タグで標識することが可能となる。このステップに続いてＤＮＡ分子が合成され、塩基配列が決定されて解析され、組織試料のありとあらゆる部分においてどの遺伝子が発現されているかが決定される。有利なことに、組織試料におけるそれぞれの細胞の個々別々の特異的トランスクリプトームを同時に得ることができる。それ故に、本発明の方法は、組織試料内の個々の細胞からの平行性の高い網羅的トランスクリプトームサインを、前記調査された組織試料内の空間的情報を失うことなく提供すると言ってもよい。本発明は、本発明の方法を実施するためのアレイ及び本発明のアレイを作製するための方法も提供する。

【０００４】

ヒト身体は１００兆個を超える細胞を含み、２５０種類を超える異なる器官及び組織に組織化されている。脳などの複雑な器官の発生及び組織化は理解からは程遠く、定量的方法を使用してそのような組織において発現される遺伝子の発現を精査し、そのような組織の発生及び機能を制御する遺伝子を調査し決定する必要性がある。器官それ自体は、栄養輸送、防御、等などのあらゆる身体機能を調整し維持することを可能にする分化細胞の混合体である。その結果、細胞機能は、特定の組織構造内での細胞の位置及びその組織内で前記細胞が他の細胞と共有する相互作用に、直接的にも間接的にも依存している。それ故に、これらの相互作用が組織内のそれぞれの細胞にどのような影響を与えているのかを転写レベルで解きほぐす必要性がある。

【０００５】

ＲＮＡディープシーケンシングによる最近の所見で、転写物の大部分がヒト株化細胞で検出できること及びヒトタンパク質コード遺伝子の大部分（７５％）が大半の組織で発現されていることが実証された。同様に、ヒトゲノムのうちの１％の詳細な研究により、染色体が偏在的に転写されていること及びすべての塩基の大部分が一次転写物に含まれていることが明らかになった。したがって、転写装置は全体レベルでは乱交雑（ｐｒｏｍｉｓｃｕｏｕｓ）であると言える。

【０００６】

ＲＮＡ翻訳、分解などの速度はいずれか１つの転写物から産生されるタンパク質の量に影響を与えることになるので、転写物はタンパク質存在量の代用に過ぎないことが周知である。この点に関して、ヒト器官及び組織の最近の抗体ベースの解析によれば、組織特異性は、空間と時間におけるタンパク質レベルの正確な調節により達成されること及び身体中の異なる組織はどのタンパク質が発現されるのかではなく、それぞれのタンパク質がどの程度産生されるかを制御することによりその独自の特徴を獲得することが示唆されている。

【０００７】

しかし、それに続く包括的研究では、トランスクリプトームとプロテオームの相関が比較されて、すべての遺伝子の大多数が発現されていることが明らかにされた。興味深いことに、個々の遺伝子産物についてはＲＮＡレベルの変化とタンパク質レベルの変化の間に

は高い相関関係が存在することが明らかにされ、タンパク質の機能的役割との関連で個々の細胞中のトランスクリプトームを研究するのが生物学的に有用であることを示している。

【０００８】

実際、組織中の組織学及び発現パターンの解析は、生体医学研究及び診断学の礎石である。組織学は、異なる染色技法を利用して、１００年以上前に先ず健康な器官の基本的構造組織及び一般病理において起こる変化を確証した。この分野の発展により、免疫組織化学によるタンパク質分布及びインサイツハイブリダイゼーションによる遺伝子発現を研究できるようになった。

【０００９】

しかし、ますます進歩する組織学技法及び遺伝子発現技法が並行して発展してきたため、画像処理とトランスクリプトーム解析が分離され、本発明の方法まで、空間分解能での包括的トランスクリプトーム解析に利用できる実行可能な方法はなかった。

【００１０】

インサイツ技法に代わるものとして、又はインサイツ技法に加えて、タンパク質及び核酸のインビトロ解析のための、すなわち全組織試料、単一細胞型又は単一細胞からでも分子を抽出し、たとえば、ＥＬＩＳＡ、ｑＰＣＲ、等により前記抽出物中の特定の分子を定量することによる方法が発達してきた。

【００１１】

遺伝子発現解析の最近の発展により、マイクロアレイ又はＲＮＡシーケンシングを使用して組織の完全なトランスクリプトームを評価することの可能性が生じ、そのような発展は生物学的過程の理解と診断学のために役立ってきた。しかし、トランスクリプトーム解析は典型的には全組織から（又は全生物からでも）抽出されるｍＲＮＡに関して実施され、トランスクリプトーム解析のために比較的小さな組織区域又は個々の細胞を収集するための方法は典型的には労働集約的で費用がかかり精度が低い。

【００１２】

それ故に、マイクロアレイ又はＲＮＡの次世代シーケンシングに基づく遺伝子発現研究の大多数が、多くの細胞を含有する代表試料を使用する。したがって、その結果は調査される遺伝子の平均発現レベルを表す。表現型的に異なる細胞の分離が、いくつかの場合には包括的遺伝子発現プラットフォームと共に使用されてきており（Ｔａｎｇ Ｆ ｅｔ ａｌ．、Ｎａｔ Ｐｒｏｔｏｃ．２０１０年；５：５１６～３５頁；Ｗａｎｇ Ｄ ＆ Ｂｏｄｏｖｉｔｚ Ｓ、Ｔｒｅｎｄｓ Ｂｉｏｔｅｃｈｎｏｌ．２０１０年；２８：２８１～９０頁）、細胞間変異について極めて正確な情報をもたらした。しかし、無傷の組織において高分解能で転写活性を研究する高処理法は、現在まで利用可能になっていない。

【００１３】

したがって、遺伝子発現パターンの解析のための既存の技法は、空間的転写情報を提供するのは一度に１個の又は一握りの遺伝子についてのみである又は位置情報を失うという代償を払って試料中の遺伝子すべての転写情報を提供する。それ故に、試料中のそれぞれの細胞のトランスクリプトームを同時に、個別に、特異的に決定する、すなわち空間的分解能を備えたトランスクリプトーム情報をもたらす組織試料中の包括的遺伝子発現解析を可能にする方法が求められていることは明らかであり、本発明はこの要求に取り組んでいる。

【００１４】

本発明の方法及び生成物の新規のアプローチは、現在確立しているアレイ及びシーケンシング技術を利用して、転写物ごとの位置情報は保持したまま、試料中の遺伝子すべての転写情報をもたらす。これは生命科学における画期的な出来事を表していることは当業者には明らかとなるであろう。前記新技術は、いわゆる「空間トランスクリプトミクス」という新分野を開き、これによりすべての多細胞生物における組織発生並びに組織及び細胞機能の理解に深みのある結果がもたらされる可能性が高い。そのような技法は、疾患状態の原因及び進行を理解すること並びにそのような疾患、たとえば癌に対する効果的な治療

を開発することに特に有用となることが明らかであろう。本発明の方法は数多くの病状の診断においても使用されることになる。

【００１５】

最初はトランスクリプトーム解析という目的を念頭において考えていたが、下で詳細に説明されるように、本発明の原理及び方法はＤＮＡの解析にも適用され、それ故にゲノム解析（「空間ゲノミクス」）にも適用することができる。したがって、本発明はもっとも広くは、核酸全般の検出及び／又は解析に関する。

【００１６】

アレイ技術、特にマイクロアレイは、スタンフォード大学での研究から生まれ、少量のＤＮＡオリゴヌクレオチドが首尾よくガラス表面に整然とした配置、いわゆる「アレイ」で付着され、そのアレイを使用して４５の遺伝子の転写がモニターされた（Ｓｃｈｅｎａ Ｍ ｅｔ ａｌ．、Ｓｃｉｅｎｃｅ．１９９５年；２７０：３６８～９、３７１頁）。

【００１７】

それ以来、世界中の研究者たちがマイクロアレイ技術を使用して３００００件を超える論文を発表してきた。様々な適用、たとえば一塩基多型（ＳＮＰ）を検出する又は変異ゲノムの遺伝子型を同定する若しくは再シーケンシングするための複数種類のマイクロアレイが開発されており、マイクロアレイ技術の重要な使用は遺伝子発現の研究を目的としていた。実際、遺伝子発現マイクロアレイは特定の試料における発現される遺伝物質のレベルを解析する手段として生み出され、実質的利益は多くの遺伝子の発現レベルを同時に比較することができる点である。いくつかの市販のマイクロアレイプラットフォームがこれらの種類の実験に利用することが可能であるが、特別注文の遺伝子発現アレイを作製することも可能になってきた。

【００１８】

遺伝子発現研究におけるマイクロアレイの使用は現在では普通であるが、新しいさらに網羅的ないわゆる「次世代ＤＮＡシーケンシング」（ＮＧＳ）技術は多くの適用、たとえば徹底的（ｉｎ－ｄｅｐｔｈ）トランスクリプトーム解析でＤＮＡマイクロアレイに取って代わり始めている。

【００１９】

超高速ゲノムシーケンシングのためのＮＧＳ技術の開発は生命科学における画期的な出来事を表している（Ｐｅｔｔｅｒｓｏｎ Ｅ ｅｔ ａｌ．、Ｇｅｎｏｍｉｃｓ．２００９年；９３：１０５～１１頁）。これらの新しい技術のおかげでＤＮＡシーケンシングの経費は劇的に減り、特定の個体のゲノムを含む高等生物のゲノムを前例のない速度で決定できるようになった（Ｗａｄｅ ＣＭ ｅｔ ａｌ．、Ｓｃｉｅｎｃｅ．２００９年；３２６：８６５～７頁；Ｒｕｂｉｎ Ｊ ｅｔ ａｌ．Ｎａｔｕｒｅ ２０１０年；４６４：５８７～９１頁）。高処理ゲノミクスの新たな前進で生物学研究風景は姿を変え、ゲノムの完全な特徴付けに加えて完全なトランスクリプトームをデジタル及び定量的形式で研究することも可能である。これらの網羅的データセットを視覚化し統合するバイオインフォマティクスツールも近年かなり改良されてきた。

【００２０】

しかし、驚くべきことに、組織学技法、マイクロアレイ技法とＮＧＳ技法を独自に組み合わせることにより、組織試料中の複数の細胞から、その情報が二次元空間分解能により特徴付けられる網羅的転写又はゲノム情報が得られることが分かってきた。したがって、一方の極では本発明の方法を使用して、細胞を組織試料中のその背景に保持したまま、試料中の１個の細胞の１個の遺伝子の発現を解析することが可能である。もう一方の極、及び本発明の好ましい態様では、前記方法を使用して、試料中のありとあらゆる細胞、又は実質的にすべての細胞におけるすべての遺伝子の発現、すなわち組織試料の包括的空間的発現パターンを同時に決定することが可能である。本発明の方法により中間体解析を実施することも可能になることは明らかであろう。

【００２１】

そのもっとも簡単な形態では、本発明は以下のように要約して説明することができる。

本発明は、「アレイ」を作製するために、物体基板、たとえば、ガラススライド上に、独自の位置タグ（ドメイン）も含む逆転写（ＲＴ）プライマーを整列配置させる必要がある。独自の位置タグはアレイ上のＲＴプライマーの場所（アレイのフィーチャー）に対応する。薄い組織切片をアレイ上に置き、逆転写反応が物体スライド上の組織切片において実施される。組織試料中のＲＮＡが結合する（又はハイブリダイズする）、ＲＴプライマーは、結合したＲＮＡを鋳型として用いて伸長されてｃＤＮＡをもたらし、したがってｃＤＮＡはアレイ表面に結合している。ＲＴプライマー中の独自の位置タグの結果として、それぞれのｃＤＮＡ鎖は組織切片中の鋳型ＲＮＡの位置についての情報を担っている。組織切片は、ｃＤＮＡ合成ステップの前又は後で、視覚化又は撮像してもよく、たとえば染色して撮像して、ｃＤＮＡ分子中の位置タグを組織試料内の位置に関連付けることができるようにしてもよい。ｃＤＮＡはシーケンシングされ、正確な位置情報を備えたトランスクリプトームが得られる。前記手順の概略図は図１に示されている。次に、配列データは組織試料中の位置とマッチさせることができ、これにより、組織切片と一緒に配列データを、たとえばコンピュータを使用して視覚化して、たとえば、組織にわたって対象のどんな遺伝子の発現パターンでも表示することが可能になる（図２）。同様に、コンピュータ画面上で組織切片の異なる区域に印をつけ、対象のどんな選択された区域間の差次的に発現された遺伝子に関する情報でも得ることが可能だと考えられる。本発明の方法は、ｍＲＮＡ集団を研究する現在の方法を使用して得られるデータと全く対照的なデータが得られることは明らかであろう。たとえば、インサイツハイブリダイゼーションに基づく方法は、単一ｍＲＮＡ転写物の相対的情報を提供するだけである。したがって、本発明の方法は現在のインサイツ技術よりもはっきりした利点を有する。本発明の方法から得られる包括的遺伝子発現情報は、同時発現情報及び転写物存在量の量的推計も与える。これは、どんな種でも、たとえば動物、植物、真菌におけるどんな組織の解析にも利用可能な一般的に適用可能な戦略であることは明らかであろう。

【００２２】

上記のように及び下にさらに詳細に説明されるように、この基本的方法論は、たとえば１つ又は複数の特定の変異を含む細胞を組織試料内で同定するためにゲノムＤＮＡの解析にまで容易に拡大することができるのは明らかであろう。たとえば、ゲノムＤＮＡを断片化してプライマー（上に記載されるＲＴプライマーと同等）にハイブリダイズさせてもよく、このプライマーは、断片化されたＤＮＡを捕捉して（たとえば、前記プライマーと相補的である配列を有するアダプターを断片化されたＤＮＡにライゲートしてもよいし、たとえば配列の端部に追加のヌクレオチド、たとえばポリＡテールを組み込む酵素を使用して断片化されたＤＮＡを伸長させてプライマーに相補的である配列を生成してもよい）、捕捉分子に相補的鎖の合成をプライミングすることができる。解析の残りのステップは上記の通りでもよい。それ故に、トランスクリプトーム解析に関連して下に記載される本発明の具体的実施形態は、必要に応じてゲノムＤＮＡを解析する方法に用いてもよい。

【００２３】

位置情報をトランスクリプトーム又はゲノム情報に結び付けることに大きな価値があることは上記の説明から見て取れるであろう。たとえば、これにより、たとえば癌研究及び診断学を含む数多くの適用にその有用性を見出せる高分解能での包括的遺伝子発現マッピングが可能になる。

【００２４】

さらに、本明細書に記載される方法は、組織試料の包括的トランスクリプトームの解析のための既に記載されている方法とは著しく異なっていることは明らかであり、これらの違いが数多くの利点をもたらす。本発明は、組織切片を使用してもアレイ表面にカップリングされているプライマー（たとえば、逆転写プライマー）によりプライミングされるＤＮＡ（たとえば、ｃＤＮＡ）の合成に干渉しないという驚くべき発見に基づいている。

【００２５】

したがって、その最初のもっとも広い態様では、本発明は、組織試料中の核酸の局在化された検出のための方法であって、

（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、
前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）前記アレイ上の捕捉プローブの前記位置と、前記組織試料における位置とが関連付けられるように前記アレイを前記組織試料に接触させて、前記組織試料の核酸を前記捕捉プローブ中の前記捕捉ドメインにハイブリダイズさせるステップと、
（ｃ）前記捕捉プローブを伸長又はライゲーションプライマーとして用いて、捕捉された核酸分子からＤＮＡ分子を生成させるステップであって、
前記伸長された又はライゲートされたＤＮＡ分子が前記位置ドメインによりタグ付けされる、ステップと、
（ｄ）場合によってタグ付けされたＤＮＡの相補鎖を生成し、及び／又は場合によってタグ付けされたＤＮＡを増幅するステップと、
（ｅ）前記タグ付けされたＤＮＡ分子及び／又はその相補体若しくはアンプリコンの少なくとも一部を前記アレイの表面から放出するステップであって、
前記一部が前記位置ドメイン又はその相補体を含む、ステップと、
（ｆ）放出されたＤＮＡ分子の配列を直接的に又は間接的に解析するステップと、
を含む方法を提供する。

【００２６】

本発明の方法は、当技術分野で公知の空間トランスクリプトミクスのための他の方法よりも著しい前進を表している。たとえば、本明細書に記載される方法により、組織試料中のすべての転写物の包括的空間的プロファイルが得られる。さらに、すべての遺伝子の発現をアレイ上の位置又はフィーチャーごとに定量することができ、これにより多数の解析を単一のアッセイからのデータに基づいて実施できるようになる。したがって、本発明の方法により、単一の組織試料中のあらゆる遺伝子の空間的発現を検出する及び／又は定量することが可能になる。さらに、標準マイクロアレイに似て、転写物の存在量が直接的に視覚化（たとえば蛍光により）されないので、前記転写物が同じ試料中に極めて異なる濃度で存在していても単一試料中の遺伝子の発現を同時に測定することができる。

【００２７】

したがって、第二のより詳細な態様では、本発明は、組織試料のトランスクリプトームを決定する、及び／又は解析するための方法であって、
（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、
前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブが逆転写酵素（ＲＴ）プライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）前記アレイ上の捕捉プローブの前記位置と、前記組織試料における位置とが関連付けられるように前記アレイを前記組織試料に接触させて、前記組織試料のＲＮＡを前記捕捉プローブ中の前記捕捉ドメインにハイブリダイズさせるステップと、
（ｃ）前記捕捉プローブをＲＴプライマーとして用いて、捕捉されたＲＮＡ分子からｃＤＮＡ分子を生成するステップであって、場合によって前記ｃＤＮＡ分子を増幅するステッ

プと、
（ｄ）ｃＤＮＡ分子及び／又は場合によってそのアンプリコンの少なくとも一部を前記アレイの表面から放出するステップであって、放出された分子は第一鎖及び／若しくは第二鎖ｃＤＮＡ分子又はそのアンプリコンであり、前記一部には前記位置ドメイン又はその相補体が含まれるステップと、
（ｅ）放出された分子の配列を直接的に又は間接的に解析するステップと
を含む方法を提供するものとみることができる。

【００２８】

下にさらに詳細に説明されているように、核酸解析のいかなる方法も解析ステップにおいて使用することができる。典型的にはこれにはシーケンシングが含まれることがあるが、実際の配列決定を実施する必要はない。たとえば、配列特異的解析方法を使用してもよい。たとえば、配列特異的増幅反応を、たとえば位置ドメインに及び／又は特定の標的配列、たとえば検出されることになる特定の標的ＤＮＡ（すなわち、特定のｃＤＮＡ／ＲＮＡ又は遺伝子、等に対応する）に特異的であるプライマーを使用して実施してもよい。例となる解析法は配列特異的ＰＣＲ反応である。

【００２９】

ステップ（ｅ）で得られる配列解析情報を使用して、試料中のＲＮＡに関する空間的情報を得てもよい。言い換えると、配列解析情報は試料中のＲＮＡの場所に関する情報を提供することができる。この空間的情報は、決定される配列解析情報の性質から導かれることがあり、たとえば、この空間的情報は使用される組織試料との関連でそれ自体が空間的情報価値のある場合がある特定のＲＮＡの存在を明らかにすることがある及び／又は空間的情報（たとえば、空間的局在化）はシーケンシング情報と相俟ってアレイ上の組織試料の位置から導かれることがある。したがって、前記方法は、たとえば、位置タグ及び組織試料中の位置とのその相関関係によって、単に配列解析情報を組織試料中の位置と関連付けることを含んでいてもよい。しかし、上記のように、空間的情報は、都合の良いことに、配列解析データを組織試料の画像と関連付けることにより得られることもあり、これは本発明の１つの好ましい実施形態を表している。したがって、好ましい実施形態では、前記方法は、
（ｆ）前記配列解析情報を前記組織試料の画像と関連付け、組織試料がステップ（ｃ）の前又は後で撮像されるステップ
も含む。

【００３０】

そのもっとも広い意味で、本発明の方法を組織試料中の核酸の局在化された検出のために使用してもよい。したがって、一実施形態では、本発明の方法は組織試料のトランスクリプトーム又はゲノムのすべてを、たとえば組織試料の包括的トランスクリプトームを決定する及び／又は解析するために使用してもよい。しかし、前記方法はこれに限定されることはなく、トランスクリプトーム又はゲノムのすべて又は一部を決定する及び／又は解析することを包含する。したがって、前記方法はトランスクリプトーム又はゲノムの一部又はサブセット、たとえば遺伝子のサブセットに対応するトランスクリプトーム、たとえば、たとえば特定の疾患又は状態、組織型、等に関連する特定の遺伝子のセットを決定する及び／又は解析することを含んでいてもよい。

【００３１】

別の態様から見ると、上で提示された方法ステップは、空間的に限定されたトランスクリプトーム又はゲノム、特に組織試料の空間的に限定された包括的トランスクリプトーム又はゲノムを得る方法を提供するものとみることが可能である。

【００３２】

別の見方をすれば、本発明の方法は、組織試料中のＤＮＡであれＲＮＡであれ、核酸の局在化された若しくは空間的検出のための、又は組織試料中の核酸（ＤＮＡ又はＲＮＡ）の局所的若しくは空間的決定及び／若しくは解析のための方法とみることもできる。特に、前記方法は、組織試料中の遺伝子発現又はゲノム変動の局在化された若しくは空間的検

出又は決定及び／若しくは解析のために使用してもよい。局在化された／空間的検出／決定／解析とは、ＲＮＡ又はＤＮＡが組織試料中の細胞又は組織内のその天然の位置又は場所に局在していることがあることを意味する。したがって、たとえば、ＲＮＡ又はＤＮＡは試料中の細胞若しくは細胞の群若しくは細胞の型に又は組織試料内の区域の特定の領域に局在していてもよい。ＲＮＡ又はＤＮＡ、たとえば発現された遺伝子又はゲノム遺伝子座の天然の場所又は位置（又は言い換えれば、組織試料中のＲＮＡ若しくはＤＮＡの場所若しくは位置）、を決定してもよい。

【００３３】

本発明は、捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備え、前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーションプライマーとして機能できるように遊離の３’端部を有する配向となっており、前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、

（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び

（ｉｉ）前記アレイに接触させる前記組織試料のＲＮＡを捕捉するための捕捉ドメイン

を有する核酸分子を含む、本発明の方法において使用するためのアレイを提供するものとみることもできる。

【００３４】

関連する態様では、本発明は、捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備え、前記捕捉プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーションプライマーとして機能できるように遊離の３’端部を有する配向となっており、前記アレイに接触させる組織試料のＲＮＡを捕捉するように、前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、

（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び

（ｉｉ）捕捉ドメイン

を有する核酸分子を含む、アレイの使用も提供する。

【００３５】

好ましくは、前記使用は、組織試料のトランスクリプトーム、特に包括的トランスクリプトームを決定する及び／又は解析することを目的とし、

（ａ）前記捕捉プローブをＲＴプライマーとして用いて捕捉されたＲＮＡ分子からｃＤＮＡ分子を生成し、場合によって前記ｃＤＮＡ分子を増殖するステップと、

（ｂ）ｃＤＮＡ分子及び／又は場合によってそのアンプリコンの少なくとも一部をアレイの表面から放出するステップであって、前記放出された分子が第一鎖及び／又は第二鎖ｃＤＮＡ分子でも又はそのアンプリコンであってもよく、前記一部が位置ドメイン又はその相補体を含むステップと、

（ｃ）放出された分子の配列を直接的に又は間接的に解析するステップと、場合によって

（ｄ）前記配列解析情報を前記組織試料の画像と関連付け、前記組織試料がステップ（ａ）の前又は後で撮像されるステップと

をさらに含む。

【００３６】

したがって、本発明のアレイを使用して、前記アレイに接触させる組織試料のＲＮＡ、たとえばｍＲＮＡを捕捉してもよいことが理解されるであろう。前記アレイは、組織試料の部分的若しくは包括的トランスクリプトームを決定する及び／若しくは解析するために又は組織試料の空間的に限定された部分的若しくは包括的トランスクリプトームを得るために使用してもよい。したがって、本発明の方法は、組織試料中の１つ又は複数の遺伝子の空間的発現を定量する方法とみることもできる。別の方法で表現すれば、本発明の方法を使用して、組織試料中の１つ又は複数の遺伝子の空間的発現を検出してもよい。さらに別の方法では、本発明の方法を使用して、組織試料内の１つ又は複数の位置で１つ又は複数の遺伝子の発現を同時に決定してもよい。さらに、前記方法は、二次元空間分解能での

組織試料の部分的又は包括的トランスクリプトーム解析のための方法とみることもできる。

【００３７】

ＲＮＡは細胞中に存在することのあるいかなるＲＮＡ分子でもよい。したがって、ＲＮＡは、ｍＲＮＡ、ｔＲＮＡ、ｒＲＮＡ、ウイルスＲＮＡ、核内低分子ＲＮＡ（ｓｎＲＮＡ）、核小体低分子ＲＮＡ（ｓｎｏＲＮＡ）、マイクロＲＮＡ（ｍｉＲＮＡ）、低分子干渉ＲＮＡ（ｓｉＲＮＡ）、ｐｉｗｉ結合ＲＮＡ（ｐｉＲＮＡ）、リボザイマルＲＮＡ、アンチセンスＲＮＡ又は非コードＲＮＡでもよい。しかし、好ましくは、ＲＮＡはｍＲＮＡである。

【００３８】

捕捉されたＲＮＡからｃＤＮＡを生成する上記の方法におけるステップ（ｃ）（上で提示された使用の好ましい記述におけるステップ（ａ）に相当する）は、ｃＤＮＡの合成に関係するとみることもできるであろう。この合成は、捕捉されたＲＮＡを鋳型として用いて、ＲＴプライマーとして機能する捕捉プローブを伸長する、捕捉されたＲＮＡの逆転写のステップを含むことになる。そのようなステップはいわゆる第一鎖ｃＤＮＡを生成する。下でさらに詳細に説明されるように、第二鎖ｃＤＮＡ合成は、場合によってアレイ上で行われてもよく、又はアレイからの第一鎖ｃＤＮＡの放出後別々のステップで行われてもよい。下でもさらに詳細に説明されるように、ある種の実施形態では、第二鎖合成は放出された第一鎖ｃＤＮＡ分子の増幅の第一ステップで行われてもよい。

【００３９】

核酸解析一般、特にＤＮＡ解析との関連において使用するためのアレイは、下で考察され説明される。ＲＮＡとの関連において使用するためのアレイ及び捕捉プローブとの関係で本明細書に記載される具体的詳細及び実施形態は、ＤＮＡを用いて使用するためのアレイを含むそのようなアレイすべてに等しく（必要に応じて）適用する。

【００４０】

本明細書で使用されるように、用語「複数の」は、２つ若しくはそれよりも多い、又は少なくとも２つ、たとえば３、５、１０、１５、２０、３０、４０、５０、６０、７０、８０、９０、１００、１５０、２００、４００、５００、１０００、２０００、５０００、１００００又はそれよりも多い、等を意味する。したがって、たとえば、捕捉プローブの数は、前述の数のうちのいずれか２つの間のどんな範囲のどんな整数でもよい。しかし、何百、何千、何万、何十万又は何百万の捕捉プローブでも有する従来型のアレイを使用できることが想定されていることは認識されるであろう。

【００４１】

したがって、本明細書で概説される方法は、組織試料、たとえば組織試料の薄切片又は「切片」内の単一細胞すべてから転写物を捕捉し標識するための「捕捉プローブ」を含む高密度核酸アレイを利用する。解析のための組織試料又は切片は、切片内の空間的情報が保持されるように、高度に平行化された形式で作製される。細胞ごとに捕捉されたＲＮＡ（好ましくはｍＲＮＡ）分子、又は「トランスクリプトーム」はｃＤＮＡに転写され、得られたｃＤＮＡ分子は、たとえば高処理シーケンシングにより解析される。得られたデータは元の組織試料、たとえば切片の画像と整列配置させた核酸プローブに取り込まれているいわゆるバーコード配列（又は、位置ドメインとして本明細書で定義されたＩＤタグ）を通じて関連付けることができる。

【００４２】

高密度核酸アレイ又はマイクロアレイは、本明細書に記載される空間的トランスクリプトーム標識化法の中核的構成要素である。マイクロアレイは分子生物学で使用されるマルチプレックス技術である。典型的マイクロアレイは、整列配置された一連のオリゴヌクレオチドの微細なスポットからなる（何十万、一般には何万のスポットを１つのアレイ上に組み込むことができる）。それぞれの核酸（オリゴヌクレオチド）スポット（オリゴヌクレオチド／核酸分子のそれぞれのスピーシーズ）の別個の位置は「フィーチャー」として知られ（それ故に、上に提示される方法においては、捕捉プローブそれぞれのスピーシー

ズはそのアレイの特異的フィーチャーとみることもでき、それぞれのフィーチャーはアレイ上の別個の位置を占める）、典型的にはそれぞれの別々のフィーチャーは、「プローブ」（又は「リポーター」）として知られる特定のＤＮＡ配列（「スピーシーズ」）をピコモル（10^{-12}モル）の範囲で含んでいる。典型的には、これらの配列は、ｃＤＮＡ又はｃＲＮＡ試料（又は「標的」）が高厳密ハイブリダイゼーション条件下でハイブリダイズすることができる遺伝子又は他の核酸エレメントの短いセクションであることが可能である。しかし、下に説明されるように、本発明のプローブは標準マイクロアレイのプローブとは異なる。

【００４３】

遺伝子発現マイクロアレイでは、プローブ－標的ハイブリダイゼーションは通常、標的のすべてに組み込まれている視覚シグナル、たとえばフルオロフォア、銀イオン又は化学発光標識の検出により検出され定量される。視覚シグナルの強度は試料中のそれぞれの標的核酸の相対的存在量と相関している。アレイは何万個ものプローブを含有することができるので、マイクロアレイ実験は多くの遺伝子試験を平行して遂行することが可能である。

【００４４】

標準マイクロアレイでは、プローブは、化学マトリックス、たとえばエポキシシラン、アミノシラン、リジン、ポリアクリルアミド、等への共有結合により固体表面又は基板に付着される。基板は典型的には、ガラス、プラスチック又はシリコンチップ若しくはスライドであるが、他のマイクロアレイプラットフォーム、たとえば微小ビーズが知られている。

【００４５】

プローブはいかなる適切な手段によって本発明のアレイに付着させてもよい。好ましい実施形態では、プローブは、化学的固定化によりアレイの基板に固定化される。この固定化は、化学反応に基づく基板（支持材）とプローブ間の相互作用であってもよい。そのような化学反応は典型的には、熱又は光を介したエネルギーの入力に頼ってはいないが、熱、たとえば化学反応のための一定の最適温度又は一定の波長の光のどちらかを適用することにより増強することができる。たとえば、化学的固定化は基板上の官能基とプローブ上の対応する機能要素間で起こることがある。プローブ中のそのような対応する機能要素は、プローブの固有の化学基、たとえばヒドロキシル基でもよく、又は追加で導入されてもよい。そのような官能基の例はアミン基である。典型的には、固定化されるプローブは機能的アミン基を含む又は機能的アミン基を含むように化学的に修飾される。そのような化学修飾のための手段と方法は周知である。

【００４６】

固定化されるプローブ内の前記官能基の局在化は、プローブの結合行動及び／又は配向性を制御し形成するために使用してもよく、たとえば官能基はプローブの５’若しくは３’端部に又はプローブの配列内に置いてもよい。固定化されるプローブのための典型的な基板は、そのようなプローブに、たとえばアミン官能性をもたせた核酸に結合することができる部分を含む。そのような基板の例は、カルボキシ、アルデヒド又はエポキシ基板である。そのような材料は当業者には公知である。官能基は、アミン基の導入により化学的反応性のあるプローブとアレイ基板間の接続反応を与えるが、当業者には公知である。

【００４７】

プローブを固定化してもよい代わりの基板は、たとえばアレイ基板上で利用可能な官能基の活性化により、化学的に活性化されなければならないことがある。用語「活性化された基板」とは、相互作用する又は反応性の化学官能基が当業者には公知である化学修飾法により確立された又は可能になった材料に関連している。たとえば、カルボキシル基を備える基板は使用する前に活性化しなければならない。さらに、核酸プローブ中に既に存在する特異的な部分と反応することができる官能基を含有する入手可能な基板が存在する。

【００４８】

代わりに、プローブを基板上で直接合成してもよい。そのようなアプローチに適した方

法は当業者には公知である。例は、Ａｇｉｌｅｎｔ Ｉｎｃ．、Ａｆｆｙｍｅｔｒｉｘ Ｉｎｃ．、Ｒｏｃｈｅ Ｎｉｍｂｌｅｇｅｎ Ｉｎｃ．又はＦｌｅｘｇｅｎ ＢＶにより開発された製造技法である。典型的には、ヌクレオチド付加物が占めることになるスポットを特異的に活性化するレーザーと１組の鏡が使用される。そのようなアプローチは、たとえば約３０μｍ又はそれよりも大きいスポットサイズ（すなわち、フィーチャー）を提供する可能性がある。

【００４９】

したがって、基板は当業者に公知のいかなる適切な基板でもよい。基板はどんな適切な形態又はフォーマットを有していてもよく、たとえば基板は、平らである、湾曲している、たとえば、組織試料と基板間の相互作用が起こる区域のほうに凸状に又は凹形に湾曲していてもよい。特に好ましいのは、基板が平らである、すなわち平面状チップ又はスライドである場所である。

【００５０】

典型的には、基板は固体支持体であり、それによって基板上のプローブの正確で追跡可能な位置決めを可能にする。基板の例は固体材料又は機能的化学基、たとえばアミン基又はアミン官能性をもった基を備える基板である。本発明により想定されている基板は非多孔性基板である。好ましい非多孔性基板はガラス、シリコン、ポリ－Ｌ－リジン被覆材料、ニトロセルロース、ポリスチレン、環状オレフィンコポリマー（ＣＯＣ）、環状オレフィンポリマー（ＣＯＰ）、ポリプロピレン、ポリエチレン及びポリカーボネートである。

【００５１】

当業者に公知のいかなる適切な材料を使用してもよい。典型的には、ガラス又はポリスチレンが使用される。ポリスチレンは通常、親水性基をほとんど含有していないので負電荷巨大分子に結合するのに適した疎水性材料である。ガラススライド上に固定化された核酸では、ガラス表面の疎水性を増加することにより核酸固定化を増加することがあることはさらに公知である。そのような増強により相対的にさらに高密度に詰まった形成が可能になることがある。ポリ－Ｌ－リジンを用いた被覆又は表面処理に加えて、基板、特にガラスは、たとえばエポキシシラン若しくはアミノシランを用いたシラン化（ｓｉｌａｎａｔｉｏｎ）により又はシリン化（ｓｉｌｙｎａｔｉｏｎ）により又はポリアクリルアミドを用いた処理により処理してもよい。

【００５２】

いくつかの標準アレイが市販されており、フィーチャーの数もサイズも変えることができる。本発明では、フィーチャーの配置は、異なる組織又は生物に存在する細胞のサイズ及び／又は密度に対応するように変更してもよい。たとえば、動物細胞は典型的には１～１００μｍの領域の断面を有し、植物細胞の断面は典型的には１～１００００μｍに及ぶ場合がある。それ故に、Ｎｉｍｂｌｅｇｅｎ（登録商標）アレイは最大２１０万フィーチャー、又は４２０万フィーチャー、及び１３マイクロメーターのフィーチャーサイズのものが入手可能であるが、動物又は真菌由来の組織試料に好まれ、たとえば８×１３０ｋフィーチャーを有する他のフォーマットは、植物組織試料には十分であることがある。市販されているアレイは、配列解析との関連で、特にＮＧＳ技術との関連で使用するためにも利用可能である又は知られている。そのようなアレイは、本発明という背景で、アレイ表面、たとえばＩｌｌｕｍｉｎａビーズアレイとして使用することもできる。それ自体を注文に応じて作製することができる市販のアレイに加えて、注文の又は非標準「自製」アレイを作製することが可能であり、アレイを作製するための方法は確立している。本発明の方法は、下に定義されるプローブを備える標準と非標準アレイの両方を利用してもよい。

【００５３】

マイクロアレイ上のプローブは、アレイの化学マトリックスに応じて、好ましくは５’又は３’端部を介してアレイに固定化される、すなわち付着される若しくは結合されてもよい。典型的には、市販のアレイでは、プローブは３’連鎖を介して付着され、それによって遊離の５’端部を残す。しかし、５’連鎖を介して基板に付着され、それによって遊離の３’端部を残すプローブを備えるアレイは利用可能であり、当技術分野で周知であり

本明細書の他所に記載されている標準技法を使用して合成してもよい。

【００５４】

核酸プローブをアレイ基板に結合させるのに使用される共有結合は、プローブは「直接」共有結合により付着するが、核酸プローブの「最初の」ヌクレオチドをたとえば、ガラス又はシリコン基板から分離する化学的部分又はリンカー、すなわち間接的連鎖が存在することがあるという点で、直接的及び間接的連鎖の両方とみることもできる。本発明の目的のために、共有結合及び／又は化学リンカーにより基板に固定化されるプローブは一般には基板に直接的に固定化される又は付着されるとみることもできる。

【００５５】

下でさらに詳細に説明されることになるように、本発明の捕捉プローブはアレイに直接的に又は間接的に、固定化されても又はアレイと相互作用してもよい。したがって、捕捉プローブはアレイに直接結合しなくてもよいが、たとえばそれ自体がアレイに直接的に又は間接的に結合する分子に結合することにより間接的に相互作用することがある（たとえば、捕捉プローブは捕捉プローブの結合パートナー、すなわちそれ自体がアレイに直接的に又は間接的に結合している表面プローブと相互作用する（たとえば、結合する又はハイブリダイズする）ことがある）。しかし、一般的に言えば、捕捉プローブは直接的に又は間接的に（１つ又は複数の媒介物により）アレイに結合している又は固定化されていることになる。

【００５６】

本発明の使用、方法及びアレイは、その５’又は３’端部を介して固定化されているプローブを含むことがある。しかし、捕捉プローブは、アレイ基板に直接的に固定化されている場合、唯一、捕捉プローブの３’端部が伸長されるように遊離している、たとえば捕捉プローブがその５’端部により固定化されているように固定化されていてもよい。捕捉プローブは、それが遊離の、すなわち伸長可能である、３’端部を有するように間接的に固定化されていてもよい。

【００５７】

伸長された又は伸長可能な３’端部とは、追加のヌクレオチドが核酸分子、たとえば捕捉プローブのもっとも３’側のヌクレオチドに付加されて核酸分子の長さを伸長すること、すなわち核酸分子を伸長するのに利用される標準重合反応、たとえばポリメラーゼにより触媒される鋳型重合を意味する。

【００５８】

したがって、一実施形態では、アレイは３’端部を介して直接的に固定化されているプローブ、いわゆる表面プローブを備え、このプローブは下に定義されている。表面プローブのそれぞれのスピーシーズは、捕捉プローブが表面プローブにハイブリダイズするように、捕捉プローブのそれぞれのスピーシーズに相補的な領域を含むので、捕捉プローブは遊離の伸長可能な３’端部を含む。本発明の好ましい態様では、アレイが表面プローブを備える場合、捕捉プローブはアレイ上でインサイツで合成される。

【００５９】

アレイプローブは、リボヌクレオチド及び／又はデオキシリボヌクレオチド並びにワトソンークリック型又は類似の塩基対相互作用に関与することができる合成ヌクレオチド残基で構成されていてもよい。したがって、核酸ドメインは、ＤＮＡ又はＲＮＡ又はその任意の改変物、たとえばＰＮＡ若しくは非ヌクレオチド骨格を含有する他の誘導体でもよい。しかし、トランスクリプトーム解析との関連では、捕捉プローブの捕捉ドメインは、捕捉されたＲＮＡ分子に相補的であるｃＤＮＡを生成する逆転写反応をプライミングすることができなければならない。下にさらに詳細に説明されるように、ゲノム解析との関連では、捕捉プローブの捕捉ドメインは、ＤＮＡ断片に結合することができなければならず、これには断片化されたＤＮＡに既に付加されている結合ドメインに結合することを含むことがある。いくつかの実施形態では、捕捉プローブの捕捉ドメインはＤＮＡ伸長（ポリメラーゼ）反応をプライミングして、捕捉されたＤＮＡ分子に相補的なＤＮＡを生成することがある。他の実施形態では、捕捉ドメインは、捕捉されたＤＮＡ分子と基板に直接的に

又は間接的に固定化されている表面プローブ間のライゲーション反応の鋳型になることがある。さらに他の実施形態では、捕捉ドメインは捕捉されたＤＮＡ分子の１つの鎖にライゲートされることがある。

【００６０】

本発明の好ましい実施形態では、捕捉プローブの少なくとも捕捉ドメインは、デオキシリボヌクレオチド（ｄＮＴＰ）を含む又はからなる。特に好ましい実施形態では、捕捉プローブの全体がデオキシリボヌクレオチドを含む又はからなる。

【００６１】

本発明の好ましい実施形態では、捕捉プローブはアレイの基板に直接的に、すなわちその５’端部により固定化されており、遊離の伸長可能な３’端部を有する。

【００６２】

本発明の捕捉プローブは少なくとも２つのドメイン、すなわち捕捉ドメインと位置ドメイン（又はフィーチャー識別タグ若しくはドメイン；位置ドメインは代わりに識別（ＩＤ）ドメイン若しくはタグ、又は位置タグと定義されることもある）を含む。捕捉プローブは、下でさらに定義されるユニバーサルドメインをさらに含むことがある。捕捉プローブが、表面プローブへのハイブリダイゼーションを介してアレイ表面に間接的に付着している場合、捕捉プローブは表面プローブに相補的な配列（たとえば、部分又はドメイン）を必要とする。そのような相補的配列は表面プローブ上の位置／識別ドメイン及び／又はユニバーサルドメインに相補的であってもよい。言い換えると、位置ドメイン及び／又はユニバーサルドメインは、表面プローブに相補的であるプローブの領域又は部分を構成することもある。しかし、捕捉プローブは、表面プローブに相補的である追加のドメイン（又は領域、部分若しくは配列）を含むこともある。合成を容易にするために、下にさらに詳細に説明されているように、そのような表面プローブ相補的領域は、捕捉ドメインの一部として又は伸長物として提供されることもある（そのような一部又は伸長物はそれ自体が、標的核酸（たとえばＲＮＡ）に結合するために使用される又は結合することができる）。

【００６３】

捕捉ドメインは典型的には捕捉プローブの３’端部に位置しており、たとえば鋳型依存性重合により伸長させることができる遊離の３’端部を含む。捕捉ドメインは、アレイに接触している組織試料の細胞に存在する核酸、たとえばＲＮＡ（好ましくはｍＲＮＡ）にハイブリダイズすることができるヌクレオチド配列を含む。

【００６４】

有利なことに、捕捉ドメインは、特定の核酸、たとえば検出する又は解析するのが望まれるＲＮＡに選択的に又は特異的に結合するように選択される又は設計されてもよい（又は、もっと一般的に言えば、結合することができてもよい）。たとえば、捕捉ドメインは、ｍＲＮＡの選択的捕捉のために選択される又は設計されてもよい。当技術分野で周知であるように、これはｍＲＮＡのポリＡテールへのハイブリダイゼーションに基づいていることがある。したがって、好ましい実施形態では、捕捉ドメインは、ポリＴ　ＤＮＡオリゴヌクレオチド、すなわちｍＲＮＡのポリＡテールにハイブリダイズすることができる、リン酸ジエステル結合により連結された一連の連続するデオキシチミジン残基を含む。代わりに、捕捉ドメインは、ポリＴに機能的に又は構造的に類似する、すなわちポリＡに選択的に結合することができるヌクレオチド、たとえばポリＵオリゴヌクレオチド又はデオキシチミジン類似体からなり、ポリＡに結合する機能特性を保持しているオリゴヌクレオチド、を含むことがある。特に好ましい実施形態では、捕捉ドメイン、又はさらに詳細には捕捉ドメインのポリＴエレメントは、少なくとも１０ヌクレオチド、好ましくは少なくとも１１、１２、１３、１４、１５、１６、１７、１８、１９又は２０ヌクレオチドを含む。追加の実施形態では、捕捉ドメイン、又はさらに詳細には捕捉ドメインのポリＴエレメントは、少なくとも２５、３０又は３５ヌクレオチドを含む。

【００６５】

当技術分野で公知であるように、ランダム配列、たとえばランダムヘキサマー又は類似

の配列も核酸の捕捉に使用することがあり、それ故にそのようなランダム配列を使用して、捕捉ドメインのすべて又は一部を形成してもよい。たとえば、ランダム配列はポリＴ（又はポリＴ類似物、等）配列と併せて使用することがある。したがって、捕捉ドメインは、ポリＴ（又は「ポリＴ様」）オリゴヌクレオチドを含む場合、ランダムオリゴヌクレオチド配列も含むことがある。これはたとえば、ポリＴ配列の５’又は３’、たとえば捕捉プローブの３’端部に位置することがあるが、そのようなランダム配列の位置決めは決定的に重要ではない。そのような構築物は、ｍＲＮＡのポリＡの最初の一部の捕捉を促進することができる。代わりに、捕捉ドメインは完全ランダム配列でもよい。当技術分野で公知の原理によれば、縮重捕捉ドメインを使用してもよい。

【００６６】

捕捉ドメインは、核酸（たとえばＲＮＡ）の所望のサブタイプ若しくはサブセット、たとえば、上に収載されているようにｍＲＮＡ若しくはｒＲＮＡ、等などの特定のタイプのＲＮＡに、又は、たとえば特定の遺伝子若しくは遺伝子群に応じて、所与のタイプのＲＮＡの特定のサブセット、たとえば特定のｍＲＮＡ種に選択的に結合することができることがある。そのような捕捉プローブは、捕捉されることが望まれるＲＮＡの配列に基づいて選択する又は設計してもよい。したがって、捕捉プローブは、特定のＲＮＡ標的又は標的の群（標的群、等）に特異的な配列特異的捕捉プローブであってもよい。したがって、捕捉プローブは、当技術分野で周知の原理に従って、特定の遺伝子配列又は特定のモチーフ配列又は共通／保存配列、等に基づいていてもよい。

【００６７】

捕捉プローブがアレイの基板に間接的に、たとえば表面プローブへのハイブリダイゼーションを介して固定化されている実施形態では、捕捉ドメインは、表面プローブの５’端部にハイブリダイズすることができる上流配列（組織試料の核酸、たとえばＲＮＡにハイブリダイズする配列の５’側）をさらに含むことがある。単独では、捕捉プローブの捕捉ドメインは、捕捉ドメインオリゴヌクレオチドとみることもでき、前記オリゴヌクレオチドは捕捉プローブがアレイ上に間接的に固定化されている実施形態において捕捉プローブの合成で使用してもよい。

【００６８】

捕捉プローブの位置ドメイン（フィーチャー識別ドメイン又はタグ）は捕捉ドメインの上流、すなわち捕捉プローブ核酸分子の５’端部により近いほうに直接的に又は間接的に位置している。好ましくは、位置ドメインは捕捉ドメインに直接隣接している、すなわち捕捉ドメインと位置ドメインの間には中間配列が存在しない。いくつかの実施形態では、位置ドメインはアレイの基板上に直接的に又は間接的に固定化されていてもよい捕捉プローブの５’端部を形成する。

【００６９】

上記のように、アレイのそれぞれのフィーチャー（別個の位置）は、それぞれのフィーチャーでの位置ドメインが独自である核酸プローブのスピーシーズのスポットを含む。したがって、捕捉プローブの「スピーシーズ」はその位置ドメインに準拠して定義され、捕捉プローブの単一スピーシーズは同じ位置ドメインを有することになる。しかし、捕捉プローブのスピーシーズのそれぞれのメンバーは全部同じ配列を有する必要はない。特に、捕捉ドメインはランダム若しくは縮重配列であってもよく又はランダム若しくは縮重配列を含んでいてもよいので、スピーシーズ内の個々のプローブの捕捉ドメインは変化してもよい。したがって、捕捉プローブの捕捉ドメインが同じであるいくつかの実施形態では、それぞれのフィーチャーは単一プローブ配列を含む。しかし、捕捉プローブが変化する他の実施形態では、プローブのスピーシーズのメンバーは全く同じ配列を有すことにはならないが、スピーシーズ内のそれぞれのメンバーの位置ドメインの配列は同じになる。求められているのは、アレイのそれぞれのフィーチャー又は位置が単一スピーシーズの捕捉プローブを担っていることである（具体的には、それぞれのフィーチャー又は位置は、同一の位置タグを有する捕捉プローブを担っている、すなわちそれぞれのフィーチャー又は位置には単一の位置ドメインが存在する）。それぞれのスピーシーズは、前記スピーシーズ

を識別する異なる位置ドメインを有する。しかし、スピーシーズのそれぞれのメンバーは、捕捉ドメインはランダムでも若しくは縮重していてもよく又はランダム若しくは縮重成分を有していてもよいので、いくつかの場合には、本明細書でさらに詳細に説明されているように、異なる捕捉ドメインを有することがある。これは、所与のフィーチャー又は位置内ではプローブの捕捉ドメインは異なることがあることを意味する。

【００７０】

したがって、必ずしもすべてではないがいくつかの実施形態では、特定のフィーチャーで固定化されたいずれか１つのプローブ分子のヌクレオチド配列は、同じフィーチャーで固定化されたその他のプローブ分子と同じであるが、それぞれのフィーチャーでのプローブのヌクレオチド配列は他のすべてのフィーチャーで固定化されたプローブとは異なっており、別であり又は区別可能である。好ましくは、それぞれのフィーチャーはプローブの異なるスピーシーズを含む。しかし、いくつかの実施形態では、フィーチャーの群がプローブの同じスピーシーズを含む、すなわち、たとえばアレイの解像度を下げるために、単一フィーチャーよりも大きなアレイの区域に及ぶフィーチャーを効果的に作製するのが有利であることがある。アレイの他の実施形態では、特定のフィーチャーで固定化されたいずれか１つのプローブ分子の位置ドメインのヌクレオチド配列は、同じフィーチャーで固定化されたその他のプローブ分子と同じでよいが、捕捉ドメインは変わることがある。にもかかわらず、捕捉ドメインは同じ種類の分子（たとえばｍＲＮＡ）全般を捕捉するように設計してもよい。

【００７１】

捕捉プローブの位置ドメイン（又はタグ）は、それぞれのフィーチャーにしか含まれない配列を含み、位置又は空間マーカー（識別タグ）として機能する。このように、組織試料のそれぞれの領域又はドメイン（たとえば組織中のそれぞれの細胞）は、核酸（たとえばある種の細胞由来のＲＮＡ（たとえば、転写物））を捕捉プローブ中の独自の位置ドメイン配列に連結しているアレイ全域で空間分解能により識別可能になる。位置ドメインのせいで、アレイ中の捕捉プローブは組織試料中の位置に関連付けられることがある、たとえば捕捉プローブは試料中の細胞に関連付けられることがある。したがって、捕捉ドメインの位置ドメインは核酸タグ（識別タグ）とみることもできる。

【００７２】

いかなる適切な配列も本発明の捕捉プローブ中の位置ドメインとして用いてもよい。適切な配列とは、位置ドメインが組織試料のＲＮＡと捕捉プローブの捕捉ドメイン間の相互作用に干渉する（すなわち、阻害する又は歪める）べきではないことを意味する。たとえば、位置ドメインは、組織試料中の核酸分子が位置ドメインと特異的にハイブリダイズしないように設計されるべきである。好ましくは、捕捉プローブの位置ドメインの核酸配列は組織試料中の核酸配列に対して８０％未満の配列同一性を有する。好ましくは、捕捉プローブの位置ドメインは組織試料中の核酸分子の実質的な部分にわたり７０％未満、６０％未満、５０％未満又は４０％未満の配列同一性を有する。配列同一性は当技術分野でいかなる公知の適切な方法でも、たとえばＢＬＡＳＴアライメントアルゴリズムにより決定してもよい。

【００７３】

好ましい実施形態では、捕捉プローブのそれぞれのスピーシーズの位置ドメインは独自のバーコード配列を含有する。バーコード配列は、ランダム配列生成を使用して生成してもよい。ランダムに生成された配列に続いて、すべての共通参照種のゲノムに位置付けることにより及び予め設定されたＴｍ間隔、ＧＣ含有量及び確実にバーコード配列が組織試料由来の核酸、たとえばＲＮＡの捕捉に干渉せず難なく互いに区別可能であるようにするその他のバーコード配列までの異なる限定された距離を用いて厳密なフィルタリングを行ってもよい。

【００７４】

上記のように及び好ましい実施形態では、捕捉プローブはユニバーサルドメイン（又はリンカードメイン若しくはタグ）も含む。捕捉プローブのユニバーサルドメインは、位置

ドメインの上流、すなわち捕捉プローブ核酸分子の５’端部により近いほうに直接的に又は間接的に位置している。好ましくは、ユニバーサルドメインは位置ドメインに直接隣接している、すなわち位置ドメインとユニバーサルドメイン間には中間配列が存在しない。捕捉プローブがユニバーサルドメインを含む実施形態では、前記ドメインは捕捉プローブの５’端部を形成し、前記端部はアレイの基板上に直接的に又は間接的に固定化されてもよい。

【００７５】

ユニバーサルドメインは本発明の方法及び使用においていくつかのやり方で利用されることがある。たとえば、本発明の方法は、合成された（すなわち、伸長された又はライゲートされた）アレイの表面由来の核酸（たとえばｃＤＮＡ）分子の少なくとも一部を放出する（たとえば、取り除く）ステップを含む。本明細書の他所で説明されるように、これはいくつかのやり方で達成されてもよく、そのうちの１つはアレイの表面由来の核酸（たとえばｃＤＮＡ）分子を切断することを含む。したがって、ユニバーサルドメインはそれ自体が、切断ドメイン、すなわち化学的に又は好ましくは酵素的にのいずれかで特異的に切断することができる配列を含むことがある。

【００７６】

したがって、切断ドメインは、核酸分子を切断することができる、すなわち２つ又はそれよりも多いヌクレオチド間のリン酸ジエステル連鎖を切断することができる１つ又は複数の酵素により認識される配列を含むことがある。たとえば、切断ドメインは制限エンドヌクレアーゼ（制限酵素）認識配列を含むことがある。制限酵素は制限部位として知られる特異的な認識ヌクレオチド配列で二本鎖又は一本鎖ＤＮＡを切断し、適切な酵素は当技術分野では周知である。たとえば、低頻度切断酵素、すなわち核酸（たとえばｃＤＮＡ）分子の他の場所で切断する可能性を抑えるために長い認識部位（少なくとも８塩基対長）を有する酵素を使用するのが特に有利である。この点に関して、核酸（たとえばｃＤＮＡ）分子の少なくとも一部を取り除く又は放出するには核酸（たとえばｃＤＮＡ）の位置ドメイン及び前記ドメインの下流の配列のすべて、すなわち位置ドメインの３’側の配列のすべてを含む一部を放出する必要があることは理解されるであろう。それ故に、核酸（たとえばｃＤＮＡ）分子の切断は位置ドメインの５’側で起こるべきである。

【００７７】

例として、切断ドメインは、ＵＳＥＲ（商標）酵素として商業上知られている、ウラシルＤＮＡグリコシラーゼ（ＵＤＧ）とＤＮＡグリコシラーゼ－リアーゼエンドヌクレアーゼＶＩＩＩの混合物により切断することができるポリＵ配列を含むことがある。

【００７８】

切断ドメインの追加の例は、捕捉プローブがアレイ基板に間接的に、すなわち表面プローブを介して固定化されている実施形態において利用することができる。切断ドメインは、１つ又は複数のミスマッチヌクレオチドを含むことがあり、すなわち表面プローブの相補的部分と捕捉プローブが１００％相補的ではない場合である。そのようなミスマッチは、たとえばＭｕｔＹ及びＴ７エンドヌクレアーゼＩ酵素により認識され、そのためミスマッチの位置で核酸分子が切断される。

【００７９】

本発明のいくつかの実施形態では、捕捉プローブの位置ドメインは切断ドメインを含み、前記切断ドメインは位置ドメインの５’端部に位置している。

【００８０】

ユニバーサルドメインは増幅ドメインも含むことがある。この増幅ドメインは切断ドメインに加えてでもよく、その代わりでもよい。本発明のいくつかの実施形態では、本明細書の他所で説明されているように、たとえばアレイ基板から放出された（たとえば、取り除かれた又は切断された）後で核酸（たとえばｃＤＮＡ）分子を増幅するのが有利なこともある。しかし、増幅の最初のサイクル又は実際、増幅のいずれのサイクルも又はすべての追加サイクルはアレイ上インサイツで起きてもよいことは認識されるであろう。増幅ドメインは増幅プライマーがハイブリダイズすることのできる別個の配列を含む。捕捉プロ

ーブのユニバーサルドメインの増幅ドメインは好ましくは、捕捉プローブのスピーシーズごとに同一である。それ故に、単回増幅反応で核酸（たとえばｃＤＮＡ）分子（増幅に先立ってアレイ基板から放出されることもあればされないこともある）のすべてを増幅するのに十分であろう。

【００８１】

いかなる適切な配列でも本発明の捕捉プローブにおいて増幅ドメインとして用いてもよい。適切な配列とは、増幅ドメインが、組織試料の核酸（たとえばＲＮＡ）と捕捉プローブの捕捉ドメイン間の相互作用に干渉する（すなわち、阻害する又は歪める）べきではないことを意味する。さらに、増幅ドメインは、増幅反応で使用されるプライマーが反応の増幅条件下で増幅ドメインのみにハイブリダイズすることができるように、組織試料の核酸（たとえばＲＮＡ）における任意の配列と同じではない又は実質的に同じではない配列を含むべきである。

【００８２】

たとえば、増幅ドメインは、組織試料中の核酸分子が増幅ドメインにも増幅ドメインの相補的配列にも特異的にハイブリダイズしないように設計されるべきである。好ましくは、捕捉プローブの増幅ドメインの核酸配列及びその相補体は組織試料中の核酸配列に対して８０％未満の配列同一性を有する。好ましくは、捕捉プローブの位置ドメインは組織試料中の核酸分子の実質的な部分にわたって７０％未満、６０％未満、５０％未満又は４０％未満の配列同一性を有する。配列同一性は当技術分野で公知の任意の適切な方法により、たとえばＢＬＡＳＴアライメントアルゴリズムを使用して決定してもよい。

【００８３】

したがって、単独では、捕捉プローブのユニバーサルドメインは、ユニバーサルドメインオリゴヌクレオチドとみることもでき、このオリゴヌクレオチドは捕捉プローブがアレイ上に間接的に固定化される実施形態では、捕捉プローブの合成において使用してもよい。

【００８４】

本発明の一代表的実施形態では、捕捉プローブのそれぞれのスピーシーズの位置ドメインのみが１つしか存在しない。それ故に、捕捉ドメイン及びユニバーサルドメイン（存在すれば）は一実施形態において、確実に組織試料由来の核酸（たとえばＲＮＡ）の捕捉がアレイ全域にわたって均一であるように、どんな特定のアレイでも捕捉プローブのスピーシーズごとに同じである。しかし、上記のように、いくつかの実施形態では、捕捉ドメインはランダム又は縮重配列を含むことによって異なることがある。

【００８５】

捕捉プローブがアレイの基板上に間接的に、たとえば表面プローブへのハイブリダイゼーションを介して、固定化されている実施形態では、捕捉プローブは下に記載される通りにアレイ上で合成されてもよい。

【００８６】

表面プローブはアレイの基板上に直接的に、たとえばその３’端部により又は３’端部で、固定化されている。表面プローブのそれぞれのスピーシーズはアレイのそれぞれのフィーチャー（個別の位置）にのみ含まれ、上で定義される捕捉プローブに部分的に相補的である。

【００８７】

それ故に、表面プローブはその５’端部に、組織試料中の核酸（たとえばＲＮＡ）に結合しない捕捉ドメインの一部に相補的であるドメイン（相補的捕捉ドメイン）を含む。言い換えると、表面プローブは、捕捉ドメインオリゴヌクレオチドの少なくとも一部にハイブリダイズすることができるドメインを含む。表面プローブは、捕捉プローブの位置ドメインに相補的であるドメイン（相補的位置ドメイン又は相補的フィーチャー識別ドメイン）をさらに含む。相補的位置ドメインは、相補的捕捉ドメインの下流（すなわち、３’端部に）直接的に又は間接的に位置している、すなわち相補的位置ドメインと相補的捕捉ドメインを分離する中間又はリンカー配列が存在することがある。捕捉プローブがアレイ表

面で合成される実施形態では、アレイの表面プローブは常に、表面プローブの３’端部に、すなわち位置ドメインの下流に直接的に又は間接的に、捕捉プローブのユニバーサルドメインに相補的であるドメイン（相補的ユニバーサルドメイン）を含む。言い換えると、表面プローブは、ユニバーサルドメインオリゴヌクレオチドの少なくとも一部にハイブリダイズすることができるドメインを含む。

【００８８】

本発明のいくつかの実施形態では、表面プローブの配列は、位置及びユニバーサルドメインに対して並びに組織試料の核酸、たとえばＲＮＡに結合しない捕捉ドメインの一部に対して１００％の相補性又は配列同一性を示す。他の実施形態では、表面プローブの配列は、捕捉プローブのドメインに対して１００％未満、たとえば９９％、９８％、９７％、９６％、９５％、９４％、９３％、９２％、９１％又は９０％未満の配列同一性を示すことがある。本発明の特に好ましい実施形態では、相補的ユニバーサルドメインは、捕捉プローブのユニバーサルドメインに対して１００％未満の配列同一性を共有する。

【００８９】

本発明の一実施形態では、捕捉プローブはアレイの基板上で合成される又は生成される。代表的実施形態では（図３参照）、アレイは上で定義された表面プローブを含む。捕捉プローブの捕捉ドメイン及びユニバーサルドメインに対応するオリゴヌクレオチドをアレイに接触させ、表面プローブの相補的ドメインにハイブリダイズさせる。過剰なオリゴヌクレオチドは、標準ハイブリダイゼーション条件下でアレイを洗浄することにより取り除いてもよい。得られたアレイは部分的に一本鎖プローブを含み、表面プローブの５’と３’端部の両方は二本鎖であり、相補的位置ドメインは一本鎖である。アレイは、捕捉プローブの位置ドメインを合成するように、ポリメラーゼ酵素で処理して、鋳型依存の様式で、ユニバーサルドメインオリゴヌクレオチドの３’端部を伸長させてもよい。次に、合成された位置ドメインの３’端部は、たとえばリガーゼ酵素を使用して捕捉ドメインオリゴヌクレオチドの５’端部にライゲートされて捕捉プローブを生成する。これに関して、捕捉ドメインオリゴヌクレオチドの５’端部はリン酸化されてライゲーションが起こるのを可能にすることは理解されるであろう。表面プローブのそれぞれのスピーシーズは独自の相補的位置ドメインを含むので、捕捉プローブのそれぞれのスピーシーズは独自の位置ドメインを含むことになる。

【００９０】

本明細書で使用される用語「ハイブリダイゼーション」又は「ハイブリダイズする」とは、ワトソン－クリック塩基対合を介して二重鎖を形成するのに十分相補的であるヌクレオチド配列間での二重鎖の形成のことである。２つのヌクレオチド配列は、その２つの分子が塩基対構成相同性を共有する場合互いに「相補的」である。「相補的」ヌクレオチド配列は、適切なハイブリダイゼーション条件下で、特異的に結合して安定な二重鎖を形成する。たとえば、それぞれの配列の３’端部がもう一方の配列の５’端部に結合し、次に１つの配列のそれぞれのＡ、Ｔ（Ｕ）、Ｇ及びＣがそれぞれもう一方の配列のＴ（Ｕ）、Ａ、Ｃ及びＧと整列する逆平行の意味で第一の配列の１セクションが第二の配列の１セクションに結合することができる場合、２つの配列は相補的である。ＲＮＡ配列は相補的Ｇ＝Ｕ又はＵ＝Ｇ塩基対も含むことができる。したがって、２つの配列は、本発明の下では「相補的」であるのに完璧な相同性を有する必要はない。通常、２つの配列は、ヌクレオチドの少なくとも約９０％（好ましくは少なくとも約９５％）が分子の限定された長さにわたって塩基対構成を共有する場合、十分に相補的である。したがって、捕捉及び表面プローブのドメインは相補性の領域を含有する。さらに、捕捉プローブの捕捉ドメインは、組織試料の核酸、たとえばＲＮＡ（好ましくはｍＲＮＡ）について相補的な領域を含有する。

【００９１】

捕捉プローブは、ポリメラーゼ伸長（上記と類似して）及び捕捉ドメインを構成することができる「テール」を付加するターミナルトランスフェラーゼ酵素を使用してアレイ基板上でも合成されてもよい。これは下の実施例７においてさらに説明される。たとえばホ

モポリマーテール、たとえばポリＴテールを導入するための、オリゴヌクレオチドの端部にヌクレオチド配列を付加するターミナルトランスフェラーゼの使用は当技術分野では公知である。したがって、そのような合成では、捕捉プローブのユニバーサルドメインに対応するオリゴヌクレオチドをアレイに接触させて、表面プローブの相補的ドメインにハイブリダイズさせてもよい。過剰なオリゴヌクレオチドは、標準ハイブリダイゼーション条件下でアレイを洗浄することにより取り除いてもよい。得られたアレイは部分的に一本鎖プローブを含み、表面プローブの５’端部は二本鎖であり、相補的位置ドメインは一本鎖である。アレイは、捕捉プローブの位置ドメインを合成するように、ポリメラーゼ酵素で処理して、鋳型依存の様式で、ユニバーサルドメインオリゴヌクレオチドの３’端部を伸長させてもよい。次に、たとえばポリＴ配列を含む捕捉ドメインを、ポリＴテールを付加するターミナルトランスフェラーゼを使用して導入して捕捉プローブを生成してもよい。

【００９２】

本発明の及び本発明の方法において使用するための典型的アレイは、複数のスポット又は「フィーチャー」を含有することがある。フィーチャーは、捕捉プローブの単一スピーシーズが固定化されているアレイ基板上の区域又は個別の位置として定義することができる。それ故に、それぞれのフィーチャーは、同じスピーシーズの複数のプローブ分子を含むことになる。同じスピーシーズのそれぞれの捕捉プローブは同じ配列を有していてもよいことが包含されるが、これは必ずしもそうである必要はないことは、この文脈で理解されるであろう。捕捉プローブのそれぞれのスピーシーズは同じ位置ドメインを有することになる（すなわち、スピーシーズのそれぞれのメンバー、それ故フィーチャー内のそれぞれのプローブは等しく「タグ」付けされることになる）が、フィーチャーのそれぞれのメンバー（スピーシーズ）の配列は、捕捉ドメインの配列が異なることがあるために、相違することがある。上記のように、ランダム又は縮重捕捉ドメインを使用してもよい。したがって、フィーチャー内の捕捉プローブは異なるランダム又は縮重配列を含むことがある。アレイ上のフィーチャーの数及び密度はアレイの解像度、すなわち組織試料のトランスクリプトーム又はゲノムを解析することができる詳細レベルを決定することになる。それ故に、フィーチャーの密度が高いほど典型的にはアレイの解像度も増すことになる。

【００９３】

上記のように、本発明のアレイ上のフィーチャーのサイズと数は、組織試料の性質と要求される解像度に依存することになる。したがって、組織試料内の細胞の領域についてのみのトランスクリプトーム又はゲノムを決定することが望ましい（又は試料が大きな細胞を含有する）場合は、アレイ上のフィーチャーの数及び／若しくは密度は減らしてもよく（すなわち、フィーチャーの考えられる最大数よりも低い）並びに／又はフィーチャーのサイズを増加させる（すなわち、それぞれのフィーチャーの面積は考えられる最小のフィーチャーよりも大きくてもよい）、たとえばアレイは少数の大きなフィーチャーを含んでいてもよい。代わりに、試料内の個々の細胞のトランスクリプトーム又はゲノムを決定するのが望ましい場合は、考えられる最大数のフィーチャーを使用することが必要になることもあり、そうなれば考えられる最小のフィーチャーサイズを使用すること、たとえばアレイは多くの小さなフィーチャーを含むことが必要になると考えられる。

【００９４】

単細胞解像度は本発明の好ましい有利な特長であってもよいが、これを達成することは不可欠ではなく、たとえば特定の細胞型又は組織領域、たとえば正常細胞対腫瘍細胞を検出する又は区別するために、細胞群レベルでの解像度も興味深い。

【００９５】

本発明の代表的実施形態では、アレイは少なくとも２、５、１０、５０、１００、５００、７５０、１０００、１５００、３０００、５０００、１００００、２００００、４００００、５００００、７５０００、１０００００、１５００００、２０００００、３００００、４０００００、５０００００、７５０００、８０００００、１０００００、１２０００００、１５０００００、１７５０００、２０００００、２１０００００、３０００００、３５０００００、４０００００、４２０００００フィーチャーを含有

してもよい。４２０００００は現在市販のアレイ上で使用することができるフィーチャーの最大数を表しているが、これを超えるフィーチャーを有するアレイを調製できることが想定されており、そのようなアレイは本発明においては興味深い。上記のように、フィーチャーサイズは減少させることもあり、こうすればさらに大きな数のフィーチャーを同じ又は類似する面積内に収容することが可能になる。例としては、これらのフィーチャーは、約２０ｃｍ^2、１０ｃｍ^2、５ｃｍ^2、１ｃｍ^2、１ｍｍ^{2}又は１００μｍ^{2}未満の面積に含まれることができる。

【００９６】

したがって、本発明のいくつかの実施形態では、それぞれのフィーチャーの面積は、約１μｍ^2、２μｍ^2、３μｍ^2、４μｍ^2、５μｍ^2、１０μｍ^2、１２μｍ^2、１５μｍ^2、２０μｍ^2、５０μｍ^2、７５μｍ^2、１００μｍ^2、１５０μｍ^2、２００μｍ^2、２５０μｍ^2、３００μｍ^2、４００μｍ^{2}又は５００μｍ^{2}からであってよい。

【００９７】

どんな生物、たとえば植物、動物又は真菌由来の組織試料でも本発明の方法において使用できることは明白であろう。本発明のアレイによれば、転写及び／又は翻訳が可能である細胞に存在するいかなる核酸、たとえばｍＲＮＡ分子の捕捉でも可能になる。本発明のアレイ及び方法は、たとえば細胞が隣接する細胞と相互接続している又は直接接触している場合にトランスクリプトーム又はゲノムの空間解像度が望ましい、試料内の細胞のトランスクリプトーム又はゲノムを単離し解析するのに特に適している。しかし、本発明の方法が、前記細胞が直接相互作用していなくても、試料、たとえば血液試料内の異なる細胞又は細胞型のトランスクリプトーム又はゲノムの解析にも有用である可能性があることは当業者には明らかになるであろう。言い換えると、細胞は組織との関連では現れる必要はなく、単一細胞（たとえば、固定されていない組織から単離される細胞）としてアレイに適用することが可能である。そのような単一細胞は、組織内の一定の位置に必ずしも固定されていないが、にもかかわらずアレイ上の一定の位置に適用され、個別に同定することができる。したがって、直接相互作用していない又は組織環境に存在していない細胞を解析することとの関連では、記載される方法の空間特性を、個々の細胞から独自の又は独立したトランスクリプトーム又はゲノムの情報を得る又は引き出すことに適用することもある。

【００９８】

したがって、試料は収穫した若しくは生検組織試料でもよく、又は場合によっては培養試料でもよい。代表的試料には、臨床試料、たとえば全血又は血液由来産物、血液細胞、組織、生検又は細胞懸濁液を含む培養組織若しくは細胞、等が含まれる。たとえば、人工組織を細胞懸濁液（たとえば、血液細胞を含む）から調製することもある。細胞はマトリックス（たとえば、ゲルマトリックス、たとえば寒天、アガロース、等）に捕捉されることがあり、次に従来のやり方で区分してもよい。そのような手順は免疫組織化学との関連で当技術分野では公知である（たとえば、Ａｎｄｅｒｓｓｏｎ　ｅｔ　ａｌ　２００６年、Ｊ．Ｈｉｓｔｏｃｈｅｍ．Ｃｙｔｏｃｈｅｍ．５４（１２）：１４１３～２３頁、Ｅｐｕｂ　２００６年　Ｓｅｐ　６参照）。

【００９９】

組織調製の様式及び得られた試料の扱い方は、本発明の方法のトランスクリプトーム又はゲノム解析に影響を及ぼすことがある。さらに、様々な組織試料は異なる物理特性を有することになり、必要な操作を実施して本発明の方法で使用するための組織試料を得ることは十分に当業者の範囲内である。しかし、試料調製のいかなる方法も本発明の方法において使用するのに適した組織試料を得るのに使用してもよいことは本明細書における開示から明らかである。たとえば、ほぼ１細胞又はそれ未満の厚みの細胞のいかなる層も本発明の方法において使用してもよい。一実施形態では、組織試料の厚みは、細胞の横断面の０．９、０．８、０．７、０．６、０．５、０．４、０．３、０．２又は０．１未満でもよい。しかし、上記の通りなので、本発明は単一細胞解像度に限定されてはおらず、それ故に、組織試料が１細胞直径又はそれ未満の厚みを有することは必要条件ではなく、さら

に厚い組織試料を必要に応じて使用してもよい。たとえば、クリオスタット切片を使用してもよく、これはたとえば、厚みが１０～２０μｍであることもある。

【０１００】

組織試料は従来の又は望みのいかなる方法で調製してもよく、本発明はいかなる特定種類の組織調製物にも制限されてはいない。新鮮な、凍結した、固定した又は非固定の組織を用いてもよい。当技術分野で記載されている及び公知であるように、いかなる望みの便利な手法でも組織試料を固定する又は包埋するために使用してもよい。したがって、いかなる公知の固定液又は包埋材料を使用してもよい。

【０１０１】

本発明において使用するための組織試料の第一の代表的例として、組織構造の完全性（すなわち、物理的特徴）を維持する又は保存するのに適した温度、たとえば－２０℃未満、好ましくは－２５、－３０、－４０、－５０、－６０、－７０、－８０℃未満での低温凍結により調製してもよい。凍結組織試料は、いかなる適切な手段によってもアレイ表面上に切片にする、すなわち薄くスライスしてもよい。たとえば、組織試料は、組織試料の構造完全性と試料中の核酸の化学的特性の両方を維持するのに適した温度で、たとえば－１５℃未満、好ましくは－２０又は－２５℃未満に設定して、急冷ミクロトーム、クリオスタットを使用して調製してもよい。したがって、試料は、組織中の核酸、たとえばＲＮＡの変性又は分解を最小限に抑えるように処理するべきである。そのような条件は当技術分野では確立しており、いかなる分解の程度も、組織試料の調製の様々な段階での核酸抽出、たとえば全ＲＮＡ抽出とそれに続く品質分析を通じてモニターしてもよい。

【０１０２】

第二の代表的例では、組織は、当技術分野で確立しているホルマリン固定とパラフィン包埋（ＦＦＰＥ）という標準法を使用して調製してもよい。組織試料の固定化とパラフィン又は樹脂ブロックへの包埋に続いて、組織試料はアレイ表面上に切片にする、すなわち薄くスライスしてもよい。上記のように、他の固定液及び／又は包埋材料を使用することは可能である。

【０１０３】

組織試料切片は、本発明の方法を実行するのに先立って、試料から包埋材料を取り除く、たとえば脱パラフィンする、すなわちパラフィン又は樹脂を取り除くための処理をする必要があることになる。この除去は、いかなる適切な方法によって達成してもよく、たとえば試料（アレイの表面上の）を適切な溶剤、たとえばキシレン中で、たとえば１０分間で２度インキュベートし、続いてエタノールすすぎ、たとえば２分間９９．５％エタノール、２分間９６％エタノール及び２分間７０％エタノールによる、パラフィン又は樹脂又は他の材料の組織試料からの除去は当技術分野では確立している。

【０１０４】

ＦＦＰＥの方法又は固定し包埋する他の方法を使用して調製される組織切片中のＲＮＡは凍結組織の場合よりも部分的に分解している可能性が高いことは当業者には明らかであろう。しかし、いかなる特定の理論にも縛られたくはないが、これは本発明の方法においては有利なことがあると考えられる。たとえば、試料中のＲＮＡが部分的に分解している場合、ＲＮＡポリヌクレオチドの平均長は非分解試料よりも少なくよりランダム化されている。したがって、部分的に分解されたＲＮＡは、本明細書の他所に記載されている様々な処理ステップ、たとえばアダプター（増幅ドメイン）のライゲーション、ｃＤＮＡ分子の増幅及びそのシーケンシングにおいて偏りが少なくなると仮定される。

【０１０５】

それ故に、本発明の一実施形態では、組織試料、すなわちアレイに接触させた組織試料の切片、はＦＦＰＥ又は固定し包埋する他の方法を使用して調製される。言い換えると、試料は固定されている、たとえば固定され包埋されていてもよい。本発明の代わりの実施形態では、組織試料は低温凍結により調製される。別の実施形態では、当技術分野で公知の手法に従って、組織のタッチインプリントを使用してもよい。他の実施形態では、非固定試料を使用してもよい。

【０１０６】
本発明の方法において使用するための組織試料切片の厚みは、試料を調製するのに使用される方法及び組織の物理的特性に依存することがある。したがって、本発明の方法においてはいかなる適切な切片厚みを使用してもよい。本発明の代表的実施形態では、組織試料切片の厚みは、少なくとも０．１μｍ、さらに好ましくは少なくとも０．２、０．３、０．４、０．５、０．７、１．０、１．５、２、３、４、５、６、７、８、９又は１０μｍになる。他の実施形態では、組織試料切片の厚みは、少なくとも１０、１２、１３、１４、１５、２０、３０、４０又は５０μｍである。しかし、厚みは決定的ではなく、これらは代表的値のみである。必要であれば又は都合がよければ、もっと厚い、たとえば７０又は１００μｍ又はさらに厚い試料を使用してもよい。典型的には、組織試料切片の厚みは１～１００μｍ、１～５０μｍ、１～３０μｍ、１～２５μｍ、１～２０μｍ、１～１５μｍ、１～１０μｍ、２～８μｍ、３～７μｍ又は４～６μｍであるが、上記のようにもっと厚い試料を使用してもよい。

【０１０７】
たとえば包埋材料の除去、たとえば脱パラフィンに続いて、組織試料切片をアレイに接触させると、組織試料中の核酸（たとえばＲＮＡ）分子はアレイ上の固定化された捕捉プローブに結合することになる。いくつかの実施形態では、核酸（たとえばＲＮＡ）分子の捕捉プローブへのハイブリダイゼーションを促進することが有利なことがある。典型的には、ハイブリダイゼーションの促進には、ハイブリダイゼーションが起こる条件を変更することが含まれる。変更することが可能な第一条件は、本明細書の他所に記載されている逆転写ステップに先立つアレイ上での組織切片のインキュベーションの時間と温度である。

【０１０８】
たとえば、組織試料切片をアレイに接触させると、アレイは少なくとも１時間インキュベートされて、核酸（たとえばＲＮＡ）を捕捉プローブにハイブリダイズさせておいてもよい。好ましくは、アレイは少なくとも２、３、５、１０、１２、１５、２０、２２若しくは２４時間又は組織試料切片が乾燥してしまうまでインキュベートされてもよい。アレイインキュベーション時間は決定的ではなく、いかなる都合の良い又は所望の時間を使用してもよい。典型的アレイインキュベーションは７２時間までででもよい。したがって、インキュベーションは、どんな適切な温度でも、たとえば室温で起こることがあるが、好ましい実施形態では、組織試料切片はアレイ上、少なくとも２５、２６、２７、２８、２９、３０、３１、３２、３３、３４、３５、３６又は３７℃の温度でインキュベートされる。５５℃までのインキュベーション温度が当技術分野では普通である。特に好ましい実施形態では、組織試料切片はアレイ上、３７℃で２４時間乾燥させておく。組織試料切片が乾燥してしまうと、アレイは、逆転写ステップを実施する前に室温で保存してもよい。組織試料切片をアレイの表面上で乾燥させておく場合、捕捉された核酸の追加の操作を達成することができる、たとえば捕捉されたＲＮＡを逆転写するステップ前に組織試料切片を再水和させる必要がないことは理解されるであろう。

【０１０９】
それ故に、本発明の方法は、試料をアレイに接触させた後に組織試料を再水和させる追加のステップを含むことがある。

【０１１０】
いくつかの実施形態では、特に組織試料中の核酸がアレイ上でのその捕捉に先立って変更の手順を受けている場合には、組織試料をアレイに接触させることに先立って捕捉プローブを遮断する（たとえば、マスクする又は改変する）のが有利なこともある。詳細には、捕捉プローブの遊離の３’端部を遮断する又は改変するのが有利なこともある。特定の実施形態では、組織試料中の核酸（たとえば断片化されたゲノムＤＮＡ）は、捕捉プローブにより捕捉されることができるように改変してもよい。たとえば及び下にさらに詳細に説明されているように、アダプター配列（捕捉プローブの捕捉ドメインに結合することができる結合ドメインを含む）を核酸（たとえば断片化されたゲノムＤＮＡ）の端部に付加

してもよい。これは、たとえば追加のヌクレオチドを配列（たとえばポリＡテール）の端部に組み込む酵素を使用して、たとえばアダプターのライゲーション又は核酸の伸長により達成してもよい。たとえば、捕捉プローブの遊離の３’端部へのポリＡテールの付加を回避するためには、組織試料をアレイに接触させて捕捉プローブの改変を回避するのに先立って、捕捉プローブ、特に捕捉プローブの遊離の３’端部を遮断する又は改変する必要がある。好ましくは、遮断ドメインは捕捉プローブが合成されるときに捕捉プローブに組み込まれてもよい。しかし、遮断ドメインは捕捉プローブの合成後に捕捉プローブに組み込まれてもよい。

【０１１１】

いくつかの実施形態では、捕捉プローブは、組織試料がアレイに接触された後で起こる組織試料の核酸の改変が進行中に捕捉ドメインの改変を妨げると考えられるどんな適切な可逆的手段により遮断されてもよい。言い換えると、捕捉プローブは、捕捉プローブの捕捉ドメインが遊離の３’端部を含まないように、すなわち３’端部が取り除かれる若しくは改変されるように可逆的にマスクされる若しくは改変される、又は捕捉プローブが、組織試料の核酸を改変するのに使用される手順、たとえばライゲーション若しくは伸長に感受性ではないように接近しにくくされてもよいし、或いは追加のヌクレオチドを取り除いて捕捉プローブの捕捉ドメインの３’端部を露呈する及び／又は回復させてもよい。

【０１１２】

たとえば、遮断プローブ（たとえばその適切な例は当技術分野で公知であるヘアピンプローブ又は部分的二本鎖プローブ）を捕捉プローブにハイブリダイズさせて捕捉ドメインの３’端部をマスクしてもよい。捕捉ドメインの遊離の３’端部を化学修飾（たとえば捕捉プローブが遊離の３’端部を含まないように化学的に可逆的なキャップ形成部分としてのアジドメチル基の付加）により遮断してもよい。適切な代わりのキャップ形成部分は当技術分野では周知であり、たとえば捕捉ドメインの末端ヌクレオチドは可逆的終止ヌクレオチドであることも可能であり、このヌクレオチドはプローブ合成中又はその後で捕捉プローブに含むこともできる。

【０１１３】

代わりに又はさらに、捕捉プローブの捕捉ドメインは、組織試料の核酸分子が改変されるときに起きる捕捉プローブのどのような修飾（たとえばヌクレオチドの追加）をも除去できるように、改変することもできる。たとえば、捕捉プローブは、捕捉ドメインの下流（すなわち捕捉ドメインの３’側）に追加の配列、すなわち遮断ドメイン、を含んでいてもよい。これは、制限エンドヌクレアーゼ認識配列又は特異的酵素活性により切断可能なヌクレオチド（たとえばウラシル）の配列の形態でも可能である。組織試料の核酸の改変に続いて、捕捉プローブは酵素的切断に供することもでき、この切断により、遮断ドメイン及び改変手順中に捕捉プローブの３’端部に付加される追加のヌクレオチドのいずれでも除去することが可能だと考えられる。遮断ドメインの除去により、捕捉プローブの捕捉ドメインが遊離の３’端部は露呈される及び／又は回復されると考えられる。遮断ドメインは捕捉プローブの一部として合成することができる又は、たとえば遮断ドメインのライゲーションにより捕捉プローブにインサイツで付加する（すなわち、既存のアレイの改変物として）こともできる。

【０１１４】

捕捉プローブは、上記の遮断機構のいかなる組合せでも使用して遮断してもよい。

【０１１５】

組織試料の核酸、たとえば断片化されたゲノムＤＮＡが改変されて前記核酸が捕捉プローブの捕捉ドメインにハイブリダイズすることが可能になると、捕捉プローブは、たとえば遮断オリゴヌクレオチドの解離、キャップ形成部分及び／又は遮断ドメインの除去により遮断を解除しなければならない。

【０１１６】

アレイのそれぞれのフィーチャーから得られる配列解析又はトランスクリプトーム若しくはゲノム情報を組織試料の領域（すなわち、区域又は細胞）と関連付けるために、組織

試料はアレイ上でフィーチャーとの関係で方向付けられる。言い換えると、組織試料は、アレイ上の捕捉プローブの位置が組織試料中の位置と関連付けられるようにアレイ上に置かれる。したがって、組織試料中のどこに捕捉プローブのそれぞれのスピーシーズの位置（又はアレイのそれぞれのフィーチャー）が対応するのかを同定することができる。言い換えると、組織試料中のどの位置に捕捉プローブのそれぞれのスピーシーズの位置が対応するのかを同定することができる。これは、下で説明されるように、アレイ上に存在する位置マーカーに基づいて実行されることができる。必ずしもそうではないが、都合の良いことに、組織試料はアレイとのその接触に続いて撮像することができる。この撮像は、組織試料の核酸が処理される前に又は後で、たとえば前記方法のｃＤＮＡ生成ステップ、特に逆転写により第一鎖ｃＤＮＡを生成するステップ前に又は後で実施してもよい。好ましい実施形態では、組織試料はアレイから捕捉され合成された（すなわち、伸長された又はライゲートされた）ＤＮＡ、たとえばｃＤＮＡの放出に先立って撮像される。特に好ましい実施形態では、組織は、組織試料の核酸が処理された後で、たとえば逆転写ステップの後で撮像され、いかなる残余の組織も、アレイから分子、たとえばｃＤＮＡの放出に先立ってアレイから取り除かれる（たとえば、洗浄される）。いくつかの実施形態では、捕捉された核酸を処理するステップ、たとえば逆転写ステップは、たとえば低温凍結による組織調製を使用する場合に、アレイ表面から残余の組織を取り除くように作用することがある。そのような場合、組織試料の撮像は、処理ステップ、たとえばｃＤＮＡ合成ステップに先立って行われてもよい。一般的に言えば、撮像は組織試料を区域に接触させた後の、しかし組織試料を分解する又は取り除く任意のステップ前のどの時期に行われてもよい。上記のように、これは組織試料に依存することがある。

【０１１７】

有利なことに、アレイは、アレイのフィーチャーとの関係での組織試料又はその画像の方向付けを促進するマーカーを含むことがある。アレイをマークするためのいかなる適切な手段も、組織試料が撮像されるとマーカーが検出可能であるように使用してもよい。たとえば、シグナル、好ましくは目に見えるシグナルを発する分子（たとえば蛍光分子）をアレイの表面に直接的に又は間接的に固定化することができる。好ましくは、アレイは、アレイ表面の別個の位置に少なくとも２個のマーカー、さらに好ましくは少なくとも３、４、５、６、７、８、９、１０、１２、１５、２０、３０、４０、５０、６０、７０、８０、９０又は１００個のマーカーを含む。都合の良いことに、数百又は数千のマーカーでさえ使用してもよい。マーカーは、パターンで提供してもよく、たとえばアレイの外縁、たとえばアレイのフィーチャーの全外列を構成する。他の情報を与えるパターン、たとえばアレイを区分する線を使用してもよい。これにより、組織試料の画像をアレイに合わせて整列させることが容易になり、又は実際には一般的にはアレイのフィーチャーを組織試料に関連付ける際に容易になることがある。したがって、マーカーは、シグナル発生分子が相互作用をしてシグナルを発生する固定化された分子であってもよい。代表的な例では、アレイは、標識された核酸がハイブリダイズすることのできるマーカーフィーチャー（たとえばアレイの基板上に固定化された核酸プローブ）を含むことがある。たとえば、標識された核酸分子又はマーカー核酸は、特定の波長（又は範囲の波長）の光を受けると、すなわち励起されると、蛍光を発することができる化学的部分に連結又はカップリングされてもよい。そのようなマーカー核酸分子は、組織試料を視覚化する又は撮像するために染色する前に、と同時に又は後でアレイに接触させてもよい。しかし、マーカーは組織試料が撮像されるときには検出可能でなければならない。したがって、好ましい実施形態では、マーカーは、組織試料を視覚化するのに使用されるのと同じ撮像条件を使用して検出してもよい。

【０１１８】

本発明の特に好ましい実施形態では、アレイは、標識された（好ましくは蛍光標識された）マーカー核酸分子（たとえばオリゴヌクレオチド）がハイブリダイズされるマーカーフィーチャーを含む。

【０１１９】

組織を撮像するステップは、当技術分野で公知のいかなる都合の良い組織学的手段（たとえば光、明視野、暗視野、位相差、蛍光、反射、干渉、共焦点顕微鏡又はその組合せ）を使用してもよい。典型的には、組織試料は、組織試料の異なる領域（たとえば細胞間）にコントラストを与える視覚化に先立って染色される。使用される染色剤の種類は、染色される組織の種類及び細胞の領域に依存することになる。そのような染色プロトコールは当技術分野では公知である。いくつかの実施形態では、１つを超える染色剤を使用して、組織試料の異なる面（たとえば組織試料の異なる領域、特定の細胞構造体（たとえば、オルガネラ）又は異なる細胞型）を視覚化（撮像）してもよい。他の実施形態では、組織試料は、たとえば組織試料が十分なコントラストを与える色素を既に含有している場合又は特定の形態の顕微鏡が使用される場合、試料を染色せずに可視化される又は撮像されてもよい。

【０１２０】

好ましい実施形態では、組織試料は蛍光顕微鏡を使用して視覚化される又は撮像される。

【０１２１】

撮像が望まれるが逆転写前に実行されなかった場合、組織試料、すなわち逆転写ステップ及び場合によって撮像に続いてアレイ基板に接触したままであるどんな残余の組織、は、好ましくはアレイからｃＤＮＡ分子を放出するステップに先立って取り除かれる。したがって、本発明の方法はアレイを洗浄するステップを含むことがある。残余の組織試料の除去は、いかなる適切な手段を使用して実施してもよく組織試料に依存することになる。もっとも簡単な実施形態では、アレイは水を用いて洗浄してもよい。水は様々な添加剤、たとえば組織の除去を促進する界面活性剤（たとえば、洗剤）、酵素等、を含有していてもよい。いくつかの実施形態では、アレイはプロテイナーゼ酵素（及び適切なバッファー）（たとえばプロテイナーゼＫ）を含む溶液を用いて洗浄される。他の実施形態では、溶液は、たとえば組織試料が植物又は真菌供給源由来の場合は、その上又は代わりに、セルラーゼ、ヘミセルラーゼ又はキチナーゼ酵素を含んでいてもよい。追加の実施形態では、アレイの洗浄に使用される溶液の温度は、たとえば少なくとも３０℃、好ましくは少なくとも３５、４０、４５、５０又は５５℃であってもよい。洗浄液は固定化された核酸分子の破壊を最小限に抑えるべきであることは明らかであろう。たとえば、いくつかの実施形態では、核酸分子は、アレイの基板に、間接的に、たとえば捕捉プローブ並びにＲＮＡ及び／又は捕捉プローブ並びに表面プローブのハイブリダイゼーションを介して固定化されることもあり、したがって洗浄ステップはアレイ上に固定化された分子間の相互作用に干渉すべきではない、すなわち核酸分子を変性させるべきではない。

【０１２２】

ハイブリダイゼーションを組織試料の核酸、たとえばＲＮＡ（好ましくは、ｍＲＮＡ）、と捕捉プローブ間で起こさせるのに十分な条件下で、アレイを組織試料に接触させるステップに続いて、ハイブリダイズした核酸を確保する（獲得する）ステップが行われる。捕捉された核酸を確保する又は獲得することは、ハイブリダイズした核酸の相補鎖が捕捉プローブに共有結合し（すなわち、ヌクレオチド結合、２つの直接隣接するヌクレオチドの並置された３’－ヒドロキシルと５’－リン酸末端の間でのリン酸ジエステル結合）、それによって、核酸が捕捉されるフィーチャーに特異的な位置ドメインで捕捉された核酸にタグ付けする又はマークすることを伴う。

【０１２３】

いくつかの実施形態では、ハイブリダイズした核酸（たとえば一本鎖核酸）を確保することは、捕捉プローブを伸長させて捕捉された核酸のコピーを作製する、たとえば捕捉された（ハイブリダイズした）ＲＮＡからｃＤＮＡを生成する、ことを伴ってもよい。これは、ハイブリダイズした核酸の相補鎖を合成する、たとえば捕捉されたＲＮＡ鋳型（捕捉プローブの捕捉ドメインにハイブリダイズしたＲＮＡ）に基づいてｃＤＮＡを生成する、ことを指すのは理解されるであろう。したがって、捕捉プローブを伸長させる最初のステップ、たとえばｃＤＮＡ生成では、捕捉された（ハイブリダイズした）核酸（たとえばＲ

ＮＡ）は伸長（たとえば逆転写）ステップのための鋳型として機能する。他の実施形態では、下に説明されるように、ハイブリダイズした核酸（たとえば部分的二本鎖ＤＮＡ）を確保することは、ハイブリダイズした核酸（たとえば断片化されたＤＮＡ）を捕捉プローブに共有結合させる、たとえばライゲーション反応において、捕捉プローブに、捕捉プローブにハイブリダイズした核酸の相補鎖をライゲートすることを伴うことがある。

【０１２４】

逆転写は、ＲＮＡ、好ましくはｍＲＮＡ（メッセンジャーＲＮＡ）から、逆転写酵素によりｃＤＮＡ（相補的又はコピーＤＮＡ）を合成するステップに関する。したがって、ｃＤＮＡは、組織試料が採取された時点で細胞中に存在していたＲＮＡのコピーとみることができる、すなわちｃＤＮＡは単離の時点で前記細胞内で発現された遺伝子のすべて又は一部を表している。

【０１２５】

捕捉プローブ、詳細には捕捉プローブの捕捉ドメインは、捕捉プローブにハイブリダイズした核酸の相補鎖を作製するためのプライマー、たとえば逆転写のためのプライマー、として機能する。それ故に、伸長反応（たとえば逆転写反応）により生成される核酸（たとえばｃＤＮＡ）は捕捉プローブの配列を組み込む、すなわち伸長反応（たとえば逆転写反応）は、アレイのそれぞれのフィーチャーと接触している組織試料の核酸（たとえば転写産物）を間接的に標識する方法とみることもできる。上記のように、捕捉プローブのそれぞれのスピーシーズは、アレイのフィーチャーごとに独自の配列を表す位置ドメイン（フィーチャー識別タグ）を含む。したがって、特定のフィーチャーで合成される核酸（たとえばｃＤＮＡ）分子のすべてが同じ核酸「タグ」を含むことになる。

【０１２６】

アレイのそれぞれのフィーチャーで合成される核酸（たとえばｃＤＮＡ）分子は、そのフィーチャーに接触している組織試料の領域又は区域（たとえば組織又は細胞型又は細胞群若しくはそのサブグループ）のゲノム又はそこから発現される遺伝子を表していることがあり、特定の条件下で、たとえば特定の時間に、特定の環境で、発生の段階で又は刺激に応答して、等で発現される遺伝子をさらに表していることがある。それ故に、いずれの単一フィーチャーのｃＤＮＡでも単一細胞で発現された遺伝子を表していることがある、又はフィーチャーが細胞間結合で試料と接触している場合、ｃＤＮＡは１つよりも多い細胞で発現された遺伝子を表していることがある。同様に、単一細胞が複数のフィーチャーと接触している場合、それぞれのフィーチャーは前記細胞で発現された遺伝子の一部を表していることがある。同様に、捕捉された核酸がＤＮＡである実施形態では、いかなる単一のフィーチャーも単一細胞又は１つよりも多い細胞のゲノムを表していることがある。代わりに、単一細胞のゲノムが複数のフィーチャーにより表されることもある。

【０１２７】

捕捉プローブを伸長するステップ、たとえば逆転写は、下に詳細に説明されるように、当技術分野においてその多くが存在するいかなる適切な酵素及びプロトコールを使用して実施してもよい。しかし、捕捉プローブの捕捉ドメインがプライマー、たとえば逆転写プライマーとして機能するので、最初の核酸（たとえばｃＤＮＡ）鎖の合成のためのプライマーを提供する必要はないことは明らかであろう。

【０１２８】

好ましくは、本発明との関連では、確保された核酸（すなわち、捕捉プローブに共有結合している核酸）（たとえばｃＤＮＡ）は二本鎖ＤＮＡを含むように処理される。しかし、いくつかの実施形態では、捕捉されたＤＮＡは、たとえば部分的二本鎖断片化されたＤＮＡが捕捉プローブにライゲートされている場合には、既に二本鎖ＤＮＡを含んでいることがある。二本鎖ＤＮＡを作製するための捕捉された核酸の処理は、第二のＤＮＡ（たとえばｃＤＮＡ）鎖のみを生成する、すなわち二本鎖ＤＮＡ分子の数を増加させずに二本鎖ＤＮＡ分子を作製する単回反応において、又は一本鎖ＤＮＡ（たとえば、線形増幅）若しくは二本鎖ＤＮＡ（たとえばｃＤＮＡ）（たとえば、指数増幅）の形態でもよい第二の鎖の複数のコピーを生成する増幅反応において達成してもよい。

【０１２９】
第二鎖ＤＮＡ（たとえばｃＤＮＡ）合成のステップは、たとえば下にさらに詳細に説明されているランダムプライマーを使用する第二鎖合成の個別のステップとして、又は増幅反応の最初のステップにおいて、アレイ上インサイツで行われてもよい。代わりに、第一鎖ＤＮＡ（たとえばｃＤＮＡ）（捕捉プローブを含む、すなわち組み込んでいる鎖）はアレイから放出されてもよく、第二鎖合成は、個別のステップとしてであれ増幅反応においてであれ、それに続いて、たとえば溶液中で実行される反応において起きてもよい。
【０１３０】
第二鎖合成がアレイ上で（すなわち、インサイツで）行われる場合、前記方法は、たとえばＲＮＡ消化酵素（リボヌクレアーゼ）、たとえばリボヌクレアーゼＨを使用して、第二鎖合成の前に捕捉された核酸（たとえばＲＮＡ）を取り除く任意のステップを含んでいてもよい。この手法は当技術分野では周知であり記載されている。しかし、これは一般的には必ずしも必要ではなく、大半の場合には前記ＲＮＡは自然に分解する。アレイからの組織試料の除去は、一般にアレイから前記ＲＮＡを取り除くことになる。必要な場合にはリボヌクレアーゼＨを使用して、ＲＮＡ除去の強さを増加することができる。
【０１３１】
たとえば、大量のＲＮＡを含む組織試料においては、二本鎖ｃＤＮＡを生成するステップは、（アレイからの放出に続いて）直接シーケンシングをすることができるのに十分な量のｃＤＮＡを生じることがある。この場合には、第二鎖ｃＤＮＡ合成は、当技術分野で公知の及び下に説明されているいかなる手段によっても達成することができる。下に説明されているように、第二鎖合成反応はアレイ上で直接、すなわちｃＤＮＡがアレイ上に固定化されている間に、又は好ましくはｃＤＮＡがアレイ基板から放出された後で実施してもよい。
【０１３２】
他の実施形態では、確保された核酸（たとえば合成されたｃＤＮＡ）の量を増強する、すなわち増幅して、ＤＮＡシーケンシングのために十分な量を生じることが必要になる。この実施形態では、確保された核酸（たとえばｃＤＮＡ）分子の第一鎖は、アレイのフィーチャーの捕捉プローブも含むが、増幅反応、たとえばポリメラーゼ連鎖反応のための鋳型として機能する。増幅の最初の反応生成物は、ＤＮＡ（たとえばｃＤＮＡ）の第二鎖になり、これは増幅反応の追加のサイクルのための鋳型として機能することになる。
【０１３３】
上記の実施形態のどちらかにおいて、ＤＮＡ（たとえばｃＤＮＡ）の第二鎖は、捕捉プローブの相補体を含むことになる。捕捉プローブがユニバーサルドメインを、特にユニバーサルドメイン内に増幅ドメインを含む場合、これはＤＮＡ（たとえばｃＤＮＡ）のそれに続く増幅のために使用してもよく、たとえば増幅反応は増幅ドメインと同じ配列を有するプライマー、すなわち増幅ドメインの相補体に相補的である（すなわち、ハイブリダイズする）プライマー、を含んでいてもよい。増幅ドメインは捕捉プローブの位置ドメインの上流にある（確保された核酸において、たとえば第一ｃＤＮＡ鎖）という事実に照らして、位置ドメインの相補体はＤＮＡ（たとえばｃＤＮＡ）分子の第二の鎖に組み込まれることになる。
【０１３４】
ＤＮＡ（たとえばｃＤＮＡ）の第二の鎖が単回反応で生成される実施形態では、第二の鎖の合成はいかなる適切な手段によっても達成することができる。たとえば、好ましくは、しかし必ずしもそうではないが、アレイ基板から放出された第一鎖ｃＤＮＡは、鋳型になったＤＮＡ合成が起こるのに十分な条件下で、ランダムプライマー（たとえばヘキサマープライマー）及びＤＮＡポリメラーゼ、好ましくは鎖置換ポリメラーゼ、たとえばクレノー（ｅｘｏ）と一緒にインキュベートしてもよい。この手順は様々な長さの二本鎖ｃＤＮＡ分子を生じることになり、完全長ｃＤＮＡ分子、すなわちそれが合成された元になる全ｍＲＮＡに対応するｃＤＮＡ分子を生じる可能性はない。ランダムプライマーはランダムな位置で、すなわち配列の端部ではなくむしろ配列内で第一鎖ｃＤＮＡ分子にハイブリ

ダイズすることになる。

【０１３５】

完全長のＤＮＡ（たとえばｃＤＮＡ）分子、すなわち捕捉された核酸（たとえばＲＮＡ）分子の全体に対応する分子を生成するのが望ましい場合（核酸、たとえばＲＮＡが組織試料内で部分的に分解している場合には、捕捉された核酸、たとえばＲＮＡ分子はゲノムＤＮＡの最初の断片と同じ長さの「完全長」転写物にはならない）、確保された核酸（たとえば第一鎖ｃＤＮＡ）分子の３’端部を改変してもよい。たとえば、リンカー又はアダプターをｃＤＮＡ分子の３’端部にライゲートしてもよい。これは、Ｔ４　ＲＮＡリガーゼ又はＣｉｒｃｌｉｇａｓｅ（商標）（Ｅｐｉｃｅｎｔｒｅ　Ｂｉｏｔｅｃｈｎｏｌｏｇｉｅｓ）などの一本鎖ライゲーション酵素を使用して達成してもよい。

【０１３６】

代わりに、ヘルパープローブ（第一鎖ｃＤＮＡ分子の３’端部にハイブリダイズすることができる部分的二本鎖ＤＮＡ分子）を、Ｔ４　ＤＮＡリガーゼなどの二本鎖ライゲーション酵素を使用して確保された核酸（たとえば第一鎖ｃＤＮＡ）分子の３’端部にライゲートしてもよい。ライゲーションステップに適した他の酵素は当技術分野では公知であり、たとえばＴｔｈ　ＤＮＡリガーゼ、Ｔａｑ　ＤＮＡリガーゼ、サーモコッカス（Ｔｈｅｒｍｏｃｏｃｃｕｓ）菌種（９°Ｎ株）ＤＮＡリガーゼ（９°Ｎ（商標）ＤＮＡリガーゼ、Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）及びＡｍｐｌｉｇａｓｅ（商標）（Ｅｐｉｃｅｎｔｒｅ　Ｂｉｏｔｅｃｈｎｏｌｏｇｉｅｓ）が含まれる。ヘルパープローブは、確保された核酸（たとえば第一ｃＤＮＡ鎖）にライゲートされているヘルパープローブの一部に相補的であるプライマーを用いて、第二鎖ＤＮＡ（たとえばｃＤＮＡ）合成をそこからプライミングすることができる特定の配列も含む。追加の代替物は、確保された核酸、たとえば第一鎖ｃＤＮＡ分子の３’端部でポリヌクレオチドテール、たとえばポリＡテール、を組み込むターミナルトランスフェラーゼ活性酵素の使用を含む。第二鎖合成はポリＴプライマーを使用してプライミングしてもよく、これには追加の増幅のための特定の増幅ドメインが含まれてもよい。「完全長」二本鎖ＤＮＡ（たとえばｃＤＮＡ）分子を生成するための他の方法（又は最長第二鎖合成）は当技術分野では確立している。

【０１３７】

いくつかの実施形態では、第二鎖合成は、たとえばＣｌｏｎｅｔｅｃｈ（登録商標）からのＳＭＡＲＴ（商標）技術を使用する鋳型切替えの方法を使用してもよい。ＳＭＡＲＴ（ＲＮＡ鋳型の５’端部での切替え機構）技術は当技術分野では確立しており、逆転写酵素、たとえばＳｕｐｅｒｓｃｒｉｐｔ（登録商標）ＩＩ（Ｉｎｖｉｔｒｏｇｅｎ）、が伸長されたｃＤＮＡ分子の３’端部で数個のヌクレオチドを付加する、すなわち一本鎖ＤＮＡオーバーハングが３’端部にあるＤＮＡ／ＲＮＡハイブリッドを作製することができるという発見に基づいている。ＤＮＡオーバーハングは、オリゴヌクレオチドプローブがハイブリダイズしてｃＤＮＡ分子のさらなる伸長のための追加の鋳型を提供することができる標的配列を提供することができる。有利なことに、ｃＤＮＡオーバーハングにハイブリダイズするオリゴヌクレオチドプローブは増幅ドメイン配列を含有しており、このドメイン配列の相補体は合成された第一鎖ｃＤＮＡ産物に組み込まれる。ｃＤＮＡ第一鎖に組み込まれている相補的増幅ドメイン配列にハイブリダイズすることになる増幅ドメイン配列を含有するプライマーを反応混合物に添加して、適切なポリメラーゼ酵素及び鋳型としてｃＤＮＡ第一鎖を使用する第二鎖合成をプライミングすることができる。この方法は、アダプターをｃＤＮＡ第一鎖の３’端部にライゲートする必要性を回避する。鋳型切替えは最初は５’キャップ構造を有する完全長ｍＲＮＡのために開発されたが、以来キャップ構造のない切断型ｍＲＮＡでも等しくうまく機能することが実証されてきた。したがって、鋳型切替えは、完全長及び／又は部分的若しくは切断型ｃＤＮＡ分子を生成する本発明の方法において使用してもよい。したがって、本発明の好ましい実施形態では、第二鎖合成は鋳型切替えを利用する、又はこれにより達成されてもよい。特に好ましい実施形態では、鋳型切替え、すなわちｃＤＮＡ第一鎖をさらに伸長して相補的増幅ドメインを組み込む反応、はインサイツで実施される（捕捉プローブが、直接的に又は間接的にアレイにまだ

結合している間に）。好ましくは、第二鎖合成反応もインサイツで実施される。

【０１３８】

ＤＮＡ（たとえばｃＤＮＡ）分子を増強する、濃縮する又は増幅することが必要である又は有利なことがある実施形態では、増幅ドメインはＤＮＡ（たとえばｃＤＮＡ）分子に組み込まれてもよい。上記のように、捕捉プローブが増幅ドメインを含むユニバーサルドメインを含む場合、第一増幅ドメインは確保された核酸分子（たとえばｃＤＮＡ分子）の第一鎖に組み込まれてもよい。これらの実施形態では、第二鎖合成は第二増幅ドメインを組み込むことができる。たとえば、第二鎖ｃＤＮＡを生成するのに使用されるプライマー（たとえばランダムヘキサマープライマー、ポリＴプライマー、ヘルパープローブに相補的であるプライマー）は、その５’端部に増幅ドメイン、すなわち増幅プライマーがハイブリダイズすることができるヌクレオチド配列を含んでいることがある。したがって、得られた二本鎖ＤＮＡは、二本鎖ＤＮＡ（たとえばｃＤＮＡ）分子のそれぞれの５’端部で又は５’端部の方に向かって増幅ドメインを含むことがある。これらの増幅ドメインは、増幅反応、たとえばＰＣＲにおいて使用されるプライマーのための標的として用いてもよい。代わりに、確保された核酸分子（たとえば第一鎖ｃＤＮＡ分子）の３’端部にライゲートされているリンカー又はアダプターは、第二増幅ドメインを含む第二ユニバーサルドメインを含むことがある。同様に、第二増幅ドメインは、鋳型切替えにより第一鎖ｃＤＮＡ分子に組み込まれていることがある。

【０１３９】

捕捉プローブが、特に増幅ドメインを含んでいるユニバーサルドメインを含まない実施形態では、ｃＤＮＡ分子の第二鎖は上記説明に従って合成されてもよい。得られた二本鎖ＤＮＡ分子は、第一ＤＮＡ（たとえばｃＤＮＡ鎖）の５’端部に（第一増幅ドメイン）、たとえばｃＤＮＡ合成ステップにおいて、第二鎖ＤＮＡに組み込まれない場合は、第二のＤＮＡ（たとえばｃＤＮＡ鎖）の５’端部に（第二増幅ドメイン）増幅ドメインを組み込むように改変することができる。そのような増幅ドメインは、たとえばライゲーティング二本鎖アダプターにより、ＤＮＡ（たとえばｃＤＮＡ）分子の端部に組み込んでもよい。ライゲーションステップに適した酵素は当技術分野では公知であり、たとえばＴｔｈ ＤＮＡリガーゼ、Ｔａｑ ＤＮＡリガーゼ、サーモコッカス（Ｔｈｅｒｍｏｃｏｃｃｕｓ）菌種（９°Ｎ株）ＤＮＡリガーゼ（９°Ｎ（商標）ＤＮＡリガーゼ、Ｎｅｗ Ｅｎｇｌａｎｄ Ｂｉｏｌａｂｓ）、Ａｍｐｌｉｇａｓｅ（商標）（Ｅｐｉｃｅｎｔｒｅ Ｂｉｏｔｅｃｈｎｏｌｏｇｉｅｓ）及びＴ４ ＤＮＡリガーゼが含まれる。好ましい実施形態では、第一及び第二増幅ドメインは異なる配列を含む。

【０１４０】

上記から、したがって、様々な方法及び技法並びに当技術分野で公知のそのような技法の組合せにより、たとえばそのようなドメインを含むプライマーの使用、アダプターのライゲーション、ターミナルトランスフェラーゼ酵素の使用により及び／又は鋳型切替え法により、増幅ドメインを含むことがあるユニバーサルドメインを、確保された（すなわち、伸長した又はライゲートされた）ＤＮＡ分子に（たとえばｃＤＮＡ分子、又はその相補体（たとえば、第二鎖）に）付加してもよいことは明らかである。本明細書の考察から明白であるように、そのようなドメインは、アレイからＤＮＡ分子を放出する前に又は後で添加してもよい。

【０１４１】

本発明の方法により合成された単一アレイ由来のＤＮＡ（たとえばｃＤＮＡ）分子のすべてが、同じ第一及び第二増幅ドメインを含んでいることがあることは上記の説明から明らかであろう。その結果として、単回増幅反応、たとえばＰＣＲは、ＤＮＡ（たとえばｃＤＮＡ）分子のすべてを増幅するのに十分であることがある。したがって、好ましい実施形態では、本発明の方法はＤＮＡ（たとえばｃＤＮＡ）分子を増幅するステップを含むことがある。一実施形態では、増幅ステップは、ＤＮＡ（たとえばｃＤＮＡ）分子のアレイの基板からの放出後に実施される。他の実施形態では、増幅はアレイ上で（すなわち、アレイ上インサイツで）実施してもよい。増幅反応はアレイ上で実施できることが当技術分

野では公知であり、そのような反応を実行するためのオンチップ熱サイクラーが存在する。したがって、一実施形態では、シーケンシングプラットフォームとして又はいかなる形態の配列解析においても（たとえば、次世代シーケンシング技術において又はそれにより）使用するための当技術分野では公知であるアレイは、本発明のアレイの基盤（たとえば、イルミナビーズアレイ、等）として用いてもよい。

【０１４２】

ＤＮＡ（たとえばｃＤＮＡ）の第二鎖の合成のためには、アレイの基板から放出されたｃＤＮＡが部分的二本鎖核酸分子を含んでいる場合には、鎖置換ポリメラーゼ（たとえば、Φ２９ＤＮＡポリメラーゼ、Ｂｓｔ（ｅｘｏ－）ＤＮＡポリメラーゼ、クレノー（ｅｘｏ－）ＤＮＡポリメラーゼ）を使用するのが好ましい。たとえば、捕捉プローブが表面プローブを介してアレイの基板に間接的に固定化され、ＤＮＡ（たとえばｃＤＮＡ）分子を放出するステップは切断ステップを含む実施形態では、放出された核酸は少なくとも部分的二本鎖（たとえば、ＤＮＡ：ＤＮＡ、ＤＮＡ：ＲＮＡ又はＤＮＡ：ＤＮＡ／ＲＮＡハイブリッド）であることになる。鎖置換ポリメラーゼは、確実に第二ｃＤＮＡ鎖合成が位置ドメイン（フィーチャー識別ドメイン）の相補体を第二ＤＮＡ、たとえばｃＤＮＡ鎖に組み込むのに必要である。

【０１４３】

アレイの表面又は基板から少なくとも一部のＤＮＡ（たとえばｃＤＮＡ分子又はそのアンプリコン）を放出するステップはいくつかの方法を使用して達成できることは明らかになるであろう。放出ステップの主目的は、ＤＮＡ（たとえばｃＤＮＡ分子又はそのアンプリコン）がアレイ上のそのフィーチャー（又は位置）に従って「タグ」付けされるように捕捉プローブ（又はその相補体）の位置ドメインが組み込まれる（又は含まれる）分子を生じることである。したがって、放出ステップは、ＤＮＡ（たとえばｃＤＮＡ分子又はそのアンプリコン）をアレイから取り除き、そのＤＮＡ（たとえばｃＤＮＡ分子又はアンプリコン）は位置ドメイン又はその相補体を含む（前記位置ドメイン又はその相補体が、たとえば捕捉プローブの伸長により確保された核酸、たとえば第一鎖ｃＤＮＡに組み込まれていた、及び場合によっては、第二鎖合成がアレイ上で起こる場合は第二鎖ＤＮＡにコピーされていた、又は増幅がアレイ上で起こる場合はアンプリコンにコピーされていたせいで）。それ故に、組織試料の様々な領域と関連付けることができる配列解析データを生じるためには、放出された分子が捕捉プローブ（又はその相補体）の位置ドメインを含むことが不可欠である。

【０１４４】

放出された分子は第一及び／又は第二鎖ＤＮＡ（たとえばｃＤＮＡ分子又はアンプリコン）である可能性があるので、及び捕捉プローブはアレイ上に間接的に固定化されていることもあるので、放出ステップがアレイからのＤＮＡ（たとえばｃＤＮＡ）分子の切断のステップを含むことがあるけれども、放出ステップは核酸切断のステップを必要としない、すなわち、ＤＮＡ（たとえばｃＤＮＡ分子又はアンプリコン）は、二本鎖分子を変性する、たとえば第二ｃＤＮＡ鎖を第一ｃＤＮＡ鎖から放出する、又はアンプリコンをその鋳型から放出する、又は第一鎖ｃＤＮＡ分子（すなわち、伸長された捕捉プローブ）を表面プローブから放出するだけで放出することができることは理解されるであろう。したがって、ＤＮＡ（たとえばｃＤＮＡ）分子は、核酸切断により及び／又は変性により（たとえば、加熱して二本鎖分子を変性することにより）アレイから放出することができる。増幅がアレイ上インサイツで実行される場合、これは当然のことながら、循環反応において変性によりアンプリコンを放出することを包含することになる。

【０１４５】

いくつかの実施形態では、ＤＮＡ（たとえばｃＤＮＡ）分子は、捕捉プローブのユニバーサルドメイン又は位置ドメインに位置していることがある切断ドメインの酵素的切断により放出される。上記のように、切断ドメインは、放出されたＤＮＡ（たとえばｃＤＮＡ）分子が位置（識別）ドメインを含むように位置ドメインの上流に（５’端部に）位置していなければならない。核酸切断のために適した酵素には、制限エンドヌクレアーゼ（た

とえばＲｓａＩ）が含まれる。他の酵素、たとえばウラシルＤＮＡグリコシラーゼ（ＵＤＧ）とＤＮＡグリコシラーゼーリアーゼエンドヌクレアーゼＶＩＩＩの混合物（ＵＳＥＲ（商標）酵素）又はＭｕｔＹとＴ７エンドヌクレアーゼＩ酵素の組合せは、本発明の方法の好ましい実施形態である。

【０１４６】

代わりの実施形態では、ＤＮＡ（たとえばｃＤＮＡ）分子は物理的手段によりアレイの表面又は基板から放出されることがある。たとえば、捕捉プローブが、たとえば表面プローブへのハイブリダイエーションを介してアレイの基板に間接的に固定化されている実施形態では、核酸分子間の相互作用を破壊するだけで十分である可能性がある。核酸分子間の相互作用を破壊する、たとえば二本鎖核酸分子を変性する、ための方法は、当技術分野では周知である。ＤＮＡ（たとえばｃＤＮＡ）分子を放出するための（すなわち、合成されたＤＮＡ（たとえばｃＤＮＡ）分子をアレイからはぎ取る）率直な方法は、二本鎖分子の水素結合に干渉する溶液を使用することである。本発明の好ましい実施形態では、ＤＮＡ（たとえばｃＤＮＡ）分子は、加熱水、たとえば少なくとも８５℃、好ましくは少なくとも９０、９１、９２、９３、９４、９５、９６、９７、９８、９９℃の水又はバッファーを適用することにより放出することができる。水素結合を破壊するのに十分な温度の使用の代替案又は追加として、前記溶液は核酸分子間の相互作用をさらに不安定化して、ＤＮＡ（たとえばｃＤＮＡ）分子を放出することができる塩、界面活性剤、等を含んでいてもよい。

【０１４７】

高温液、たとえば９０～９９℃水の適用で、捕捉プローブ又は表面プローブをアレイ基板に固定化するのに使用される共有結合を破壊するのに十分でもありうることは理解されるであろう。それ故に、好ましい実施形態では、ＤＮＡ（たとえばｃＤＮＡ）分子は、熱水をアレイに適用して共有結合的に固定化された捕捉又は表面プローブを破壊することにより放出してもよい。

【０１４８】

放出されたＤＮＡ、たとえばｃＤＮＡ分子（放出されたＤＮＡ、たとえばｃＤＮＡ分子を含む溶液）は、さらなる操作、たとえば第二鎖合成及び／又は増幅、のために収集されることは暗黙的に含まれる。にもかかわらず、本発明の方法は放出されたＤＮＡ（たとえばｃＤＮＡ）分子を収集する又は回収するステップを含むとみることもできる。上記のように、インサイツ増幅との関連では、放出された分子は確保された核酸（たとえばｃＤＮＡ）のアンプリコンを含んでいてもよい。

【０１４９】

本発明の方法の実施形態では、どんな伸長されていない又はライゲートされていない捕捉プローブも取り除くのが望ましいことがある。これは、たとえば、アレイからＤＮＡ分子を放出するステップの後であってもよい。たとえば伸長されていない又はライゲートされていないプローブの分解のための酵素（たとえばエキソヌクレアーゼ）の使用を含む、いかなる望ましい又は都合のよい方法でも、そのような除去のために使用してもよい。

【０１５０】

アレイから放出されており、上記の通りに改変されている可能性があるＤＮＡ（たとえばｃＤＮＡ分子又はアンプリコン）は、研究するために解析される（たとえば、その配列を決定するが、上記のように、実際の配列決定は必要ではなく、配列を解析するいかなる方法を使用してもよい）。したがって、核酸解析のいかなる方法を使用してもよい。配列解析のステップにより、位置付けられたドメインを同定することができ、それ故に解析された分子を組織試料内の位置に局在化することが可能になる。同様に、解析された分子の性質又は同一性を決定することができる。このようにして、アレイ中（それ故に組織試料中）の所与の位置の核酸（たとえばＲＮＡ）を決定することができる。それ故に、解析ステップは、解析された分子（及びそれ故に「標的」分子）及びその位置ドメインを同定するいかなる方法も含む又は使用してもよい。一般的に、そのような方法は配列特異的方法になる。たとえば、前記方法は配列特異的プライマー又はプローブ、特に位置ドメインに

及び／又は検出される若しくは解析される特定の核酸分子（たとえば検出される核酸（たとえばＲＮＡ若しくはｃＤＮＡ分子）に対応するＤＮＡ分子）に特異的であるプライマー又はプローブを使用することがある。典型的には、そのような方法では、配列特異的増幅プライマー、たとえばＰＣＲプライマーを使用してもよい。

【０１５１】

いくつかの実施形態では、標的関連分子のサブセット又はファミリー（たとえば配列類似性及び／又は保存されたドメインを共有するタンパク質の特定の群（たとえば受容体のファミリー）をコードする配列のすべて）を解析するのが望ましいことがある。それ故に、本明細書に記載される増幅及び／又は解析法は、捕捉された核酸又はそれに由来する核酸（たとえばアンプリコン）のサブセットにハイブリダイズする縮重又は遺伝子ファミリー特異的プライマー又はプローブを使用してもよい。特に好ましい実施形態では、増幅及び／又は解析法は、ユニバーサルプライマー（すなわち、捕捉された配列のすべてに共通するプライマー）を標的分子のサブセットに特異的な縮重又は遺伝子ファミリー特異的プライマーと組み合わせて利用してもよい。

【０１５２】

したがって、一実施形態では、配列解析の増幅ベースの、特にＰＣＲベースの方法が使用される。

【０１５３】

しかし、放出されたＤＮＡ（たとえばｃＤＮＡ）分子を改変する及び／又は増幅するステップは試料に追加の成分、たとえば酵素、プライマー、ヌクレオチド、等を導入してもよい。それ故に、本発明の方法は、たとえばシーケンシング反応に干渉することがあるオリゴヌクレオチドプライマー、ヌクレオチド、塩、等を取り除くために、配列解析に先立って放出されたＤＮＡ（たとえばｃＤＮＡ分子又はアンプリコン）を含む試料を精製するステップをさらに含むことがある。ＤＮＡ（たとえばｃＤＮＡ）分子を精製するいかなる適切な方法を使用してもよい。

【０１５４】

上記のように、放出されたＤＮＡ分子の配列解析は直接的でも間接的でもよい。したがって、配列解析基質（配列解析ステップ又は手順を受ける分子とみることもできる）はアレイから放出される分子そのままでもよく、前記分子に由来する分子であってもよい。したがって、たとえば、シーケンシング反応を含む配列解析ステップとの関連ではシーケンシング鋳型は、アレイから放出される分子でもよく、前記分子に由来する分子であってもよい。たとえば、アレイから放出される第一及び／又は第二鎖ＤＮＡ、たとえばｃＤＮＡ分子、は配列解析を直接受けてもよく（たとえば、シーケンシング）、すなわち配列解析反応又は手順に直接関与してもよい（たとえば、シーケンシング反応若しくはシーケンシング手順、又はシーケンシングされる若しくは他の方法で同定される分子である）。インサイツ増幅との関連では、放出された分子はアンプリコンであってもよい。代わりに、放出された分子は、配列解析（たとえば、シーケンシング又は他の手段による同定）の前に第二鎖合成又は増幅のステップを受けてもよい。したがって、配列解析基質（たとえば、鋳型）は、アレイから直接放出される分子のアンプリコン又は第二鎖でもよい。

【０１５５】

二本鎖分子の両鎖は配列解析（たとえば、シーケンシングされる）を受けてもよいが、本発明はこれに限定されず、一本鎖分子（たとえば、ｃＤＮＡ）が解析され（たとえば、シーケンシングされ）てもよい。たとえば、様々なシーケンシング技術（たとえばＨｅｌｉｃｏｓ　ｏｒ　Ｐａｃｂｉｏ技術又は開発中のナノポアシーケンシング技術）を単一分子シーケンシングのために使用してもよい。したがって、一実施形態では、ＤＮＡ（たとえばｃＤＮＡ）の第一鎖がシーケンシングを受けてもよい。第一鎖ＤＮＡ（たとえばｃＤＮＡ）は、単一分子シーケンシングを可能にするために３’端部で改変される必要があることもある。これは、第二ＤＮＡ（たとえばｃＤＮＡ）鎖を取り扱うための手順に類似する手法によって実行することができる。そのような手法は当技術分野では公知である。

【０１５６】

本発明の好ましい態様では、配列解析は捕捉された核酸（たとえばＲＮＡ）配列の一部及び位置ドメインの配列を同定する又は明らかにすることになる。位置ドメイン（又はタグ）の配列は、核酸（たとえばｍＲＮＡ）分子が捕捉されたフィーチャーを同定することになる。捕捉された核酸（たとえばＲＮＡ）分子の配列は、それが対応する遺伝子を決定するために、試料が由来する生物の配列データベースと比較されることがある。組織試料のどの領域（たとえば、細胞）がフィーチャーと接触していたのかを決定することにより、組織試料のどの領域が前記遺伝子を発現していた（又は、たとえば空間ゲノミクスの場合では、遺伝子を含有していた）のかを決定することが可能である。この解析は、本発明の方法により生成されるＤＮＡ（たとえばｃＤＮＡ）分子のすべてについて達成され、組織試料の空間的トランスクリプトーム又はゲノムが得られる。

【０１５７】

代表的な例として、シーケンシングデータは解析されて、その配列を捕捉プローブの特定のスピーシーズに（すなわち位置ドメインの配列に従って）分類してもよい。これは、たとえばそれぞれの捕捉プローブ位置ドメイン（タグ）配列について配列を個々のファイルに分類するＦａｓｔＸツールキットＦＡＳＴＱ　Ｂａｒｃｏｄｅスプリッターツールを使用することにより達成することができる。それぞれのスピーシーズの（すなわちそれぞれのフィーチャー由来の）配列は解析されて、転写物の同一性を決定することができる。たとえば、配列は、配列を１つ又は複数のゲノムデータベース、好ましくは組織試料が得られた生物のデータベースと比較するＢｌａｓｔｎソフトウェアを使用して同定してもよい。本発明の方法により生成される配列に最大の類似性のあるデータベース配列の同一性は前記配列に割り当てることができる。一般に、少なくとも$1e^{-6}$、好ましくは$1e^{-7}$、$1e^{-8}$又は$1e^{-9}$の確実性を有するヒットのみが、首尾よく同定されたとみることができる。

【０１５８】

本発明の方法ではいかなる核酸シーケンシング法でも使用できることは明らかであろう。しかし、いわゆる「次世代シーケンシング」技法は、本発明において特に有用になる。高処理シーケンシングは、これにより多数の核酸を極めて短期間で部分的にシーケンシングすることができるので、本発明の方法においては特に有用である。完全に又は部分的にシーケンシングされたゲノムの数が最近激増したことに照らして、それぞれの分子が対応する遺伝子を決定するために生成されたＤＮＡ（たとえばｃＤＮＡ）分子の完全長をシーケンシングすることは不可欠ではない。たとえば、ＤＮＡ（たとえばｃＤＮＡ）分子のそれぞれの端部から最初の１００ヌクレオチドで、その核酸（たとえばｍＲＮＡ）が捕捉されたフィーチャー（すなわち、アレイ上のその位置）と発現される遺伝子の両方を同定するのに十分であるはずである。ＤＮＡ（たとえばｃＤＮＡ）分子の「捕捉プローブ端部」からの配列反応により、位置ドメインの配列及び少なくとも約２０塩基、好ましくは３０又は４０塩基の転写物特異的配列データが得られる。「非捕捉プローブ端部」からの配列反応により、少なくとも約７０塩基、好ましくは８０、９０又は１００塩基の転写物特異的配列データが得られることがある。

【０１５９】

代表的な例として、シーケンシング反応は、Ｉｌｌｕｍｉｎａ（商標）技術において使用されるなどの可逆的ダイターミネーターに基づいていてもよい。たとえば、ＤＮＡ分子は、たとえばガラス又はシリコンスライド上のプライマーに先ず結合され、局所的クローンコロニーが形成されるように増幅される（ブリッジ増幅）。４種類のｄｄＮＴＰが添加され、組み込まれなかったヌクレオチドは洗い流される。ピロシーケンスとは異なり、ＤＮＡは一度に１つのヌクレオチドのみを伸長することができる。蛍光標識されたヌクレオチドの画像がカメラで取られ、次に色素は終端３’ブロッカーと共にＤＮＡから化学的に取り除かれ、次のサイクルが可能になる。これは必要な配列データが得られるまで繰り返すことができる。この技術を使用すれば、数千の核酸を１つのスライド上で同時にシーケンシングすることができる。

【０１６０】

他の高処理シーケンシング技術、たとえばピロシーケンスは本発明の方法において等しく適切である可能性がある。この方法では、ＤＮＡはオイル溶液中の水滴内で増幅され（エマルジョンＰＣＲ）、それぞれの水滴には単一プライマー被覆ビーズに付着した単一ＤＮＡ鋳型が含有されており、このビーズは次にクローンコロニーを形成する。シーケンシング装置には多くのピコリットル容積のウェルが含有され、それぞれのウェルは単一ビーズとシーケンシング酵素を含有している。ピロシーケンスは、新生ＤＮＡに付加される個々のヌクレオチドの検出のために光を発するルシフェラーゼを使用し、組合せデータを使用して配列読出しを生む。

【０１６１】

開発中の技術の例は、ＤＮＡの重合中に放出される水素イオンの検出に基づいている。シーケンシングされる鋳型ＤＮＡ鎖を含有するマイクロウェルは１種類のヌクレオチドで満たされる。導入されたヌクレオチドが主要な鋳型ヌクレオチドに相補的である場合、前記ヌクレオチドは成長中の相補鎖に組み込まれる。このせいで、超感受性イオンセンサーを始動させる水素イオンが放出されるが、これは反応が起きたことを示している。ホモポリマーリピートが鋳型配列に存在する場合、複数のヌクレオチドが一回のサイクルで組み込まれることになる。このせいで、対応する数の水素イオンが放出されて電子シグナルは比例して高くなる。

【０１６２】

したがって、次世代のシーケンシングフォーマットは徐々に利用されるようになっていることは明白であり、そうしたプラットフォームの主な特長の１つとして実行時間が短くなるにしたがって、他のシーケンシング技術が本発明の方法において有用になることは明らかとなろう。

【０１６３】

本発明の不可欠な特長は、上記のように、捕捉された核酸分子の相補鎖を捕捉プローブに固定する（たとえば捕捉されたＲＮＡ分子を逆転写する）ステップである。逆転写反応は当技術分野では周知であり、代表的な逆転写反応では、反応混合物には逆転写酵素、ｄＮＴＰ及び適切なバッファーが含まれる。反応混合物には、他の成分（たとえばリボヌクレアーゼ阻害剤（複数可））が含まれることもある。プライマー及び鋳型は捕捉プローブの捕捉ドメインであり、捕捉されたＲＮＡ分子は上に記載されている。主題方法では、それぞれのｄＮＴＰは典型的には、約１０～５０００μＭ、通常は約２０～１０００μＭに及ぶ量で存在していることになる。ＤＮＡポリメラーゼ活性のある酵素を使用して、同等の反応を実施して捕捉されたＤＮＡ分子の相補鎖を生成してもよいことは明らかであろう。この種の反応は当技術分野では周知であり、下にさらに詳細に説明されている。

【０１６４】

望ましい逆転写酵素活性は、１つ又は複数の別個の酵素によって提供されることがあり、適切な例は、Ｍ－ＭＬＶ、ＭｕＬＶ、ＡＭＶ、ＨＩＶ、ＡｒｒａｙＳｃｒｉｐｔ（商標）、ＭｕｌｔｉＳｃｒｉｂｅ（商標）、ＴｈｅｒｍｏＳｃｒｉｐｔ（商標）並びにＳｕｐｅｒＳｃｒｉｐｔ（登録商標）Ｉ、ＩＩ及びＩＩＩ酵素である。

【０１６５】

逆転写酵素反応はいかなる適切な温度でも実行でき、その温度は酵素の特性に依存することになる。典型的には、逆転写酵素反応は３７～５５℃で実施されるが、この範囲外の温度も適切であることもある。反応時間は、わずか１、２、３、４若しくは５分又は４８時間にもなることがある。典型的には、反応は選択に従って、５～１２０分、好ましくは５～６０、５～４５若しくは５～３０分又は１～１０若しくは１～５分間実行されることになる。反応時間は決定的ではなく、いかなる所望の反応時間を使用してもよい。

【０１６６】

前述の通り、方法のある種の実施形態には増幅ステップが含まれ、このステップでは、たとえば試料を濃縮して核酸（たとえば組織試料から捕捉された転写物）のさらに良好な表出を得るために生成されたＤＮＡ（たとえばｃＤＮＡ）分子のコピー数が増加される。増幅は所望により、線形でも指数関数的でもよく、興味深い代表的増幅プロトコールは、

ポリメラーゼ連鎖反応（ＰＣＲ）、等温増幅、等が含まれるが、これらに限定されない。

【０１６７】

ポリメラーゼ連鎖反応（ＰＣＲ）は当技術分野では周知であり、米国特許第４６８３２０２号、米国特許第４６８３１９５号、米国特許第４８００１５９号、米国特許第４９６５１８８号及び米国特許第５５１２４６２号に記載されており、その開示は参照により本明細書に組み込まれる。代表的ＰＣＲ増幅反応では、反応混合物はアレイからの上記の放出されたＤＮＡ（たとえばｃＤＮＡ）分子を含み、この分子はプライマー伸長反応において用いられる１つ又は複数のプライマー、たとえば第一及び／又は第二増幅ドメインにハイブリダイズするＰＣＲプライマー（たとえば、幾何学的（又は指数関数的）増幅において用いられるフォワード及びリバースプライマー又は線形増幅において用いられる単一プライマー）と組み合わされる。放出されたＤＮＡ（たとえばｃＤＮＡ）分子（以下、便宜上鋳型ＤＮＡと呼ばれる）が接触されるオリゴヌクレオチドプライマーは、アニーリング条件下（下にさらに極めて詳細に説明されている）で相補的鋳型ＤＮＡへのハイブリダイゼーションを提供するのに十分な長さになる。プライマーの長さは増幅ドメインの長さに依存することになるが、一般的には少なくとも１０ｂｐ長、通常は少なくとも１５ｂｐ長、さらに通常では少なくとも１６ｂｐ長になり、３０ｂｐ長にも又はさらに長くなることもあり、プライマーの長さは一般的には１８～５０ｂｐ長、通常は約２０～３５ｂｐ長に及ぶことになる。鋳型ＤＮＡは、鋳型ＤＮＡのプライマー伸長線形又は指数関数的増幅が望まれるかどうかに応じて、単一プライマー又は２つのプライマー（フォワード及びリバースプライマー）のセットに接触させてもよい。

【０１６８】

上記の成分に加えて、主題方法において作製された反応混合物は典型的には、ポリメラーゼ及びデオキシリボヌクレオシド三リン酸（ｄＮＴＰ）を含む。望ましいポリメラーゼ活性は、１つ又は複数の別個のポリメラーゼ酵素により提供されることがある。多くの実施形態では、反応混合物は少なくともファミリーＡポリメラーゼを含み、興味深い代表的ファミリーＡポリメラーゼには、天然に存在するポリメラーゼ（Ｔａｑ）並びにＫｌｅｎｔａｑ（Ｂａｒｎｅｓ　ｅｔ　ａｌ．Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ　ＵＳＡ（１９９４）９１：２２１６～２２２０頁に記載されている）などのその誘導体及び類似体を含むサーマス・アクアチカス（Ｔｈｅｒｍｕｓ　ａｑｕａｔｉｃｕｓ）ポリメラーゼ；天然に存在するポリメラーゼ（Ｔｔｈ）並びにその誘導体及び類似体を含むサーマス・サーモフィラス（Ｔｈｅｒｍｕｓ　ｔｈｅｒｍｏｐｈｉｌｕｓ）ポリメラーゼ、等が含まれるがこれらに限定されない。実行される増幅反応が高忠実度反応である、ある種の実施形態では、反応混合物は、たとえばファミリーＢポリメラーゼにより提供されることのあるような３’－５’エキソヌクレアーゼ活性を有するポリメラーゼ酵素をさらに含んでいてもよく、興味深いファミリーＢポリメラーゼには、Ｐｅｒｉｅｒ　ｅｔ　ａｌ．、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ　ＵＳＡ（１９９２）８９：５５７７～５５８１頁に記載されているサーモコッカス・リトラリス（Ｔｈｅｒｍｏｃｏｃｃｕｓ　ｌｉｔｏｒａｌｉｓ）ＤＮＡポリメラーゼ（Ｖｅｎｔ）；Ｌｕｎｄｂｅｒｇ　ｅｔ　ａｌ．、Ｇｅｎｅ（１９９１）１０８：１～６頁に記載されているパイロコッカス・フリオサス（Ｐｙｒｏｃｏｃｃｕｓ　ｆｕｒｉｏｓｕｓ）ＤＮＡポリメラーゼ（Ｐｆｕ）、パイロコッカス・ヴェッセイ（Ｐｙｒｏｃｏｃｃｕｓ　ｗｏｅｓｅｉ（Ｐｗｏ））、等が含まれるがこれらに限定されない。反応混合物がファミリーＡとファミリーＢポリメラーゼの両方を含む場合、ファミリーＡポリメラーゼはファミリーＢポリメラーゼよりも多い量で反応混合物に存在していてもよく、活性の違いは通常少なくとも１０倍、さらに通常では少なくとも約１００倍になる。通常、反応混合物は、存在する４種の天然に存在する塩基に対応して４つの異なる種類のｄＮＴＰ（すなわちｄＡＴＰ、ｄＴＴＰ、ｄＣＴＰ及びｄＧＴＰ）を含むことになる。主題方法では、それぞれのｄＮＴＰは典型的には、約１０～５０００μＭ、通常は約２０～１０００μＭに及ぶ量で存在することになる。

【０１６９】

主題方法の逆転写酵素及び／又は増幅ステップにおいて調製された反応混合物は、一価

イオンの供給源、二価陽イオンの供給源及び緩衝剤を含む水性緩衝媒体をさらに含んでいることがある。ＫＣｌ、Ｋ－アセテート、NH_4－アセテート、Ｋ－グルタミン酸、NH_4Cl、硫酸アンモニウム、等などの一価イオンのいかなる都合の良い供給源も用いることができる。二価陽イオンは、マグネシウム、マンガン、亜鉛、等であってもよく、陽イオンは典型的にはマグネシウムになる。$MgCl_2$、Ｍｇ－アセテート、等を含むマグネシウム陽イオンのいかなる都合の良い供給源も用いることができる。バッファー中に存在するMg^{2+}の量は０．５～１０ｍＭに及ぶことがあるが、好ましくは約３～６ｍＭに及ぶことになり、理想的には約５ｍＭになる。バッファー中に存在していてもよい代表的な緩衝剤又は塩には、Ｔｒｉｓ、Ｔｒｉｃｉｎｅ、ＨＥＰＥＳ、ＭＯＰＳ、等が含まれ、緩衝剤の量は典型的には、約５～１５０ｍＭ、通常は約１０～１００ｍＭ、さらに通常は約２０～５０ｍＭに及ぶことになり、ある種の好ましい実施形態では、緩衝剤は約６．０～９．５に及ぶｐＨを与えるのに十分な量で存在することになり、もっとも好ましくは７２℃でｐＨ７．３である。緩衝媒体に存在していてもよい他の薬剤には、ＥＤＴＡ、ＥＧＴＡ、等などのキレート剤が含まれる。

【０１７０】

主題方法のステップの逆転写酵素、ＤＮＡ伸長又は増幅反応混合物を調製する際に、様々な構成成分はいかなる都合の良い順番で組み合わせてもよい。たとえば、増幅反応では、バッファーはプライマー、ポリメラーゼと、次に鋳型ＤＮＡと組み合わせてもよく、又は様々な構成成分のすべてを同時に組み合わせて反応混合物を作製してもよい。

【０１７１】

上記のように、本発明の好ましい実施形態では、ＤＮＡ（たとえばｃＤＮＡ）分子は核酸分子の端部に増幅ドメインを付加することにより改変してもよく、これにはライゲーション反応を含むことがある。ライゲーション反応は、捕捉プローブがアレイ表面に間接的に固定化されている場合には、アレイ上での捕捉プローブのインサイツ合成にも必要とされる。

【０１７２】

当技術分野では公知であるように、リガーゼは、２つの直接隣接している核酸の並置された３’ヒドロキシルと５’リン酸終端間でのリン酸ジエステル結合の形成を触媒する。いかなる都合の良いリガーゼでも用いてよく、興味深い代表的リガーゼには、温度感受性及び熱安定性リガーゼが含まれるがこれらに限定されない。温度感受性リガーゼには、バクテリオファージＴ４　ＤＮＡリガーゼ、バクテリオファージＴ７リガーゼ及びイー・コリ（Ｅ．ｃｏｌｉ）リガーゼが含まれるがこれらに限定されない。熱安定性リガーゼには、Ｔａｑリガーゼ、Ｔｔｈリガーゼ及びＰｆｕリガーゼが含まれるがこれらに限定されない。熱安定性リガーゼは、原核、真核又は古細菌生物を含むがこれらに限定されない好熱性又は超好熱性生物から得られる。ある種のＲＮＡリガーゼも本発明の方法において用いることができる。

【０１７３】

このライゲーションステップでは、適切なリガーゼ並びに必要である及び／又は望ましいどんな試薬でも反応混合物と組み合わされて、関連オリゴヌクレオチドのライゲーションが起こるのに十分な条件下で維持される。ライゲーション反応条件は当業者には周知である。ライゲーション中、ある種の実施形態における反応混合物は、約４℃～約５０℃、たとえば約２０℃～約３７℃に及ぶ温度で、約５秒～約１６時間、たとえば約１分～約１時間に及ぶ期間維持されてもよい。さらに他の実施形態では、反応混合物は約３５℃～約４５℃、たとえば約３７℃～約４２℃に及ぶ温度で、たとえば３８℃、３９℃、４０℃又は４１℃で又は約３８℃、３９℃、４０℃又は４１℃で、約２分～約８時間を含む、約５秒～約１６時間、たとえば約１分～約１時間に及ぶ期間維持されてもよい。代表的な実施形態では、ライゲーション反応混合物には、５０ｍＭ　Ｔｒｉｓ　ｐＨ７．５、１０ｍＭ　$MgCl_2$、１０ｍＭ　ＤＴＴ、１ｍＭ　ＡＴＰ、２５ｍｇ／ｍｌ　ＢＳＡ、０．２５ユニット／ｍｌリボヌクレアーゼ阻害剤及びＴ４　ＤＮＡリガーゼが０．１２５ユニット／ｍｌで含まれる。さらに別の代表的実施形態では、２．１２５ｍＭマグネシウムイオン

、０．２ユニット／ｍｌリボヌクレアーゼ阻害剤及び０．１２５ユニット／ｍｌ　ＤＮＡリガーゼが用いられる。反応におけるアダプターの量は試料中のＤＮＡ、たとえばｃＤＮＡの濃度に依存することになり、一般にはＤＮＡ、たとえばｃＤＮＡのモル量の１０～１００倍の間で存在することになる。

【０１７４】

代表的な例として、本発明の方法は、以下の

（ａ）アレイを組織試料に接触させるステップであって、前記アレイは捕捉プローブの複数のスピーシーズが、直接的に又は間接的に固定化されている基板を備え、それぞれのスピーシーズはアレイ上の別個の位置を占め、かつ前記プローブが逆転写酵素（ＲＴ）プライマーとして機能できるように遊離の３’端部を有する配向となっており、前記捕捉プローブのそれぞれのスピーシーズが、組織試料のＲＮＡが前記捕捉プローブにハイブリダイズするように、５’から３’に向けて、

（ｉ）アレイ上の捕捉プローブの位置に対応する位置ドメイン、及び

（ｉｉ）捕捉ドメイン

を有する核酸を含む、ステップと、

（ｂ）アレイ上の組織試料を撮像するステップと、

（ｃ）捕捉されたｍＲＮＡ分子を逆転写してｃＤＮＡ分子を生成するステップと、

（ｄ）アレイを洗浄して残余の組織を取り除くステップと、

（ｅ）前記ｃＤＮＡ分子の少なくとも一部をアレイの表面化から放出するステップと、

（ｆ）放出されたｃＤＮＡ分子上で第二鎖ｃＤＮＡ合成を実施するステップと、

（ｇ）前記ｃＤＮＡ分子の配列を解析する（たとえば、シーケンシングする）ステップと

を含むことがある。

【０１７５】

代わりの代表的な例として、本発明の方法は、以下の

（ａ）アレイを組織試料に接触させるステップであって、前記アレイは捕捉プローブの少なくとも２つのスピーシーズが、直接的に又は間接的に固定化されている基板を備え、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブが逆転写酵素（ＲＴ）プライマーとして機能できるように遊離の３’端部を有する配向となっており、前記捕捉プローブのそれぞれのスピーシーズが、組織試料のＲＮＡが前記捕捉プローブにハイブリダイズするように、５’から３’に向けて、

（ｉ）アレイ上の捕捉プローブの位置に対応する位置ドメイン、及び

（ｉｉ）捕捉ドメイン

を有する核酸を含む、ステップと、

（ｂ）場合によって組織試料を再水和するステップと、

（ｃ）捕捉されたｍＲＮＡ分子を逆転写して第一鎖ｃＤＮＡ分子を生成し、場合によって第二鎖ｃＤＮＡ分子を合成するステップと、

（ｄ）アレイ上の組織試料を撮像するステップと、

（ｅ）アレイを洗浄して残余の組織を取り除くステップと、

（ｆ）前記ｃＤＮＡ分子の少なくとも一部をアレイの表面化から放出するステップと、

（ｇ）放出されたｃＤＮＡ分子を増幅するステップと、

（ｈ）前記増幅されたｃＤＮＡ分子の配列を解析する（たとえば、シーケンシングする）ステップと

を含むことがある。

【０１７６】

さらに追加の代表的な例として、本発明の方法は、以下の

（ａ）アレイを組織試料に接触させるステップであって、前記アレイは捕捉プローブの複数のスピーシーズが、直接的に又は間接的に固定化されている基板を備え、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブが逆転写酵素（ＲＴ）プライマーとして機能できるように遊離の３’端部を有する配向となっており、前記捕捉プ

ローブのそれぞれのスピーシーズが、組織試料のＲＮＡが前記捕捉プローブにハイブリダイズするように、５’から３’に向けて、
（ｉ）アレイ上の捕捉プローブの位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）場合によってアレイ上の組織試料を撮像するステップと、
（ｃ）捕捉されたｍＲＮＡ分子を逆転写してｃＤＮＡ分子を生成するステップと、
（ｄ）場合によって、ステップ（ｂ）としてまだ実施されていなければ、アレイ上の組織試料を撮像するステップと、
（ｅ）アレイを洗浄して残余の組織を取り除くステップと、
（ｆ）前記ｃＤＮＡ分子の少なくとも一部をアレイの表面から放出するステップと、
（ｇ）放出されたｃＤＮＡ分子上で第二鎖ｃＤＮＡ合成を実施するステップと、
（ｈ）二本鎖ｃＤＮＡ分子を増幅するステップと、
（ｉ）場合によってｃＤＮＡ分子を精製して、シーケンシング反応に干渉することがある成分を取り除くステップと、
（ｊ）前記増幅されたｃＤＮＡ分子の配列を解析する（たとえば、シーケンシングする）ステップと
を含むことがある。

【０１７７】

本発明は上記の方法におけるステップのいかなる適切な組合せも含む。本発明が、たとえば増幅がアレイ上インサイツで実施される場合に、これらの方法の変動も包含することは理解されるであろう。撮像ステップを省く方法も包含されている。

【０１７８】

本発明は、（ｉ）アレイに接触させる組織試料からｍＲＮＡを捕捉するのに使用するための、又は（ｉｉ）組織試料の（たとえば、一部の若しくは包括的）トランスクリプトームを決定する及び／若しくは解析するのに使用するためのアレイを作る又は作製するための方法であって、捕捉プローブの複数のスピーシーズをアレイ基板に直接的に又は間接的に固定化することを含み、前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）アレイ上の捕捉プローブの位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む前記方法を含むとみることも可能である。

【０１７９】

本発明のアレイを作製する方法は、捕捉プローブのそれぞれのスピーシーズがアレイ上のフィーチャーとして固定化されるようにさらに定義にしてもよい。

【０１８０】

アレイ上に捕捉プローブを固定化する方法は、本明細書に記載されるいかなる適切な方法でも使用して達成してもよい。捕捉プローブがアレイ上に間接的に固定化されている場合、捕捉プローブはアレイ上で合成されてもよい。前記方法は、以下の
（ａ）複数の表面プローブをアレイ基板に直接的又は間接的に固定化するステップであって、前記表面プローブが
（ｉ）捕捉ドメインオリゴヌクレオチドの一部（核酸、たとえばＲＮＡを捕捉することに関与していない部分）にハイブリダイズすることができるドメイン、
（ｉｉ）相補的位置ドメイン、及び
（ｉｉｉ）相補的ユニバーサルドメイン
を含むステップと、
（ｂ）アレイ上に固定化された表面プローブに、捕捉ドメインオリゴヌクレオチド及びユニバーサルドメインオリゴヌクレオチドをハイブリダイズさせるステップと、
（ｃ）鋳型重合によりユニバーサルドメインオリゴヌクレオチドを伸長して、捕捉プローブの位置ドメインを生成するステップと、

（ｄ）位置ドメインを捕捉ドメインオリゴヌクレオチドにライゲートして、捕捉オリゴヌクレオチドを作製するステップ
のうちのいずれか１つ又は複数を含んでいてもよい。

【０１８１】

ステップ（ｄ）のライゲーションは、ステップ（ｃ）の伸長と同時に起こってもよい。したがって、ステップ（ｄ）のライゲーションは別々のステップにおいて実行される必要はないが、当然のことながら、これは必要な場合には包含されている。

【０１８２】

本発明のアレイを作製する上記方法により作製されたアレイのフィーチャーは、上記説明に従ってさらに定義されてもよい。

【０１８３】

本発明は、ＲＮＡの検出又は解析及びトランスクリプトーム解析又は検出に関して上に説明されているが、記載されている原理は細胞中のＤＮＡの検出又は解析に及びゲノム研究に同様に適用することが可能であることは認識されるであろう。したがって、さらに広く見渡せば、本発明は核酸全般の検出に一般的に適用可能であると、さらにより詳細な態様ではＤＮＡの解析又は検出のための方法を提供するとみることができる。空間情報はゲノミクス関連、すなわち空間分解能でのＤＮＡ分子の検出及び／又は解析において価値があることもある。これは、本発明に従ってゲノムタグ付けにより達成することができる。そのような局在化された又は空間的検出法は、たとえば組織の異なる細胞若しくは領域のゲノム変動を研究する、たとえば正常細胞若しくは組織と疾患細胞若しくは組織（たとえば、正常細胞若しくは組織対腫瘍細胞若しくは組織）を比較するという状況において、又は疾患進行におけるゲノム変化を研究する、等において有用であることがある。たとえば、腫瘍組織は、前記組織が含有するゲノム変動（たとえば、変異及び／又は他の遺伝子異常、たとえば染色体再編、染色体増幅／欠失／挿入、等）が異なることがある異種細胞集団を含むことがある。異なる細胞におけるゲノム変動又は異なるゲノム遺伝子座の局所的な形態での検出は、そのような状況において、たとえばゲノム変動の空間的分布を研究するのに有用であることがある。そのような方法の主な有用性は、腫瘍解析にあると考えられる。本発明の状況では、たとえば１つのフィーチャー上の細胞全体のゲノムを捕捉するように設計されているアレイを調製してもよい。したがって、組織試料中の異なる細胞を比較することができる。当然のことながら、本発明はそのような設計に限定されてはおらず、他の変動も可能なことがあり、その場合ＤＮＡは局在化された方法で検出され、アレイで捕捉されたＤＮＡの位置は組織試料中の位置又は場所と関連付けられる。

【０１８４】

したがって、さらに一般的な態様では、本発明は、組織試料中の核酸の局在化された検出のための方法であって、

（ａ）
捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、

前記補足プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、

前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、

（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び

（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、

（ｂ）アレイ上の捕捉プローブの位置と、組織試料中の位置とが関連付けられるように前記アレイを組織試料に接触させ、組織試料の核酸を前記捕捉プローブの捕捉ドメインにハイブリダイズさせるステップと、

（ｃ）前記捕捉プローブを伸長又はライゲーションプライマーとして用いて捕捉された核酸分子からＤＮＡ分子を生成するステップであって、前記伸長された又はライゲートさ

れたＤＮＡ分子が位置ドメインによりタグ付けされるステップと、
（ｄ）場合によって前記タグ付けされたＤＮＡの相補鎖を生成する及び／又は場合によって前記タグ付けされたＤＮＡを増幅するステップと、
（ｅ）アレイの表面から前記タグ付けされたＤＮＡ分子及び／又はその相補体若しくはアンプリコンの少なくとも一部を放出し、前記一部が位置ドメイン又はその相補体を含むステップと、
（ｆ）前記放出されたＤＮＡ分子の配列を直接的に又は間接的に解析する（たとえば、シーケンシングする）ステップと
を含む方法を提供するとみることができる。

【０１８５】

上でさらに詳細に説明されたように、核酸解析のいかなる方法でも解析ステップにおいて使用してもよい。典型的には、これはシーケンシングを含むことがあるが、実際の配列決定を必ずしも実施する必要はない。たとえば、配列特異的解析方法を使用してもよい。たとえば、配列特異的増幅反応を、たとえば、位置ドメインに及び／又は特定の標的配列、たとえば検出される特定の標的ＤＮＡ（すなわち、特定のｃＤＮＡ／ＲＮＡ又は遺伝子又は遺伝子バリアント又はゲノム遺伝子座又はゲノムバリアント、等に対応する）に特異的なプライマーを使用して実施してもよい。例となる解析法は配列特異的ＰＣＲ反応である。

【０１８６】

ステップ（ｆ）において得られる配列解析（たとえば、シーケンシング）情報を使用して、試料中の核酸に関する空間情報を得ることもできる。言い換えると、配列解析情報は試料中の核酸の位置に関する情報を提供することができる。この空間情報は、たとえば決定された又は同定された配列から得られた配列解析情報の性質から導かれることがあり、たとえば前記空間情報は、使用される組織試料との関連ではそれ自体が空間的な情報を与えることができる特定の核酸分子の存在を明らかにすることができる、及び／又は空間情報（たとえば、空間的局在化）は、配列解析情報と相俟ってアレイ上の組織試料の位置から導かれることがある。しかし、上記のように、空間情報は、配列解析データを組織試料の画像と関連付けることにより都合よく得られることがあり、これは本発明の１つの好ましい実施形態を表す。

【０１８７】

したがって、好ましい実施形態では、前記方法は、
（ｇ）前記配列解析情報を前記組織試料の画像と関連付け、前記組織試料はステップ（ｃ）の前に又は後で撮像されるステップも含む。

【０１８８】

ステップ（ａ）において言及されるプライマー伸長反応は、ポリメラーゼ触媒伸長反応と定義してもよく、すなわちプライマーとして捕捉プローブを鋳型として捕捉された核酸を利用して相補鎖を合成することにより、捕捉プローブに共有結合している捕捉された核酸分子の相補鎖を獲得するように作用する。言い換えると、プライマー伸長反応は、どんなポリメラーゼ酵素により実行されるいかなるプライマー伸長反応であってもよい。核酸はＲＮＡであることもあり、又はＤＮＡであることもある。したがって、ポリメラーゼはいかなるポリメラーゼでもよい。ポリメラーゼは逆転写酵素であることもあり、又はＤＮＡポリメラーゼであることもある。ライゲーション反応はいかなるリガーゼにより実行されてもよく、捕捉プローブに捕捉された核酸分子の相補鎖を固定するように作用する、すなわち捕捉された核酸分子（捕捉プローブにハイブリダイズした）は部分的に二本鎖であり、相補鎖は捕捉プローブにライゲートされている。

【０１８９】

そのような方法の一つの好ましい実施形態は、トランスクリプトームの決定及び／若しくは解析のために、又はＲＮＡの検出のために上に記載されている方法である。代わりの好ましい実施形態では、検出される核酸分子はＤＮＡである。そのような実施形態では、本発明は、組織試料中のＤＮＡの局在化された検出のための方法であって、

（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、
前記補足プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、

（ｂ）アレイ上の捕捉プローブの位置と、組織試料中の位置とが関連付けられるように前記アレイを組織試料に接触させ、組織試料のＤＮＡを前記捕捉プローブ中の捕捉ドメインにハイブリダイズさせるステップと、
（ｃ）前記組織試料中のＤＮＡを断片化するステップであって、前記断片化がステップ（ｂ）において前記アレイを組織試料に接触させる前に、間に又は後で実行される、ステップと、
（ｄ）捕捉されたＤＮＡ断片を鋳型として用いてプライマー伸長反応において前記捕捉プローブを伸長させて伸長されたＤＮＡ分子を生成する、又は前記捕捉されたＤＮＡ断片をライゲーション反応において捕捉プローブにライゲートしてライゲートされたＤＮＡ分子を生成するステップであって、前記伸長された又はライゲートされたＤＮＡ分子が位置ドメインによりタグ付けされる、ステップと、
（ｅ）場合によって前記タグ付けされたＤＮＡの相補鎖を生成する及び／又は場合によって前記タグ付けされたＤＮＡを増幅するステップと、
（ｆ）アレイの表面から前記タグ付けされたＤＮＡ分子並びに／又はその相補体及び／若しくはアンプリコンの少なくとも一部を放出するステップであって、前記一部が位置ドメイン又はその相補体を含む、ステップと、
（ｇ）前記放出されたＤＮＡ分子の配列を直接的に又は間接的に解析するステップと
を含む方法を提供する。

【０１９０】
前記方法は、
（ｈ）前記配列解析情報を前記組織試料の画像と関連付け、前記組織試料がステップ（ｄ）の前に又は後で撮像されるステップを
さらに含むこともある。

【０１９１】
標的核酸がＤＮＡである空間ゲノミクスとの関連では、撮像と画像関連付けステップを含むことは、いくつかの状況では好ましいことがある。

【０１９２】
ＤＮＡが捕捉される実施形態では、前記ＤＮＡは細胞中に存在することができるいかなるＤＮＡ分子でもよい。したがって、前記ＤＮＡはゲノム（すなわち核ＤＮＡ、ミトコンドリアＤＮＡ又はプラスチドＤＮＡ（たとえば葉緑体ＤＮＡ））でもよい。好ましい実施形態では、前記ＤＮＡはゲノムＤＮＡである。

【０１９３】
ステップ（ｂ）における接触後、すなわち組織試料がアレイ上に置かれた後に断片化が実行される場合、断片化はＤＮＡが捕捉ドメインにハイブリダイズされる前に起こることが理解されるであろう。言い換えると、ＤＮＡ断片は、前記捕捉プローブ中の捕捉ドメインにハイブリダイズされる（又はさらに詳細には、ハイブリダイズさせる）。

【０１９４】
有利なことに、しかし必ずしもそうではないが、本発明のこの態様の特定の実施形態では、組織試料のＤＮＡ断片に、アレイ上の捕捉プローブによるその捕捉を可能にする又は促進する結合ドメインを与えてもよい。したがって、結合ドメインは捕捉プローブの捕捉

ドメインにハイブリダイズすることができる。したがって、そのような結合ドメインは捕捉ドメインの相補体とみることもできるが（すなわち、結合ドメインは相補的捕捉ドメインとみることもできる）、捕捉ドメインと結合ドメイン間の絶対的相補性は必要ではなく、結合ドメインが、生産的ハイブリダイゼーションを起こすのに十分相補的であること、すなわち組織試料中のＤＮＡ断片が捕捉プローブの捕捉ドメインにハイブリダイズすることができることだけが求められる。そのような結合ドメインの提供により、試料中のＤＮＡが断片化ステップの後まで捕捉プローブに結合しないことが保証される。結合ドメインは、当技術分野で周知の手順により、たとえば結合ドメインを含有することができるアダプター又はリンカー配列のライゲーションにより、ＤＮＡ断片に与えてもよい。たとえば、はみ出ている端部を有するリンカー配列を使用してもよい。結合ドメインは、ＤＮＡ断片へのリンカーのライゲーションに続いて、結合ドメインを含有する一本鎖部分が捕捉プローブの捕捉ドメインへのハイブリダイゼーションに利用可能であるように、そのようなリンカーの一本鎖部分に存在していてもよい。代わりに及び好ましい実施形態では、結合ドメインは、ポリヌクレオチドテール、たとえばポリＡドメインなどのホモポリマーテールを導入するターミナルトランスフェラーゼ酵素を使用することにより導入してもよい。これは、ＲＮＡ法との関連においてユニバーサルドメインを導入するために上に記載されている手順に類似する手順を使用して実行してもよい。したがって、有利な実施形態では、共通の結合ドメインを導入することができる。言い換えると、すべてのＤＮＡ断片に共通であり、アレイ上への断片の捕捉を達成するのに使用することができる結合ドメインである。

【０１９５】

テーリング反応が実行されて（共通の）結合ドメインを導入する場合、アレイ上の捕捉プローブをテーリング反応から保護することができる、すなわち捕捉プローブを上記の通りに遮断する又はマスクすることができる。これは、たとえば遮断オリゴヌクレオチドを捕捉プローブに、たとえば捕捉プローブのはみ出ている端部（たとえば、一本鎖部分）にハイブリダイズさせることにより達成してもよい。たとえば、捕捉ドメインがポリＴ配列を含む場合、そのような遮断オリゴヌクレオチドはポリＡオリゴヌクレオチドであってもよい。遮断オリゴヌクレオチドは遮断された３’端部（すなわち、伸長する又はテールすることができない端部）を有していてもよい。捕捉プローブも、上で詳細に説明しているように、化学的及び／又は酵素的改変により保護される、すなわち遮断されてもよい。

【０１９６】

結合ドメインが上記のリンカーのライゲーションにより与えられる場合、捕捉プローブを伸長して捕捉プローブプライマーの位置タグを含む捕捉されたＤＮＡ断片の相補的コピーを生成するのではなく、ＤＮＡ断片は捕捉プローブの３’端部にライゲートされてもよいことは理解されるであろう。上記のように、ライゲーションにはライゲートされる５’端部がリン酸化される必要がある。したがって、一実施形態では、付加されるリンカーの５’端部、すなわち捕捉プローブにライゲートされることになる端部（すなわち、ＤＮＡ断片に付加されるリンカーのはみ出ていない端部）がリン酸化されることになる。そのようなライゲーション実施形態では、したがって、リンカーを二本鎖ＤＮＡ断片にライゲートしてもよく、前記リンカーは結合ドメインを含有する一本鎖の突出した３’端部を有することが分かることになる。アレイに接触すると、突出した端部は捕捉プローブの捕捉ドメインにハイブリダイズする。このハイブリダイゼーションにより、付加されるリンカーの５’端部（はみ出ていない）へのライゲーションのために捕捉プローブの３’端部が並置される。捕捉プローブ、それ故に位置ドメインは、このようにしてこのライゲーションにより捕捉されたＤＮＡ断片に組み込まれる。そのような実施形態は図２１に図式的に示されている。

【０１９７】

したがって、本発明のこの態様の方法は、さらに詳細な実施形態では、

（ａ）捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備えるアレイを提供するステップであって、

前記補足プローブの複数のスピーシーズが、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、
前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、
（ｉ）前記アレイ上の前記捕捉プローブの前記位置に対応する位置ドメイン、及び
（ｉｉ）捕捉ドメイン
を有する核酸分子を含む、ステップと、
（ｂ）アレイ上の捕捉プローブの位置と、組織試料中の位置とが関連付けられるように前記アレイを組織試料に接触させるステップと、
（ｃ）前記組織試料中のＤＮＡを断片化するステップであって、前記断片化がステップ（ｂ）において前記アレイを組織試料に接触させる前に、間に又は後で実行される、ステップと、
（ｄ）前記捕捉ドメインにハイブリダイズすることができる結合ドメインを前記ＤＮＡ断片に与えるステップと、
（ｅ）前記ＤＮＡ断片を前記捕捉プローブ中の捕捉ドメインにハイブリダイズさせるステップと、
（ｆ）捕捉されたＤＮＡ断片を鋳型として用いてプライマー伸長反応において前記捕捉プローブを伸長させて伸長されたＤＮＡ分子を生成する、又は前記捕捉されたＤＮＡ断片をライゲーション反応において捕捉プローブにライゲートしてライゲートされたＤＮＡ分子を生成するステップであって、前記伸長された又はライゲートされたＤＮＡ分子が位置ドメインによりタグ付けされる、ステップと、
（ｇ）場合によって前記タグ付けされたＤＮＡの相補鎖を生成する及び／又は場合によって前記タグ付けされたＤＮＡを増幅するステップと、
（ｈ）アレイの表面から前記タグ付けされたＤＮＡ分子並びに／又はその相補体及び／若しくはアンプリコンの少なくとも一部を放出するステップであって、前記一部が位置ドメイン又はその相補体を含む、ステップと、
（ｉ）前記放出されたＤＮＡ分子の配列を直接的に又は間接的に解析するステップと
を含むこともある。

【０１９８】

前記方法は、場合によって
（ｊ）前記配列解析情報を前記組織試料の画像と関連付け、前記組織試料がステップ（ｆ）の前に又は後で撮像される追加のステップを
含むこともある。

【０１９９】

上記の核酸又はＤＮＡ検出の方法において、タグ付けされた核酸／ＤＮＡの相補的コピーを生成する又はタグ付けされたＤＮＡを増幅する任意のステップは、ＲＮＡ／トランスクリプトーム解析／検出法との関連で上で説明された原理に従って、鎖置換ポリメラーゼ酵素の使用を伴うことがある。適切な鎖置換ポリメラーゼは上で考察されている。これは、位置ドメインが相補的コピー又はアンプリコンにコピーされることを保証するためである。これは特に捕捉プローブが、表面プローブへのハイビリダイゼーションによりアレイに固定化される場合に当てはまることになる。

【０２００】

しかし、このステップにおける鎖置換ポリメラーゼの使用は不可欠ではない。たとえば、非鎖置換ポリメラーゼを、位置ドメインにハイブリダイズするオリゴヌクレオチドのライゲーションと一緒に使用してもよい。そのような手順はアレイ上での捕捉プローブの合成について上で説明された手順に類似している。

【０２０１】

一実施形態では、本発明の方法は、組織試料のゲノムすべて（たとえば組織試料の包括的ゲノム）を決定する及び／又は解析するために使用することができる。しかし、前記方法はこれに限定されず、前記ゲノムのすべて又は一部を決定する及び／又は解析すること

を包含している。したがって、前記方法は、ゲノムの一部又はサブセット（たとえば遺伝子の又は染色体のサブセット又は群に対応する部分的ゲノム（たとえば（たとえば特定の疾患又は状態、組織型、等に関連する）特定の遺伝子若しくは染色体のセット又はゲノムの特定の領域若しくは部分））を決定する及び／又は解析することを含むことがある。したがって、前記方法を使用して、正常組織と比べた場合の腫瘍組織からのゲノム配列又はゲノム遺伝子座を検出する又は解析することができる、又は組織試料中の異なる細胞型内でさえ、ゲノム配列又はゲノム遺伝子座を検出する又は解析することができる。異なる細胞、細胞の群、組織又は組織の一部若しくは型における異なるゲノムバリアント又は遺伝子座の存在若しくは非存在又は分布若しくは位置を調べることもできる。

【０２０２】

別の態様から眺めると、上記の方法ステップは、組織試料の核酸（たとえばゲノム配列、バリアント又は遺伝子座）に関する空間情報を得る方法を提供するとみることができる。別の言い方をすれば、本発明の方法は、ゲノム、特に個々の又は空間的に分布するゲノム、の標識化（又はタグ付け）のために使用してもよい。

【０２０３】

別の眺め方をすれば、本発明の方法は、組織試料中のＤＮＡの空間的検出のための方法、或いは空間分解能でＤＮＡを検出するための、又は組織試料中のＤＮＡの局所的若しくは空間的決定及び／若しくは解析のための方法とみることもできる。特に、前記方法は組織試料中の遺伝子若しくはゲノム配列又はゲノムバリアント若しくは遺伝子座（たとえば、ゲノムバリアント又は遺伝子座の分布）の局在化された若しくは空間的検出又は決定及び／若しくは解析のために使用してもよい。局在化された／空間的検出／決定／解析は、ＤＮＡが組織試料において細胞又は組織内のその天然の位置又は場所に局在化していてもよいことを意味する。したがって、たとえば、ＤＮＡは試料中の細胞若しくは細胞の群若しくは細胞の型に、又は組織試料内の区域の特定の領域に局在化していてもよい。ＤＮＡの天然の場所又は位置（言い換えると、組織試料中のＤＮＡの場所又は位置）、たとえばゲノムバリアント又は遺伝子座を決定することができる。

【０２０４】

したがって、本発明のアレイを使用すれば、前記アレイに接触される組織試料の核酸（たとえばＤＮＡ）を捕捉することができることが理解されるであろう。前記アレイは、組織試料の部分的若しくは包括的ゲノムを決定する及び／若しくは解析するために又は組織試料の空間的に限定された部分的若しくは包括的ゲノムを得るために使用してもよい。したがって、本発明の方法は、組織試料中の１つ又は複数のゲノム配列（又はバリアント又は遺伝子座）の空間的分布を定量する方法とみることもできる。別の表現をすれば、本発明の方法を使用して、組織試料中の１つ又は複数のゲノム配列又はゲノムバリアント又はゲノム遺伝子座の空間的分布を検出することができる。さらに別のやり方では、本発明の方法を使用して、組織試料内の１つ又は複数の位置での１つ又は複数のゲノム配列又はゲノムバリアント又はゲノム遺伝子座の場所又は分布を同時に決定することができる。さらに、前記方法は、空間分解能、たとえば二次元空間分解能での組織試料の核酸（たとえばＤＮＡ）の部分的又は包括的解析のための方法とみることもできる。

【０２０５】

本発明は、捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備え、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブが伸長又はライゲーションプライマーとして機能できるように遊離の３’端部を有する配向となっており、前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、

（ｉ）アレイ上の捕捉プローブの位置に対応する位置ドメイン、及び

（ｉｉ）前記アレイに接触させる組織試料の核酸を捕捉するための捕捉ドメイン

を有する核酸分子を含む、本発明の方法において使用するアレイを提供するとみることもできる。

【０２０６】

一態様では、捕捉される核酸分子はＤＮＡである。上記のＲＮＡ検出との関連において説明された方法への類似により、たとえば標的ＤＮＡ中のモチーフの特定の配列（たとえば保存された配列）への特異的ハイブリダイゼーションによって、捕捉ドメインは検出される特定のＤＮＡに、又はＤＮＡの特定の種類若しくは群に特異的であってもよい。代わりに、捕捉されるＤＮＡには、捕捉プローブの捕捉ドメインにより認識されることがある結合ドメイン（たとえば上記の共通結合ドメイン）を与えてもよい。したがって、上記のように、結合ドメインは、たとえばホモポリマー配列（たとえばポリＡ）であってもよい。さらに、そのような結合ドメインは、ＲＮＡ／トランスクリプトーム解析又は検出のための方法と関連して上で説明された原理及び方法に従って又は類似して与えてもよい。そのような場合、捕捉ドメインは、組織試料のＤＮＡ分子に導入される結合ドメインに相補的であってもよい。

【０２０７】

上のＲＮＡ関連でも記載されたように、捕捉ドメインはランダム配列でも縮重配列でもよい。したがって、ＤＮＡは、ランダム若しくは縮重捕捉ドメインに又は少なくとも部分的にランダム若しくは縮重配列を含む捕捉ドメインに結合することにより非特異的に捕捉されてもよい。

【０２０８】

関連する態様では、本発明は、捕捉プローブの複数のスピーシーズが、直接的に又は間接的に、その上に固定化された基板を備え、それぞれのスピーシーズがアレイ上の別個の位置を占め、かつ前記プローブがプライマー伸長又はライゲーション反応のためのプライマーとして機能できるように遊離の３’端部を有する配向となっており、前記アレイに接触させる組織試料の核酸、たとえばＤＮＡ又はＲＮＡを捕捉するために、前記捕捉プローブのそれぞれのスピーシーズが、５’から３’に向けて、

（ｉ）アレイ上の捕捉プローブの位置に対応する位置ドメイン、及び

（ｉｉ）捕捉ドメイン

を有する核酸分子を含む、

アレイの使用も提供する。

【０２０９】

好ましくは、前記使用は組織試料中の核酸の局在化された検出を目的とし、

（ａ）前記捕捉プローブを伸長又はライゲーションプライマーとして用いて捕捉された核酸分子からＤＮＡ分子を生成するステップであって、前記伸長された又はライゲートされた分子が位置ドメインによってタグ付けされるステップと、

（ｂ）場合によって前記タグ付けされた核酸の相補鎖を生成する及び／又は前記タグ付けされた核酸を増幅するステップと、

（ｃ）アレイの表面から前記タグ付けされたＤＮＡ分子及び／又はその相補体若しくはアンプリコンの少なくとも一部を放出するステップであって、前記一部が位置ドメイン又はその相補体を含むステップと、

（ｄ）放出されたＤＮＡ分子の配列を直接的に又は間接的に解析するステップと、場合によって

（ｅ）前記配列解析情報を前記組織試料の画像と関連付け、前記組織試料がステップ（ａ）の前に又は後で撮像されるステップと

をさらに含む。

【０２１０】

組織試料中のＤＮＡを断片化するステップは、当技術分野で公知のいかなる望ましい手順を使用して実行してもよい。したがって、断片化の物理的方法（たとえば音波破砕又は超音波処理）を使用してもよい。化学的方法も知られている。たとえば、エンドヌクレアーゼ（たとえば制限酵素）を用いた断片化の酵素的方法を使用してもよい。さらに、このための方法と酵素は当技術分野では周知である。断片化は、アレイ上に置くための組織試料を調製する、たとえば組織切片を調製する前、間又は後に実行してもよい。都合の良いことに、断片化は組織を固定するステップにおいて達成してもよい。したがって、たとえ

ば、ホルマリン固定により、ＤＮＡは断片化されることになる。他の固定液でも同様の結果を生じることができる。

【０２１１】

本発明のこれらの態様においてアレイを調製し使用する詳細に関しては、ＲＮＡ法との関連において上で与えられた説明及び詳細な点は、本明細書に記載されるさらに一般的な核酸検出及びＤＮＡ検出法と同様に適用されることは理解されるであろう。したがって、上記のすべての態様及び詳細が同様に適用される。たとえば、逆転写酵素プライマー及び反応、等の考察は、上で言及される伸長プライマー、ポリメラーゼ反応、等のいかなる態様と同様に適用することができる。同じように、第一及び第二鎖ｃＤＮＡ合成への言及は、タグ付けされたＤＮＡ分子及びその相補体に同様に適用することができる。上記の配列解析の方法を使用してもよい。

【０２１２】

例として、捕捉ドメインは上記の捕捉プローブについて記載される通りでもよい。ポリＴ又はポリＴ含有捕捉ドメインは、たとえばＤＮＡ断片にポリＡ配列を含む結合ドメインが与えられる場合に使用してもよい。

【０２１３】

たとえば、増幅及び／又は切断のために、捕捉プローブ／タグ付けされたＤＮＡ分子（すなわち、タグ付けされた伸長された又はライゲートされた分子）に上記のユニバーサルドメインを与えてもよい。

【０２１４】

本発明は、以下の図を参照し、以下の非限定的実施例に準拠してさらに説明されることになる。

【図面の簡単な説明】

【０２１５】

【図１】トランスクリプトーム解析のために組織切片からｍＲＮＡを捕捉するための整列配置された「バーコード化」オリゴ－ｄＴプローブを使用する全体的構想を示す図である。

【図２】対応する組織切片についての転写物存在量の視覚化の模式図を示す図である。

【図３】３’から５’表面プローブ組成及びアレイ表面に間接的に固定化されている５’から３’配向捕捉プローブの合成を示す図である。

【図４】プローブ放出後にアレイ表面への蛍光標識されたプローブのハイブリダイゼーションにより測定された、自製アレイからの酵素的切断（ＵＳＥＲ又はＲｓａｌ）の効率及びＡｇｉｌｅｎｔ製アレイからの９９℃水による効率を示す棒グラフを示す図である。

【図５】Ａｇｉｌｅｎｔ社製市販のアレイからのＤＮＡ表面プローブの９９℃水媒介放出後に捕捉された蛍光画像を示す図である。蛍光検出プローブは熱水処理後にハイブリダイズされた。上のアレイは非処理対照である。

【図６】ｃＤＮＡ合成後にそれぞれ細胞質（上）及び核染色（中）で処理されたトランスクリプトーム捕捉アレイ上の固定されたマウス脳組織切片並びに両方の染色を示す混合画像（下）を示す図である。

【図７】模式図に見られる低密度自製ＤＮＡ捕捉アレイにわたりその起源について分類された読取りを収載する表を示す図である。

【図８】バーコード化マイクロアレイを使用して核及びＭａｐ２を特異的に染色したＦＦＰＥマウス脳組織を示す図である。

【図９】核染色（白）及び可視形態学を用いたＦＦＰＥマウス脳嗅球を示す図である。

【図１０】低解像度アレイのために理論的スポッティングパターンを重ねた、核を染色（白）したＦＦＰＥマウス脳嗅球（約２×２ｍｍ）を示す図である。

【図１１】中－高解像度アレイのために理論的スポッティングパターンを重ねた、核を染色（白）したＦＦＰＥマウス脳嗅球（約２×２ｍｍ）を示す図である。

【図１２】糸球体領域（図９の右上）に焦点を合わせて画像を拡大したＦＦＰＥマウス脳嗅球を示す図である。

【図１３】増幅中にＢ＿ハンドル（Ｂ＿Ｒ６）に連結されたランダムヘキサマープライマー（Ｒ６）を使用するＵＳＥＲ放出から得られた生成物を示す図である；バイオアナライザー上に表されている生成物。
【図１４】増幅中にＢ＿ハンドル（Ｂ＿Ｒ８）に連結されたランダムオクタマープライマー（Ｒ８）を使用するＵＳＥＲ放出から得られた生成物を示す図である；バイオアナライザー上に表されている生成物。
【図１５】アレイ全体で覆われたＦＦＰＥ脳組織上で実施された実験の結果を示す図である。ｃＤＮＡの合成及び表面からの放出後のＩＤ特異的及び遺伝子特異的プライマー（Ｂ２Ｍ　エクソン４）を用いて増幅されたＩＤ５（左）及びＩＤ２０（右）；増幅されたＩＤ５及びＩＤ２０。
【図１６】実施例４に記載される方法の原理、すなわち空間標識タグ配列（位置ドメイン）を担持している固定化されたＤＮＡオリゴ（捕捉プローブ）を用いるマイクロアレイの使用、の略図を示す図である。マイクロアレイのオリゴのそれぞれのフィーチャーは、１）独自の標識タグ（位置ドメイン）及び２）捕捉配列（捕捉ドメイン）を担っている。
【図１７】平均サイズ２００ｂｐまで前もって断片化されたゲノムＤＮＡを用いて実行された実施例５に記載されている空間ゲノミクスプロトコールの結果を示す図である。アレイ上で増幅された内部生成物はＤＮＡを標識し合成した。検出されたピークは予測されたサイズである。
【図１８】平均サイズ７００ｂｐまで前もって断片化されたゲノムＤＮＡを用いて実行された実施例５に記載されている空間ゲノミクスプロトコールの結果を示す図である。アレイ上で増幅された内部生成物はＤＮＡを標識し合成した。検出されたピークは予測されたサイズである。
【図１９】平均サイズ２００ｂｐまで前もって断片化されたゲノムＤＮＡを用いて実行された実施例５に記載されている空間ゲノミクスプロトコールの結果を示す図である。表面オリゴに含有される１つの内部プライマー及び１つのユニバーサル配列を用いて増幅された生成物。アレイ上で実行された増幅はＤＮＡを標識し合成した。ゲノムＤＮＡのランダム断片化及びターミナルトランスフェラーゼ標識化は極めて多様な試料プールを生成すると仮定すると、予測される生成物はスメアである。
【図２０】平均サイズ７００ｂｐまで前もって断片化されたゲノムＤＮＡを用いて実行された実施例５に記載されている空間ゲノミクスプロトコールの結果を示す図である。表面オリゴに含有される１つの内部プライマー及び１つのユニバーサル配列を用いて増幅された生成物。アレイ上で実行された増幅はＤＮＡを標識し合成した。ゲノムＤＮＡのランダム断片化及びターミナルトランスフェラーゼ標識化は極めて多様な試料プールを生成すると仮定すると、予測される生成物はスメアである。
【図２１】ポリＴ捕捉ドメインへのハイブリダイゼーションのために結合ドメインを導入するためのＤＮＡ断片へのリンカーのライゲーション及び捕捉プローブへのそれに続くライゲーションの略図を示す図である。
【図２２】高密度捕捉アレイ上で使用される５’から３’配向捕捉プローブの組成を示す図である。
【図２３】高密度アレイの枠を示す図であり、枠は組織試料を方向付けるのに使用され、蛍光マーカープローブのハイブリダイゼーションにより視覚化される。
【図２４】高密度アレイから切断された及び切断されていない捕捉プローブを示す図であり、枠プローブはウラシル塩基を含有していないので切断されない。捕捉プローブはポリＡオリゴヌクレオチドに連結されたフルオロフォアで標識された。
【図２５】マウス嗅球から捕捉された転写物を用いて調製されたシーケンシングライブラリーのバイオアナライザー画像を示す図である。
【図２６】マウス嗅球から抽出された全ＲＮＡから捕捉された転写物のＭａｔｌａｂ視覚化を示す図である。
【図２７】マウス嗅球組織からの捕捉後Ｍａｔｌａｂ視覚化を使用して捕捉アレイにわたり視覚化されたＯｌｆｒ（臭覚受容体）転写物を示す図である。

【図２８】自製４１－ＩＤ－タグマイクロアレイについてのプリントのパターンを示す図である。
【図２９】捕捉アレイ上でのポリＡテールゲノム断片の捕捉後のＡ４３１特異的転座から生成された空間ゲノミクスライブラリーを示す図である。
【図３０】捕捉アレイ上のポリＡテールＵ２ＯＳゲノム断片へのスパイクド１０％及び５０％ポリＡテールＡ４３１ゲノム断片の捕捉後のＡ４３１特異的転座の検出を示す図である。
【図３１】組織画像で表面を覆われた４１－ＩＤ－タグ自製アレイ上のマウス嗅球組織から捕捉されたＩＤ－タグ付けされた転写物のＭａｔｌａｂ視覚化を示す図である。明確にするために、特定の遺伝子が同定された特異的フィーチャーは円で囲まれている。
【０２１６】
実施例１
アレイの調製
以下の実験は、オリゴヌクレオチドプローブが５’又は３’端部のどちらかによりアレイ基板に付着されて、ｍＲＮＡにハイブリダイズすることができる捕捉プローブを有するアレイを生じる方法を示す。
【０２１７】
５’から３’配向プローブをプリントした自製マイクロアレイの調製
個々のタグ配列を有する２０個のＲＮＡ捕捉オリゴヌクレオチド（Ｔａｇ１～２０、表１）は、捕捉プローブとして機能するようにガラススライド上にスポットされた。前記プローブはＣ６スペーサーを有する５’－終端アミノリンカーを用いて合成された。すべてのプローブがＳｉｇｍａ－Ａｌｄｒｉｃｈ（Ｓｔ．Ｌｏｕｉｓ、ＭＯ、ＵＳＡ）により合成された。ＲＮＡ捕捉プローブは、１５０ｍＭリン酸ナトリウム中、ｐＨ８．５、濃度２０μＭで懸濁され、ＣｏｄｅＬｉｎｋ（商標）活性化マイクロアレイスライド（７．５ｃｍ×２．５ｃｍ；Ｓｕｒｍｏｄｉｃｓ、Ｅｄｅｎ　Ｐｒａｉｒｉｅ、ＭＮ、ＵＳＡ）上にナノプロッターＮＰ２．１／Ｅ（Ｇｅｓｉｍ、Ｇｒｏｓｓｅｒｋｍａｎｎｓｄｏｒｆ、Ｇｅｒｍａｎｙ）を使用してスポットされた。プリント後、表面ブロッキングは製造業者の使用説明書に従って実施された。プローブはスライド上の１６個の同一アレイにプリントされ、それぞれのアレイは予め定義されたプリントパターンを含有していた。１６個のサブアレイは、１６－パッドマスク（ＣｈｉｐＣｌｉｐ（商標）Ｓｃｈｌｅｉｃｈｅｒ＆Ｓｃｈｕｅｌｌ　Ｂｉｏｓｃｉｅｎｃｅ、Ｋｅｅｎｅ、ＮＨ、ＵＳＡ）によりハイブリダイゼーション中は、分離されていた。
【０２１８】

【表１】

表1

名称	配列	5' 修飾	3' 修飾	長さ
遊離3'捕捉プローブの配列				
TAP-ID1	UUAAGTACAAATCTCGACTGCCACTCTGAACCTTCTCCTTCACCTTT

ID15	AATCCTCTCCTTGCCAGTTC (配列番号20)		20
ID16	GATGCCTCCACCTGTAGAAC (配列番号21)		20
ID17	GAAGGAATGGAGGATATCGC (配列番号22)		20
ID18	GATCCAAGGACCATCGACTG (配列番号23)		20
ID19	CCACTGGAACCTGACAACCG (配列番号24)		20
ID20	CTGCTTCTTCCTGGAACTCA (配列番号25)		20

遊離5'表面プローブの配列及びオンチップ遊離3'捕捉プローブ合成

遊離5'表面プローブ - A	GCGTTCAGAGTGGCAGTCGAGATCACGCGGCAATCATATCGGACAGATCGGAAGAGCGTAGTGTAG (配列番号26) アミノC7		66
遊離5'表面プローブ - U	GCGTTCAGAGTGGCAGTCGAGATCACGCGGCAATCATATCGGACGGCTGCTGGTAAATAGAGATCA (配列番号27) アミノC7		66
ニック	GCG		3
LP'	TTCAGAGTGGCAGTCGAGATCAC (配列番号28)		23
ID'	GCGGCAATCATATCGGAC (配列番号29)		18
A' 22bp MutYミスマッチ	AGATCGGAAGAGCGTAGTGTAG (配列番号30)		22
U' 22bp MutYミスマッチ	GGCTGCTGGTAAATAGAGATCA (配列番号31)		

捕捉プローブ合成のためのハイブリダイズされた配列

イルミナ増幅ハンドルA	ACACTCTTTCCCTACACGACGCTCTTCCGATCT (配列番号32)		33
ユニバーサル増幅ハンドルU	AAGTGTGGAAAGTTGATCGCTATTTACCAGCAGCC (配列番号33)		35
捕捉_LP_ポリ-dTVN	GTGATCTCGACTGCCACTCTGAATTTTTTTTTTTTTTTTTTTVN (配列番号34)	リン酸化	45
捕捉_LP_ポリ-d24T	GTGATCTCGACTGCCACTCTGAATTTTTTTTTTTTTTTTTTTTTTT (配列番号35)	リン酸化	47

追加の二次ユニバーサル増幅ハンドル

イルミナ増幅ハンドルB	AGACGTGTGCTCTTCCGATCT (配列番号36)		21
ユニバーサル増幅ハンドルX	ACGTCTGTGAATAGCCGCAT (配列番号37)		20

B_R6ハンドル(又はX)	AGACGTGTGCTCTTCCGATCTNNNNNNNNN (配列番号38)	27 (26)
B_R8ハンドル(又はX)	AGACGTGTGCTCTTCCGATCTNNNNNNNNNNN (配列番号39)	29 (28)
B_ポリTVN (又はX)	AGACGTGTGCTCTTCCGATCTTT

び２μＭのスレッド結合プライマー（捕捉ドメインオリゴヌクレオチド）を含有するハイブリダイゼーション溶液が５０℃で４分間インキュベートされた。その間、自製アレイはＣｈｉｐＣｌｉｐ（Ｗｈａｔｍａｎ）に装着された。前記アレイは引き続き、ウェルあたり５０μＬのハイブリダイゼーション溶液を用いて、５０℃、３０分間、３００ｒｐｍ振盪でインキュベートされた。

【０２２１】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃で０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水され、再びＣｈｉｐＣｌｉｐに戻された。

【０２２２】

伸長及びライゲーション反応のために（捕捉プローブの位置ドメインを生成するため）、１０×Ａｍｐｌｉｇａｓｅバッファー、２．５Ｕ　ＡｍｐｌｉＴａｑ　ＤＮＡポリメラーゼＳｔｏｆｆｅｌ断片（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）、１０Ｕ　Ａｍｐｌｉｇａｓｅ（Ｅｐｉｃｅｎｔｒｅ　Ｂｉｏｔｅｃｈｎｏｌｏｇｉｅｓ）、ｄＮＴＰ各々２ｍＭ（Ｆｅｒｍｅｎｔａｓ）及び水を含有する５０μＬの酵素混合物がそれぞれのウェルにピペットで移された。それに続いて、前記アレイは５５℃で３０分間インキュベートされた。インキュベーション後、前記アレイは、前述のアレイ洗浄法に従って洗浄されたが、第一のステップは６分間ではなく１０分間の持続時間を有する。

【０２２３】

前記方法は図３に描かれている。

【０２２４】

組織調製

以下の実験は、組織試料切片を本発明の方法において使用するために調製することができる方法を示す。

【０２２５】

新鮮な凍結組織の調製及び捕捉プローブアレイ上への切片作り

新鮮な固定されていないマウス脳組織は必要ならば切り取って－４０℃冷イソペンタン中で凍結させ、続いてクリオスタットを用いて１０μｍの切片化のためにマウントされた。組織切片は、使用されるそれぞれの捕捉プローブアレイ上に適用された。

【０２２６】

ホルマリン固定パラフィン包埋（ＦＦＰＥ）組織の調製

マウス脳組織は４％ホルマリン中で４℃で２４時間固定された。その後、前記組織は以下の通りにインキュベートされた：７０％エタノール中１時間、３×インキュベーション；８０％エタノール中１時間、１×インキュベーション；９６％エタノール中１時間、１×インキュベーション；１００％エタノール中１時間、３×インキュベーション；及びキシレン中室温で１時間、２×インキュベーション。

【０２２７】

次に、脱水された試料は液体低融点パラフィン中、５２～５４℃で最長３時間インキュベートされ、その間パラフィンは１度交換され、残余キシレンを洗い落とした。次に、完了した組織塊は室温で保存された。次に、切片は、使用されるそれぞれの捕捉プローブアレイ上に、ミクロトームを用いてパラフィン中４μｍでカットされた。

【０２２８】

切片はアレイスライド上で３７℃、２４時間乾燥され、室温で保存された。

【０２２９】

ＦＦＰＥ組織の脱パラフィン

ＣｏｄｅＬｉｎｋスライドに付着したホルマリン固定パラフィン化マウス脳１０μｍ切片は、キシレン中１０分間で２度、９９．５％エタノール２分間；９６％エタノール２分間；７０％エタノール２分間脱パラフィンされ、次に空気乾燥された。

【０２３０】
ｃＤＮＡ合成
以下の実験は、組織試料切片からアレイ上に捕捉されたｍＲＮＡをｃＤＮＡ合成のための鋳型として使用することができることを示す。
【０２３１】
チップ上でのｃＤＮＡ合成
Ｗｈａｔｍａｎ社製の１６ウェルマスク及びＣｈｉｐＣｌｉｐスライドホルダーをＣｏｄｅＬｉｎｋスライドに装着させた。ｃＤＮＡ合成を実施する場合はＩｎｖｉｔｒｏｇｅｎ社製Ｐｌａｔｉｎｕｍ（登録商標）Ｔａｑ　ＤＮＡポリメラーゼを用いるＳｕｐｅｒＳｃｒｉｐｔ（商標）ＩＩＩ　Ｏｎｅ－ｓｔｅｐ　ＲＴ－ＰＣＲ　Ｓｙｓｔｅｍを使用した。反応ごとに、２５μｌ　２×反応混合物（Ｐｌａｔｉｎｕｍ（登録商標）Ｔａｑ　ＤＮＡポリメラーゼを用いるＳｕｐｅｒＳｃｒｉｐｔ（商標）ＩＩＩ　Ｏｎｅ－ｓｔｅｐ　ＲＴ－ＰＣＲ　Ｓｙｓｔｅｍ、Ｉｎｖｉｔｒｏｇｅｎ）、２２．５μｌ　H_2O及び０．５μｌ　１００×ＢＳＡが混合され５０℃まで加熱された。ＳｕｐｅｒＳｃｒｉｐｔＩＩＩ／Ｐｌａｔｉｎｕｍ　Ｔａｑ酵素混合物が反応あたり２μｌで反応混合物に添加され、５０μｌの反応混合物がチップ上のそれぞれのウェルに添加された。前記チップは５０℃で３０分間インキュベートされた（サーモミキサーコンフォート、Ｅｐｐｅｎｄｏｒｆ）。
反応混合物はウェルから取り除かれ、スライドは、２×ＳＳＣ、０．１％ＳＤＳ、５０℃で１０分間；０．２×ＳＳＣ、室温で１分間；及び０．１×ＳＳＣ、室温で１分間を用いて洗浄された。次に、チップは遠心乾燥された。
【０２３２】
ＦＦＰＥ組織切片の場合、切片は組織を取り除く前に、この時点で染色し視覚化することができた、下の視覚化のセクションを参照されたい。
【０２３３】
視覚化
染色に先立つ蛍光マーカープローブのハイブリダイゼーション
組織の適用に先立って、蛍光マーカープローブは、捕捉プローブアレイ上にプリントされたマーカーオリゴヌクレオチドを含むフィーチャーにハイブリダイズされた。蛍光マーカープローブは、組織視覚化後の得られた画像を方向付けるのに役立ち、画像をシーケンシング後に得られる個々の捕捉プローブ「タグ」（位置ドメイン）配列についての得られた発現プロファイルと組み合わせることが可能になる。蛍光プローブをハイブリダイズさせるため、４×ＳＳＣ及び０．１％ＳＤＳ、２μＭの検出プローブ（Ｐ）を含有するハイブリダイゼーション溶液は５０℃で４分間インキュベートされた。その間、自家アレイはＣｈｉｐＣｌｉｐ（Ｗｈａｔｍａｎ）に装着された。アレイはそれに続いて、ウェルあたり５０μＬのハイブリダイゼーション溶液と一緒に５０℃、３０分間、３００ｒｐｍ振盪でインキュベートされた。
【０２３４】
インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃、０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水された。
【０２３５】
ｃＤＮＡ合成に先立つ又は後のＦＦＰＥ組織切片の一般的組織学的染色
捕捉プローブアレイ上に固定化されたＦＦＰＥ組織切片は、前述のようにｃＤＮＡ合成に先立って脱パラフィン後に洗浄され再水和された、又は前述のようにｃＤＮＡ合成後に洗浄された。次に、前記切片は以下の通りに処理された；ヘマトキシリン中で３分間インキュベートする、脱イオン水を用いてすすぐ、水道水中で５分間インキュベートする、酸性エタノール中８～１２回素早く浸す、水道水中２×１分間すすぐ、脱イオン水中２分間すすぐ、エオシン中３０秒間インキュベートする、９５％エタノール中３×５分間洗浄する、１００％エタノール中３×５分間洗浄する、キシレン中３×１０分間洗浄する（一晩

実行することができる）、ＤＰＸを使用してスライド上にカバーガラスを置く、一晩フード内でスライドを乾燥させる。

【０２３６】

ｃＤＮＡ合成に先立つ又は後のＦＦＰＥ組織切片中の標的タンパク質の一般的免疫組織化学染色

捕捉プローブアレイ上に固定化されたＦＦＰＥ組織切片は、前述のようにｃＤＮＡ合成に先立って脱パラフィン後に洗浄され再水和された、又は前述のようにｃＤＮＡ合成後に洗浄された。次に、前記切片は、全染色作業中乾燥させておくことなく以下の通りに処理された；切片は室温で一晩湿潤チャンバー内で一次抗体（１×Ｔｒｉｓ緩衝食塩水（５０ｍＭ　Ｔｒｉｓ、１５０ｍＭ　ＮａＣｌ、ｐＨ７．６）、４％ロバ血清及び０．１％ｔｒｉｔｏｎ－Ｘを含むブロッキング溶液中の希釈一次抗体）と一緒にインキュベートされる；１×ＴＢＳを用いて３回すすぐ；室温で１時間湿潤チャンバー内で蛍光色素（ＦＩＴＣ、Ｃｙ３又はＣｙ５）にコンジュゲートされた適合する二次抗体と一緒に切片をインキュベートする。１×ＴＢＳを用いて３回すすぎ、できる限り多くのＴＢＳを取り除き、切片をＰｒｏＬｏｎｇ　Ｇｏｌｄ＋ＤＡＰＩ（Ｉｎｖｉｔｒｏｇｅｎ）と一緒にマウントし、蛍光顕微鏡及び適合するフィルターセットを用いて解析する。

【０２３７】

残余組織の除去

凍結組織

新鮮な凍結マウス脳組織では、ｃＤＮＡ合成に直に続く洗浄ステップだけで組織を完全に取り除くのに十分であった。

【０２３８】

ＦＦＰＥ組織

付着したホルマリン固定パラフィン化マウス脳組織切片を有するスライドは、ＣｈｉｐＣｌｉｐスライドホルダー及び１６ウェルマスク（Ｗｈａｔｍａｎ）に装着された。ＲＮｅａｓｙ　ＦＦＰＥキット（Ｑｉａｇｅｎ）由来の１５０μｌのプロテイナーゼＫダイジェストバッファーごとに、１０μｌのプロテイナーゼＫ溶液（Ｑｉａｇｅｎ）が添加された。５０μｌの最終混合液がそれぞれのウェルに添加され、スライドは５６℃で３０分間インキュベートされた。

【０２３９】

捕捉プローブ（ｃＤＮＡ）放出

ＰＣＲバッファー中ウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ放出（共有結合プローブ）

１６ウェルマスク及びＣｏｄｅＬｉｎｋスライドはＣｈｉｐＣｌｉｐホルダー（Ｗｈａｔｍａｎ）に装着された。１×ＦａｓｔＳｔａｒｔ高忠実度反応バッファーを１．８ｍＭ $MgCl_2$（Ｒｏｃｈｅ）と一緒に、２００μＭ　ｄＮＴＰ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）及び０．１Ｕ／１μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有する５０μｌの混合物を３７℃まで加熱し、それぞれのウェルに添加し、混ぜながら（３００ｒｐｍで３秒間、静止して６秒間）（サーモミキサーコンフォート；Ｅｐｐｅｎｄｏｒｆ）３７℃で３０分間インキュベートした。次に、放出されたｃＤＮＡ及びプローブを含有する反応混合物は、ピペットを用いてウェルから回収された。

【０２４０】

ＴｄＴ（ターミナルトランスフェラーゼ）バッファー中ウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ放出（共有結合プローブ）

１×ＴｄＴバッファー（２０ｍＭ　Ｔｒｉｓ－アセテート（ｐＨ７．９）、５０ｍＭ酢酸カリウム及び１０ｍＭ酢酸マグネシウム）（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）；０．１μｇ／μｌ　ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）；及び０．１Ｕ／μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有する５０μｌの混合物は３７℃まで予加熱され、それぞれのウェルに添加され、混ぜながら（３００ｒｐｍで３秒間、静止して６秒間）（サーモミキサーコンフ

ォート；Ｅｐｐｅｎｄｏｒｆ）３７℃で３０分間インキュベートされた。次に、放出されたｃＤＮＡ及びプローブを含有する反応混合物はピペットを用いてウェルから回収された。

【０２４１】

熱湯を用いた捕捉プローブ放出（共有結合プローブ）

１６ウェルマスク及びＣｏｄｅＬｉｎｋスライドはＣｈｉｐＣｌｉｐホルダー（Ｗｈａｔｍａｎ）に装着された。５０μｌの９９℃水はピペットでそれぞれのウェルに移された。９９℃水は３０分間反応させておいた。次に、放出されたｃＤＮＡ及びプローブを含有する反応混合物はピペットを用いてウェルから回収された。

【０２４２】

加熱ＰＣＲバッファーを用いた捕捉プローブ放出（ハイブリダイズされたインサイツ合成捕捉プローブ、すなわち表面プローブにハイブリダイズされた捕捉プローブ）

１×ＴｄＴバッファー（２０ｍＭ　Ｔｒｉｓ－アセテート（ｐＨ７．９）、５０ｍＭ酢酸カリウム及び１０ｍＭ酢酸マグネシウム）（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）；０．１μｇ／μｌ　ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）；及び０．１Ｕ／μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有する５０μｌの混合物は９５℃まで予熱された。混合物はそれぞれのウェルに添加され、混ぜながら（３００ｒｐｍで３秒間、静止して６秒間）（サーモミキサーコンフォート；Ｅｐｐｅｎｄｏｒｆ）９５℃で５分間インキュベートされた。次に、放出されたプローブを含有する反応混合物はウェルから回収された。

【０２４３】

加熱したＴｄＴ（ターミナルトランスフェラーゼ）バッファーを用いた捕捉プローブ放出（ハイブリダイズされたインサイツ合成された捕捉プローブ、すなわち表面プローブにハイブリダイズされた捕捉プローブ）

１×ＴｄＴバッファー（２０ｍＭ　Ｔｒｉｓ－アセテート（ｐＨ７．９）、５０ｍＭ酢酸カリウム及び１０ｍＭ酢酸マグネシウム）（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）；０．１μｇ／μｌ　ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）；及び０．１Ｕ／μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有する５０μｌの混合物は９５℃まで予熱された。次に、混合物はそれぞれのウェルに添加され、混ぜながら（３００ｒｐｍで３秒間、静止して６秒間）（サーモミキサーコンフォート；Ｅｐｐｅｎｄｏｒｆ）９５℃で５分間インキュベートされた。次に、放出されたプローブを含有する反応混合物はウェルから回収された。

【０２４４】

ＵＳＥＲ酵素及び９９℃まで加熱された水を用いてアレイを処理する有効性は図３に見ることができる。ＵＳＥＲ酵素及びＲｓａｌ酵素を使用する酵素的切断は、上記の「自製」アレイを使用して実施された（図４）。ＤＮＡ表面プローブの熱水媒介放出は、Ａｇｉｌｅｎｔ社製の市販のアレイを使用して実施された（図５参照）。

【０２４５】

プローブ収集及びリンカー導入

実験は、アレイ表面から放出された第一鎖ｃＤＮＡを改変して、二本鎖ＤＮＡを作製し、続いて増幅できることを示している。

【０２４６】

Ｐｉｃｏｐｌｅｘ全ゲノム増幅キットによる全トランスクリプトーム増幅（得られたｄｓＤＮＡの端に保持されていない位置ドメイン（タグ）配列を含む捕捉プローブ配列）

捕捉プローブは、ＰＣＲバッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いて（共有結合捕捉プローブ）、又は加熱ＰＣＲバッファーを用いて（ハイブリダイズされたインサイツ合成された捕捉プローブ、すなわち表面プローブにハイブリダイズされた捕捉プローブ）放出された。

【０２４７】

放出されたｃＤＮＡは、Ｐｉｃｏｐｌｅｘ（Ｒｕｂｉｃｏｎ　Ｇｅｎｏｍｉｃｓ）ラン

ダムプライマー全ゲノム増幅法を使用して増幅され、前記方法は製造業者の使用説明書に従って実行された。

【０２４８】

ターミナルトランスフェラーゼ（ＴｄＴ）を用いるｄＡテーリングによる全トランスクリプトーム増幅（得られたｄｓＤＮＡの端に保持されている位置ドメイン（タグ）配列を含む捕捉プローブ配列）

捕捉プローブは、ＴｄＴ（ターミナルトランスフェラーゼ）バッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いて（共有結合捕捉プローブ）、又は加熱ＴｄＴ（ターミナルトランスフェラーゼ）バッファーを用いて（ハイブリダイズされたインサイツ合成された捕捉プローブ、すなわち表面プローブにハイブリダイズされた捕捉プローブ）放出された。

【０２４９】

３８μｌの切断混合物は清潔な０．２ｍｌのＰＣＲ管に入れられた。混合物は、１×ＴｄＴバッファー（２０ｍＭ　Ｔｒｉｓ－アセテート（ｐＨ７．９）、５０ｍＭ酢酸カリウム及び１０ｍＭ酢酸マグネシウム）（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）；０．１μｇ／μｌ　ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）；０．１Ｕ／μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）（加熱された放出のためではない）；放出されたｃＤＮＡ（表面プローブから伸長された）；及び放出された表面プローブを含有していた。ＰＣＲ管に、０．５μｌのリボヌクレアーゼＨ（５Ｕ／μｌ、最終濃度０．０６Ｕ／μｌ）、１μｌのＴｄＴ（２０Ｕ／μｌ、最終濃度０．５Ｕ／μｌ）及び０．５μｌのｄＡＴＰ（１００ｍＭ、最終濃度１．２５ｍＭ）が添加された。ｄＡテーリングでは、管は３７℃、１５分間熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中でインキュベートされ、続いて７０℃で１０分間ＴｄＴを不活化した。ｄＡテーリング後、ＰＣＲマスター混合物が調製された。前記混合物は、１．８ｍＭ　$MgCl_2$を有する１×Ｆａｓｔｓｔａｒｔ　ＨｉＦｉ　ＰＣＲバッファー（ｐＨ８．３）（Ｒｏｃｈｅ）；０．２ｍＭのそれぞれのｄＮＴＰ（Ｆｅｒｍｅｎｔａｓ）；０．２μＭのそれぞれのプライマー、Ａ（捕捉プローブの増幅ドメインに相補的）及びＢ＿（ｄＴ）２４（Ｅｕｒｏｆｉｎｓ　ＭＷＧ　Ｏｐｅｒｏｎ）（第一ｃＤＮＡ鎖の３’端部にカップリングされることになるポリＡテールに相補的）；並びに０．１Ｕ／μｌ　Ｆａｓｔｓｔａｒｔ　ＨｉＦｉ　ＤＮＡポリメラーゼ（Ｒｏｃｈｅ）を含有していた。２３μｌのＰＣＲマスター混合物は９個の清潔な０．２ｍｌ　ＰＣＲ管に入れられた。２μｌのｄＡテーリング混合物は前記管のうちの８個に添加され、２μｌの水（リボヌクレアーゼ／デオキシリボヌクレアーゼなし）が最後の管に添加された（陰性対照）。ＰＣＲ増幅は以下のプログラムで実行され、ホットスタート、９５℃で２分間；第二鎖合成、５０℃で２分間及び７２℃で３分間、３０ＰＣＲサイクルでの増幅、９５℃で３０秒間、６５℃で１分間、７２℃で３分間並びに最終伸長、７２℃で１０分間。

【０２５０】

反応後精製及び解析

４つの増幅生成物はまとめてプールされ、Ｑｉａｑｕｉｃｋ　ＰＣＲ精製カラム（Ｑｉａｇｅｎ）を通して処理され、３０μｌのＥＢ（１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５）中に溶出された。生成物はＢｉｏａｎａｌｙｚｅｒ（Ａｇｉｌｅｎｔ）上で解析された。ＤＮＡ　１０００キットは製造業者の説明書に従って使用された。

【０２５１】

シーケンシング

Ｉｌｌｕｍｉｎａシーケンシング

試料インデックス化を使用するＩｌｌｕｍｉｎａシーケンシングのためのｄｓＤＮＡライブラリーは製造業者の説明書に従って実行された。シーケンシングはＨｉＳｅｑ２０００プラットフォーム（Ｉｌｌｕｍｉｎａ）上で実行された。

【０２５２】

バイオインフォマティクス

ｄＡテーリングターミナルトランスフェラーゼアプローチを使用して増幅される全トランスクリプトームライブラリー由来のシーケンシングデータからデジタルトランスクリプトーム情報を得る

シーケンシングデータは、ＦａｓｔＸツールキットＦＡＳＴＱバーコードスプリッターツールを通じて、それぞれの捕捉プローブ位置ドメイン（タグ）配列の個々のファイルに分類された。次に、個々にタグ付けされたシーケンシングデータは、Ｔｏｐｈａｔマッピングツールを用いたマウスゲノムへのマッピングを通じて解析された。得られたＳＡＭファイルは、ＨＴｓｅｑカウントソフトウェアを通じて転写物数について処理された。

【０２５３】

Ｐｉｃｏｐｌｅｘ全ゲノム増幅キットアプローチを使用して増幅される全トランスクリプトームライブラリー由来のシーケンシングデータからデジタルトランスクリプトーム情報を得る

シーケンシングデータは、ＦａｓｔＸツールキットＦＡＳＴＱ－ｔｏ－ＦＡＳＴＡ変換機を使用してＦＡＳＴＱフォーマットからＦＡＳＴＡフォーマットに変換された。シーケンシング読取りはＢｌａｓｔｎを使用して捕捉プローブ位置ドメイン（タグ）配列に整列させ、タグ配列のうちの１つに対して$1e^{-6}$よりもよいヒットを有する読取りは、それぞれタグ配列ごとに個々のファイルに選り分けられた。次に、タグ配列読取りのファイルは、Ｂｌａｓｔｎを使用してマウストランスクリプトームに整列させ、ヒットが収集された。

【０２５４】

視覚化データと発現プロファイルを組み合わせる

個々の捕捉プローブ位置ドメイン（タグ）配列の発現プロファイルは、染色を通じて組織切片から得られる空間情報と組み合わされる。それによって組織切片の細胞コンパートメントからのトランスクリプトームデータは、所与の構造背景における異なる細胞亜型についての別個の発現フィーチャーを区別する利用可能性のある直接比較の様式で解析することができる。

【０２５５】

実施例２

図８～１２は、実施例１に記載される一般手順に従って、バーコードトランスクリプトーム捕捉アレイ上で染色されたＦＦＰＥマウス脳組織（嗅球）切片の視覚化に成功していることを示している。実施例１における新鮮な凍結組織を用いた実験と比較すると、図８のほうがＦＦＰＥ組織について良好な形態を示している。図９及び図１０は、異なる種類のプローブ密度アレイ上に組織が配置されている様子を示している。

【０２５６】

実施例３

ランダムプライマー第二鎖合成とそれに続くユニバーサルハンドル増幅による全トランスクリプトーム増幅（得られたｄｓＤＮＡの端に保持されているタグ配列を含む捕捉プローブ配列）

ＰＣＲバッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ放出に続いて（共有結合プローブ）

又は

加熱ＰＣＲバッファーを用いた捕捉プローブ放出に続いて（ハイブリダイズされたインサイツ合成された捕捉プローブ）

１μｌリボヌクレアーゼＨ（５Ｕ／μｌ）は、１．８ｍＭ $MgCl_2$ を有する４０μｌの１×Ｆａｓｔｓｔａｒｔ ＨｉＦｉ ＰＣＲバッファー（ｐＨ８．３）（Ｒｏｃｈｅ、ｗｗｗ．ｒｏｃｈｅ－ａｐｐｌｉｅｄ－ｓｃｉｅｎｃｅ．ｃｏｍ）、０．２ｍＭのそれぞれのｄＮＴＰ（Ｆｅｒｍｅｎｔａｓ、ｗｗｗ．ｆｅｒｍｅｎｔａｓ．ｃｏｍ）、０．１μｇ／μｌ ＢＳＡ（Ｎｅｗ Ｅｎｇｌａｎｄ Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）、０．１Ｕ／μｌ ＵＳＥＲ酵素（Ｎｅｗ Ｅｎｇｌａｎｄ Ｂｉｏｌａｂｓ）、放出されたｃＤＮＡ（表面プローブから伸長された）及び放出された表面プローブを含有して

いる２つの管のそれぞれに、最終濃度０．１２Ｕ／μｌで添加された。管は、熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ、ｗｗｗ．ａｐｐｌｉｅｄｂｉｏｓｙｓｔｅｍｓ．ｃｏｍ）中、３７℃で３０分間、続いて７０℃で２０分間インキュベートされた。１μｌのクレノーフラグメント（３’から５’ｅｘｏマイナス）（Ｉｌｌｕｍｉｎａ、ｗｗｗ．ｉｌｌｕｍｉｎａ．ｃｏｍ）及び１μｌのハンドル連結ランダムプライマー（１０μＭ）（Ｅｕｒｏｆｉｎｓ　ＭＷＧ　Ｏｐｅｒｏｎ、ｗｗｗ．ｅｕｒｏｆｉｎｓｄｎａ．ｃｏｍ）は、最終濃度０．２３μＭで、２つの管（管のうちの１つにＢ＿Ｒ８（オクタマー）及び残りの管にＢ＿Ｒ６（ヘキサマー））に添加された。２つの管は、熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中で、１５℃で１５分間、２５℃で１５分間、３７℃で１５分間及び最後に７５℃で２０分間インキュベートされた。インキュベーション後、１μｌのそれぞれのプライマー、Ａ＿Ｐ及びＢ（１０μＭ）（Ｅｕｒｏｆｉｎｓ　ＭＷＧ　Ｏｐｅｒｏｎ）は、最終濃度それぞれ０．２２μＭで両方の管に添加された。１μｌのＦａｓｔｓｔａｒｔ　ＨｉＦｉ　ＤＮＡポリメラーゼ（５Ｕ／μｌ）（Ｒｏｃｈｅ）も、最終濃度０．１１Ｕ／μｌで両方の管に添加された。ＰＣＲ増幅は熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中で、以下のプログラム：ホットスタート、９４℃で２分間、続いて５０サイクルを９４℃で１５秒間、５５℃で３０秒間、６８℃で１分間及び最終伸長、６８℃で５分間により実行された。増幅後、２つの管のそれぞれ由来の４０μｌはＱｉａｑｕｉｃｋ　ＰＣＲ精製カラム（Ｑｉａｇｅｎ、ｗｗｗ．ｑｉａｇｅｎ．ｃｏｍ）を用いて精製され、３０μｌのＥＢ（１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５）中に溶出された。精製された生成物はバイオアナライザー（Ａｇｉｌｅｎｔ、ｗｗｗ．ｈｏｍｅ．ａｇｉｌｅｎｔ．ｃｏｍ）を用いて解析され、ＤＮＡ　７５００キットが使用された。結果は図１３及び１４に示されている。

【０２５７】

この実施例は、ランダムヘキサマー及びランダムオクタマー第二鎖合成の使用、それに続く放出されたｃＤＮＡ分子から集団を生成する増幅を実証している。

【０２５８】

実施例４

ｃＤＮＡ合成及びプローブ収集後のＩＤ特異的及び遺伝子特異的生成物の増幅

ＰＣＲバッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ放出に続く（共有結合プローブ）。

【０２５９】

切断されたｃＤＮＡは、最終反応容積１０μｌで増幅された。１．８ｍＭ　$MgCl_2$を有する１．４×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー中、７μｌの切断された鋳型、１μｌのＩＤ特異的フォワードプライマー（２μＭ）、１μｌの遺伝子特異的リバースプライマー（２μＭ）及び１μｌのＦａｓｔＳｔａｒｔ高忠実度酵素ブレンドは、１．８ｍＭ　$MgCl_2$を有する１×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー及び１ＵのＦａｓｔＳｔａｒｔ高忠実度酵素ブレンドとの１０μｌの最終反応容積を与える。ＰＣＲ増幅は熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中で、以下のプログラム：ホットスタート、９４℃で２分間、続いて５０サイクルを９４℃で１５秒間、５５℃で３０秒間、６８℃で１分間及び最終伸長、６８℃で５分間により実行された。

【０２６０】

プライマー配列、ほぼ２５０ｂｐの生成物になる。

ベーター２マイクログロブリン（Ｂ２Ｍ）プライマー

５’－ＴＧＧＧＧＧＴＧＡＧＡＡＴＴＧＣＴＡＡＧ－３’（配列番号４３）

ＩＤ－１プライマー

５’－ＣＣＴＴＣＴＣＣＴＴＣＴＣＣＴＴＣＡＣＣ－３’（配列番号４４）

ＩＤ－５プライマー

５’－ＧＴＣＣＴＣＴＡＴＴＣＣＧＴＣＡＣＣＡＴ－３’（配列番号４５）

ＩＤ－２０プライマー

５’－ＣＴＧＣＴＴＣＴＴＣＣＴＧＧＡＡＣＴＣＡ－３’（配列番号４６）

【０２６１】
結果は図１５に示されている。これは２つの異なるＩＤプライマー（すなわち、マイクロアレイ上の異なる位置に配置されたＩＤタグに特異的である）及びすべてのプローブを覆う脳組織由来の同じ遺伝子特異的プライマーを使用してＩＤ特異的及び遺伝子特異的生成物の増幅に成功したことを示している。したがって、この実験は、生成物がＩＤタグ特異的又は標的核酸特異的増幅反応により同定できることを確立している。異なるＩＤタグを区別することができることがさらに確立されている。第二の実験では、組織がアレイ上のＩＤプローブ（すなわち、捕捉プローブ）の半分だけを覆うが、組織に覆われたスポットで陽性結果（ＰＣＲ生成物）が得られた。

【０２６２】
実施例５
空間ゲノミクス
背景。前記方法はその目的として、組織試料からＤＮＡ分子を保持された空間分解能で捕捉し、特定のＤＮＡ断片が組織のどの部分から生じているのかを決定することができなければならない。

【０２６３】
方法。前記方法の原理は、空間標識タグ配列（位置ドメイン）を担持する固定化されたＤＮＡオリゴ（捕捉プローブ）を有するマイクロアレイを使用することである。マイクロアレイのオリゴのそれぞれのフィーチャーは、１）独自の標識タグ（位置ドメイン）及び２）捕捉配列（捕捉ドメイン）を担っている。どの標識タグが地理的にアレイ表面のどこに置かれているのかを追跡すれば、それぞれの標識タグから二次元で位置情報を引き出すことが可能になる。断片化されたゲノムＤＮＡが、たとえばＦＦＰＥ処理された組織の切片の添加を通じて、マイクロアレイに添加される。この組織切片中のゲノムＤＮＡは、固定処理により予め断片化されている。

【０２６４】
組織片がアレイ上に置かれると、ユニバーサルテーリング反応が、ターミナルトランスフェラーゼ酵素の使用を通じて実行される。テーリング反応により、組織中のゲノムＤＮＡ断片の突出した３’端部にポリｄＡテールが付加される。表面上のオリゴは、ハイブリダイズされ３’遮断されたポリｄＡプローブを介してターミナルトランスフェラーゼによるテーリングから遮断される。

【０２６５】
ターミナルトランスフェラーゼテーリングに続いて、ゲノムＤＮＡ断片は、ポリｄＡテールが表面オリゴ上のポリｄＴ捕捉配列に出会うことを通じてその近傍の空間的にタグ付けされたオリゴにハイブリダイズすることができる。ハイブリダイゼーションが終了した後、クレノーｅｘｏ－などの鎖置換ポリメラーゼは、ハイブリダイズしたゲノムＤＮＡ断片に相補的な新しいＤＮＡ鎖を作製するためのプライマーとして表面上のオリゴを使用することができる。新しいＤＮＡ鎖は、この時点で表面オリゴの標識タグの位置情報も含有することになる。

【０２６６】
最後のステップとして、新たに生成され標識されたＤＮＡ鎖は、酵素的手段、変性又は物理的手段のいずれかを通じて表面から切断される。次に、鎖は収集され、ユニバーサルハンドルの導入、特異的アンプリコンの増幅及び／又はシーケンシングを通じて鎖の全セットの下流増幅を受けることができる。

【０２６７】
図１６はこの手順の模式図である。

【０２６８】
材料及び方法
５’から３’配向プローブをプリントした自製マイクロアレイの調製
個々のタグ配列を有する２０個のＤＮＡ捕捉オリゴ（表１）は、捕捉プローブとして機能するようにガラススライド上にスポットされた。プローブは、Ｃ６スペーサーを有する

５’終端アミノリンカーを用いて合成された。すべてのプローブがＳｉｇｍａ－Ａｌｄｒｉｃｈ（Ｓｔ．Ｌｏｕｉｓ、ＭＯ、ＵＳＡ）により合成された。ＤＮＡ捕捉プローブは、１５０ｍＭリン酸ナトリウム中に、ｐＨ８．５、濃度２０μＭで懸濁され、ＣｏｄｅＬｉｎｋ（商標）活性化マイクロアレイスライド（７．５ｃｍ×２．５ｃｍ；Ｓｕｒｍｏｄｉｃｓ、Ｅｄｅｎ　Ｐｒａｉｒｉｅ、ＭＮ、ＵＳＡ）上にナノプロッターＮＰ２．１／Ｅ（Ｇｅｓｉｍ、Ｇｒｏｓｓｅｒｋｍａｎｎｓｄｏｒｆ、Ｇｅｒｍａｎｙ）を使用してスポットされた。プリント後、表面ブロッキングは製造業者の使用説明書に従って実施された。プローブはスライド上の１６個の同一アレイにプリントされ、それぞれのアレイは予め定義されたプリントパターンを含有していた。１６個のサブアレイは１６－パッドマスク（ＣｈｉｐＣｌｉｐ（商標）Ｓｃｈｌｅｉｃｈｅｒ＆Ｓｃｈｕｅｌｌ　Ｂｉｏｓｃｉｅｎｃｅ、Ｋｅｅｎｅ、ＮＨ、ＵＳＡ）によりハイブリダイゼーション中は、分離されていた。

【０２６９】

３’から５’配向プローブをプリントした自製マイクロアレイの調製及び５’から３’配向捕捉プローブの合成

オリゴのプリントは、上記の５’から３’配向プローブを用いた場合のように実施された。

【０２７０】

捕捉プローブ合成のためのプライマーをハイブリダイズさせるため、４×ＳＳＣ及び０．１％ＳＤＳ、２μＭの伸長プライマー（Ａ＿プライマー）及び２μＭのスレッド結合プライマー（ｐ＿ポリ＿ｄＴ）を含有するハイブリダイゼーション溶液が５０℃で４分間インキュベートされた。その間、自製アレイはＣｈｉｐＣｌｉｐ（Ｗｈａｔｍａｎ）に装着された。前記アレイは引き続き、ウェルあたり５０μＬのハイブリダイゼーション溶液を用いて、５０℃、３０分間、３００ｒｐｍ振盪でインキュベートされた。

【０２７１】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃で０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水され、再びＣｈｉｐＣｌｉｐに戻された。

【０２７２】

伸長及びライゲーションのために、１０×Ａｍｐｌｉｇａｓｅバッファー、２．５Ｕ　ＡｍｐｌｉＴａｑ　ＤＮＡポリメラーゼＳｔｏｆｆｅｌ断片（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）、１０Ｕ　Ａｍｐｌｉｇａｓｅ（Ｅｐｉｃｅｎｔｒｅ　Ｂｉｏｔｅｃｈｎｏｌｏｇｉｅｓ）、ｄＮＴＰ各々２ｍＭ（Ｆｅｒｍｅｎｔａｓ）及び水を含有する５５μＬの酵素混合物がそれぞれのウェルにピペットで移される。それに続いて、前記アレイは５５℃で３０分間インキュベートされる。インキュベーション後、前記アレイは、前述のアレイ洗浄法に従って洗浄されるが、第一のステップは６分間ではなく１０分間の持続時間を有する。

【０２７３】

ｄＡテーリングからの表面オリゴ捕捉配列の保護のためのポリｄＡプローブのハイブリダイゼーション

表面オリゴ捕捉配列の保護のために３’ビオチン遮断ポリｄＡプローブをハイブリダイズさせるため、４×ＳＳＣ及び０．１％ＳＤＳ、２μＭの３’ビオチン－ポリｄＡを含有するハイブリダイゼーション溶液は５０℃で４分間インキュベートされた。その間、自製アレイはＣｈｉｐＣｌｉｐ（Ｗｈａｔｍａｎ）に装着された。前記アレイは引き続き、ウェルあたり５０μＬのハイブリダイゼーション溶液を用いて、５０℃、３０分間、３００ｒｐｍ振盪でインキュベートされた。

【０２７４】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃で０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で

、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水され、再びＣｈｉｐＣｌｉｐに戻された。

【０２７５】

ホルマリン固定パラフィン包埋（ＦＦＰＥ）組織の調製

マウス脳組織は４％ホルマリン中で４℃で２４時間固定された。その後、前記組織は以下の通りにインキュベートされた：７０％エタノール中１時間、３×インキュベーション；８０％エタノール中１時間、１×インキュベーション；９６％エタノール中１時間、１×インキュベーション；１００％エタノール中１時間、３×インキュベーション；キシレン中室温で１時間、２×インキュベーション。

【０２７６】

次に、脱水された試料は液体低融点パラフィン中、５２～５４℃で最長３時間インキュベートされ、その間パラフィンは１度交換され、残余キシレンを洗い落とした。次に、完了した組織塊は室温で保存された。次に、切片は、使用されるそれぞれの捕捉プローブアレイ上に、ミクロトームを用いてパラフィン中４μｍでカットされた。

【０２７７】

切片はアレイスライド上で３７℃、２４時間乾燥され、室温で保存される。

【０２７８】

ＦＦＰＥ組織の脱パラフィン

ＣｏｄｅＬｉｎｋスライドに付着したホルマリン固定パラフィン化マウス脳１０μｍ切片は、キシレン中１０分間で２度；９９．５％エタノール２分間；９６％エタノール２分間；７０％エタノール２分間脱パラフィンされ、次に空気乾燥された。

【０２７９】

ゲノムＤＮＡのユニバーサルテーリング

ｄＡテーリングのために、１×ＴｄＴバッファー（２０ｍＭ　Ｔｒｉｓ－アセテート（ｐＨ７．９）、５０ｍＭ酢酸カリウム及び１０ｍＭ酢酸マグネシウム）（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）、０．１μｇ／μｌ　ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）、１μｌのＴｄＴ（２０Ｕ／μｌ）及び０．５μｌのｄＡＴＰ（１００ｍＭ）を含有する５０μｌの反応混合物が調製された。混合物はアレイ表面に添加され、アレイは熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中３７℃で１５分間インキュベートされ、続いてＴｄＴは７０℃で１０分間不活化された。この後、温度は再び５０℃まで下げられ、ｄＡテール付きゲノム断片の表面オリゴ捕捉配列へのハイブリダイゼーションを可能にした。

【０２８０】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃で０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水された。

【０２８１】

標識ＤＮＡの伸長

１×クレノーバッファー、２００μＭのｄＮＴＰ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）及び１μｌのクレノー断片（３’から５’ｅｘｏマイナス）を含有する混合物５０μｌを含有する反応混合物の５０μｌは３７℃まで加熱され、それぞれのウェルに添加されて混ぜながら（３００ｒｐｍで３秒、静止して６秒）３７℃で３０分間インキュベートされた（サーモミキサーコンフォート；Ｅｐｐｅｎｄｏｒｆ）。

【０２８２】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃で０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水された。

【０２８３】
残余組織の除去
付着したホルマリン固定パラフィン化マウス脳組織切片を有するスライドは、ＣｈｉｐＣｌｉｐスライドホルダー及び１６ウェルマスク（Ｗｈａｔｍａｎ）に装着された。ＲＮｅａｓｙ　ＦＦＰＥキット（Ｑｉａｇｅｎ）由来の１５０μｌのプロテイナーゼＫダイジェストバッファーごとに、１０μｌのプロテイナーゼＫ溶液（Ｑｉａｇｅｎ）が添加された。５０μｌの最終混合液がそれぞれのウェルに添加され、スライドは５６℃で３０分間インキュベートされた。
【０２８４】
ＰＣＲバッファー中ウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ放出（共有結合プローブ）
１６ウェルマスク及びＣｏｄｅＬｉｎｋスライドはＣｈｉｐＣｌｉｐホルダー（Ｗｈａｔｍａｎ）に装着された。１．８ｍＭ　$MgCl_2$を有する１×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー（Ｒｏｃｈｅ）、２００μＭ　ｄＮＴＰ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）及び０．１Ｕ／１μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有する５０μｌの混合物を３７℃まで加熱し、それぞれのウェルに添加し、混ぜながら（３００ｒｐｍで３秒間、静止して６秒間）（サーモミキサーコンフォート；Ｅｐｐｅｎｄｏｒｆ）３７℃で３０分間インキュベートした。次に、放出されたｃＤＮＡ及びプローブを含有する反応混合物は、ピペットを用いてウェルから回収された。
【０２８５】
標識ＤＮＡの合成及びプローブ収集後のＩＤ特異的及び遺伝子特異的生成物の増幅
ＰＣＲバッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ放出に続く（共有結合プローブ）。
【０２８６】
切断されたＤＮＡは、最終反応容積１０μｌで増幅された。１．８ｍＭ　$MgCl_2$を有する１．４×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー中、７μｌの切断された鋳型、１μｌのＩＤ特異的フォワードプライマー（２μＭ）、１μｌの遺伝子特異的リバースプライマー（２μＭ）及び１μｌのＦａｓｔＳｔａｒｔ高忠実度酵素ブレンドは、１．８ｍＭ　$MgCl_2$を有する１×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー及び１ＵのＦａｓｔＳｔａｒｔ高忠実度酵素ブレンドとの１０μｌの最終反応容積を与える。ＰＣＲ増幅は熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中で、以下のプログラム：ホットスタート、９４℃で２分間、続いて５０サイクルを９４℃で１５秒間、５５℃で３０秒間、６８℃で１分間及び最終伸長、６８℃で５分間により実行された。
【０２８７】
ランダムプライマー第二鎖合成とそれに続くユニバーサルハンドル増幅による全ゲノム増幅（得られたｄｓＤＮＡの端に保持されているタグ配列を含む捕捉プローブ配列）
ＰＣＲバッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ放出に続く（共有結合プローブ）。
【０２８８】
反応混合物は、１．８ｍＭ　$MgCl_2$を有する４０μｌの１×Ｆａｓｔｓｔａｒｔ　ＨｉＦｉ　ＰＣＲバッファー（ｐＨ８．３）（Ｒｏｃｈｅ、ｗｗｗ．ｒｏｃｈｅ－ａｐｐｌｉｅｄ－ｓｃｉｅｎｃｅ．ｃｏｍ）、０．２ｍＭのそれぞれのｄＮＴＰ（Ｆｅｒｍｅｎｔａｓ、ｗｗｗ．ｆｅｒｍｅｎｔａｓ．ｃｏｍ）、０．１μｇ／μｌ　ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）、０．１Ｕ／μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）、放出されたＤＮＡ（表面プローブから伸長された）及び放出された表面プローブを含有していた。管は、熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ、ｗｗｗ．ａｐｐｌｉｅｄｂｉｏｓｙｓｔｅｍｓ．ｃｏｍ）中、３７℃で３０分間、続いて７０℃で２０分間インキュベートされた。１μｌのクレノー断片（３’から５’ｅｘｏマイナス）（Ｉｌｌｕｍｉｎａ、ｗｗｗ．ｉｌｌｕｍｉｎａ．ｃｏｍ）及び１μｌのハンドル連結ランダムプライマー（１０μＭ）（Ｅｕｒ

ｏｆｉｎｓ ＭＷＧ Ｏｐｅｒｏｎ、ｗｗｗ．ｅｕｒｏｆｉｎｓｄｎａ．ｃｏｍ）は管に添加された。管は、熱サイクラー（Ａｐｐｌｉｅｄ Ｂｉｏｓｙｓｔｅｍｓ）中で、１５℃で１５分間、２５℃で１５分間、３７℃で１５分間及び最後に７５℃で２０分間インキュベートされた。インキュベーション後、１μｌのそれぞれのプライマー、Ａ＿Ｐ及びＢ（１０μＭ）（Ｅｕｒｏｆｉｎｓ ＭＷＧ Ｏｐｅｒｏｎ）は管に添加された。１μｌのＦａｓｔｓｔａｒｔ ＨｉＦｉ ＤＮＡポリメラーゼ（５Ｕ／μｌ）（Ｒｏｃｈｅ）も管に添加された。ＰＣＲ増幅は熱サイクラー（Ａｐｐｌｉｅｄ Ｂｉｏｓｙｓｔｅｍｓ）中で、以下のプログラム：ホットスタート、９４℃で２分間、続いて５０サイクルを９４℃で１５秒間、５５℃で３０秒間、６８℃で１分間及び最終伸長、６８℃で５分間により実行された。増幅後、管由来の４０μｌはＱｉａｑｕｉｃｋ ＰＣＲ精製カラム（Ｑｉａｇｅｎ、ｗｗｗ．ｑｉａｇｅｎ．ｃｏｍ）を用いて精製され、３０μｌのＥＢ（１０ｍＭ Ｔｒｉｓ－Ｃｌ、ｐＨ８．５）中に溶出された。精製された生成物はバイオアナライザー（Ａｇｉｌｅｎｔ、ｗｗｗ．ｈｏｍｅ．ａｇｉｌｅｎｔ．ｃｏｍ）を用いて解析され、ＤＮＡ ７５００キットが使用された。

【０２８９】

視覚化

染色に先立つ蛍光マーカープローブのハイブリダイゼーション

組織の適用に先立って、蛍光マーカープローブは、捕捉プローブアレイ上にプリントされた指定されたマーカー配列にハイブリダイズされる。蛍光マーカープローブは、組織視覚化後の得られた画像を方向付けるのに役立ち、画像をシーケンシング後に得られる個々の捕捉プローブ「タグ」配列についての得られた発現プロファイルと組み合わせることが可能になる。蛍光プローブをハイブリダイズさせるため、４×ＳＳＣ及び０．１％ＳＤＳ、２μＭの検出プローブ（Ｐ）を含有するハイブリダイゼーション溶液は５０℃で４分間インキュベートされた。その間、自製アレイはＣｈｉｐＣｌｉｐ（Ｗｈａｔｍａｎ）に装着された。アレイはそれに続いて、ウェルあたり５０μＬのハイブリダイゼーション溶液と一緒に５０℃、３０分間、３００ｒｐｍ振盪でインキュベートされた。

【０２９０】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃、０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水された。

【０２９１】

標識ＤＮＡの合成に先立つ又は後のＦＦＰＥ組織切片の一般的組織学的染色

捕捉プローブアレイ上に固定化されたＦＦＰＥ組織切片は、前述のように標識ＤＮＡ合成に先立って脱パラフィン後に洗浄され再水和される、又は前述のように標識ＤＮＡ合成後に洗浄される。次に、前記切片は以下の通りに処理された；ヘマトキシリン中で３分間インキュベートする、脱イオン水を用いてすすぐ、水道水中で５分間インキュベートする、酸性エタノール中８～１２回素早く浸す、水道水中２×１分間すすぐ、脱イオン水中２分間すすぐ、エオシン中３０秒間インキュベートする、９５％エタノール中３×５分間洗浄する、１００％エタノール中３×５分間洗浄する、キシレン中３×１０分間洗浄する（一晩実行することができる）、ＤＰＸを使用してスライド上にカバーガラスを置く、一晩フード内でスライドを乾燥させる。

【０２９２】

標識ＤＮＡの合成に先立つ又は後のＦＦＰＥ組織切片における標的タンパク質の一般的免疫組織化学染色

捕捉プローブアレイ上に固定化されたＦＦＰＥ組織切片は、前述のように標識ＤＮＡ合成に先立って脱パラフィン後に洗浄され再水和される、又は前述のように標識ＤＮＡ合成後に洗浄される。次に、前記切片は、全染色作業中乾燥させておくことなく以下の通りに処理された；一次抗体をブロッキング溶液（１×ＴＢＳ（Ｔｒｉｓ緩衝食塩水（５０ｍＭ Ｔｒｉｓ、１５０ｍＭ ＮａＣｌ、ｐＨ７．６）、４％ロバ血清及び０．１％ｔｒｉｔ

ｏｎ－Ｘ）に希釈する、切片を湿潤チャンバー中室温で一晩一次抗体と一緒にインキュベートする、１×ＴＢＳを用いて３回すすぐ、切片を室温で１時間湿潤チャンバー内で蛍光色素（ＦＩＴＣ、Ｃｙ３又はＣｙ５）にコンジュゲートされた適合する二次抗体と一緒にインキュベートする、１×ＴＢＳを用いて３回すすぐ、できる限り多くのＴＢＳを取り除き、切片をＰｒｏＬｏｎｇ　Ｇｏｌｄ＋ＤＡＰＩ（Ｉｎｖｉｔｒｏｇｅｎ）と一緒にマウントし、蛍光顕微鏡及び適合するフィルターセットを用いて解析する。

【０２９３】

実施例６

この実験は実施例５の原理に従って、しかし組織ではなくアレイ上の断片化されたゲノムＤＮＡを使用して行われた。ゲノムＤＮＡはそれぞれ平均サイズ２００ｂｐと７００ｂｐまで予め断片化された。この実験は、前記原理が機能することを明らかにしている。断片化されたゲノムＤＮＡはＦＦＰＥ組織に極めて類似している。

【０２９４】

標識ＤＮＡの合成及びプローブ収集後の内部遺伝子特異的生成物の増幅

１．８ｍＭ　$MgCl_2$を有する１×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー（Ｒｏｃｈｅ）、２００μＭのｄＮＴＰ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）及び０．１Ｕ／１μｌのＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有するＰＣＲバッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ（共有結合プローブ）放出に続く。

【０２９５】

切断されたＤＮＡは、最終反応容積５０μｌで増幅された。４７μｌの切断された鋳型に、１μｌのＩＤ特異的フォワードプライマー（１０μＭ）、１μｌの遺伝子特異的リバースプライマー（１０μＭ）及び１μｌのＦａｓｔＳｔａｒｔ高忠実度酵素ブレンドが添加された。ＰＣＲ増幅は熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中で、以下のプログラム：ホットスタート、９４℃で２分間、続いて５０サイクルを９４℃で１５秒間、５５℃で３０秒間、６８℃で１分間及び最終伸長、６８℃で５分間により実行された。

【０２９６】

標識ＤＮＡの合成及びプローブ収集後の標識特異的及び遺伝子特異的生成物の増幅

１．８ｍＭ　$MgCl_2$を有する１×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー（Ｒｏｃｈｅ）、２００μＭのｄＮＴＰ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）及び０．１Ｕ／１μｌのＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有するＰＣＲバッファー中のウラシル切断ＵＳＥＲ酵素混合物を用いた捕捉プローブ（共有結合プローブ）放出に続く。

【０２９７】

切断されたＤＮＡは、最終反応容積５０μｌで増幅された。４７μｌの切断された鋳型に、１μｌの標識特異的フォワードプライマー（１０μＭ）、１μｌの遺伝子特異的リバースプライマー（１０μＭ）及び１μｌのＦａｓｔＳｔａｒｔ高忠実度酵素ブレンドが添加された。ＰＣＲ増幅は熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中で、以下のプログラム：ホットスタート、９４℃で２分間、続いて５０サイクルを９４℃で１５秒間、５５℃で３０秒間、６８℃で１分間及び最終伸長、６８℃で５分間により実行された。

フォワード－ゲノムＤＮＡヒトプライマー

５’－ＧＡＣＴＧＣＴＣＴＴＴＴＣＡＣＣＣＡＴＣ－３’（配列番号４７）

リバース－ゲノムＤＮＡヒトプライマー

５’－ＧＧＡＧＣＴＧＣＴＧＧＴＧＣＡＧＧＧ－３’（配列番号４８）

Ｐ－標識特異的プライマー

５’－ＡＴＣＴＣＧＡＣＴＧＣＣＡＣＴＣＴＧＡＡ－３’（配列番号４９）

【０２９８】

結果は図１７及び２０に示されている。前記図はアレイ上で増幅された内部生成物を示

しており、図１７及び１８における検出されたピークは予想されたサイズである。したがって、これはゲノムＤＮＡを捕捉し増幅できることを実証している。図１９及び２０では、ゲノムＤＮＡのランダム断片化及びターミナルトランスフェラーゼ標識化が極めて多様な試料プールを生成することを考えると、スメアである。

【０２９９】

実施例７

ポリメラーゼ伸長及びターミナルトランスフェラーゼテーリングを使用する５’から３’配向捕捉プローブの代替合成

捕捉プローブ合成のためのプライマーをハイブリダイズさせるため、４×ＳＳＣ及び０．１％ＳＤＳ及び２μＭの伸長プリマー（Ａ＿プライマー）を含有するハイブリダイゼーション溶液が５０℃で４分間インキュベートされた。その間、自製アレイ（実施例１参照）はＣｈｉｐＣｌｉｐ（Ｗｈａｔｍａｎ）に装着された。前記アレイは引き続き、ウェルあたり５０μＬのハイブリダイゼーション溶液を用いて、５０℃、３０分間、３００ｒｐｍ振盪でインキュベートされた。

【０３００】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃で０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水され、再びＣｈｉｐＣｌｉｐに戻された。

【０３０１】

１μｌのクレノー断片（３’から５’ｅｘｏマイナス）（Ｉｌｌｕｍｉｎａ、ｗｗｗ．ｉｌｌｕｍｉｎａ．ｃｏｍ）は１０×クレノーバッファーと共に、ｄＮＴＰそれぞれ２ｍＭ（Ｆｅｒｍｅｎｔａｓ）及び水は５０μｌの反応に混合され、それぞれのウェルにピペットで移された。

【０３０２】

アレイはＥｐｐｅｎｄｏｒｆサーモミキサーにおいて、１５℃で１５分間、２５℃で１５分間、３７℃で１５分間及び最後に７５℃で２０分間インキュベートされた。

【０３０３】

インキュベーション後、アレイはＣｈｉｐＣｌｉｐから取り除かれ、以下の３つのステップ、１）５０℃で０．１％ＳＤＳを有する２×ＳＳＣ液、６分間、３００ｒｐｍ振盪で、２）０．２×ＳＳＣ、１分間、３００ｒｐｍ振盪で、及び３）０．１×ＳＳＣ、１分間、３００ｒｐｍ振盪で洗浄された。次にアレイは、遠心脱水され、再びＣｈｉｐＣｌｉｐに戻された。

【０３０４】

ｄＴテーリングのために、１×ＴｄＴバッファー（２０ｍＭ　Ｔｒｉｓ－アセテート（ｐＨ７．９）、５０ｍＭ酢酸カリウム及び１０ｍＭ酢酸マグネシウム）（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、ｗｗｗ．ｎｅｂ．ｃｏｍ）、０．１μｇ／μｌ　ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）、０．５μｌのリボヌクレアーゼＨ（５Ｕ／μｌ）、１μｌのＴｄＴ（２０Ｕ／μｌ）及び０．５μｌのｄＴＴＰ（１００ｍＭ）を含有する５０μｌの混合物が調製された。混合物はアレイ表面に添加され、アレイは熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）中３７℃で１５分間インキュベートされ、続いてＴｄＴは７０℃で１０分間不活化された。

【０３０５】

実施例８

ＵＳＥＲシステム切断及びターミナルトランスフェラーゼを介した増幅を用いて５’から３’高プローブ密度アレイ及びホルマリン固定凍結（ＦＦ凍結）組織を使用する空間トランスクリプトーム

アレイ調製

予め製作された高密度マイクロアレイチップはＲｏｃｈｅ　Ｎｉｍｂｌｅｇｅｎ（Ｍａ

ｄｉｓｏｎ、ＷＩ、ＵＳＡ）に発注した。それぞれの捕捉プローブアレイは１３５０００のフィーチャーを含有しており、そのうちの１３２６４０フィーチャーが、独自のＩＤタグ配列（位置ドメイン）及び捕捉領域（捕捉ドメイン）を含む捕捉プローブを担持していた。それぞれのフィーチャーは１３×１３μｍのサイズであった。捕捉プローブは、５’から３’方向に、５つのｄＵＴＰ塩基（切断ドメイン）及びジェネラル増幅ドメインを含有するユニバーサルドメイン、ＩＤタグ（位置ドメイン）並びに捕捉領域（捕捉ドメイン）で構成されていた（図２２及び表２）。それぞれのアレイは、アレイ視覚化中に方向付けを助ける蛍光プローブのハイブリダイゼーションを可能にするジェネリック３０ｂｐ配列（表２）を担持するマーカープローブの枠（図２３）も取り付けられていた。

【０３０６】

組織調製－ホルマリン固定凍結組織の調製

動物（マウス）は５０ｍｌのＰＢＳ及び１００ｍｌの４％ホルマリン液を灌流された。嗅球の切除後、組織は後固定のために２４時間、４％ホルマリン浴に漬けられた。次に、組織はＰＢＳに溶解させた３０％ショ糖中で２４時間ショ糖処理され、形態を安定化させ過剰なホルマリンを取り除いた。組織は制御された速度で－４０℃まで凍結降下させ、実験間は－２０℃で保管した。３時間の組織後固定又は後固定なしの類似の調製は平行標本について実行された。後固定のない２％ホルマリンを用いた灌流も首尾よく使用された。同様に、ショ糖処理ステップは省くことができた。組織は１０μｍの切片を作るためにクリオスタットにマウントされた。組織片は、使用されるそれぞれの捕捉プローブアレイ上に貼り付けられた。場合によってさらに良好な組織付着性のために、アレイチップは５０℃で１５分間置かれた。

【０３０７】

随意の対照－切片組織からの全ＲＮＡ調製

全ＲＮＡは、製造業者の使用説明書に従ってＲＮｅａｓｙＦＦＰＥキット（Ｑｉａｇｅｎ）を使用して単一の組織切片（１０μｍ）から抽出された。組織切片から得られた全ＲＮＡは、ＲＮＡが組織切片から直接アレイ上に捕捉される実験との比較のために対照実験で使用された。したがって、全ＲＮＡがアレイに適用される場合には、組織ステップの染色、視覚化及び分解は省かれた。

【０３０８】

オンチップ反応

マーカープローブの枠プローブへのハイブリダイゼーション、逆転写、核染色、組織消化及びプローブ切断反応はすべて、ウェルあたり５０μｌの反応容積で１６ウェルシリコンガスケット（Ａｒｒａｙｉｔ、Ｓｕｎｎｙｖａｌｅ、ＣＡ、ＵＳＡ）において実施された。蒸発を防ぐために、カセットはプレートシーラー（ｉｎ Ｖｉｔｒｏ ＡＢ、Ｓｔｏｃｋｈｏｌｍ、Ｓｗｅｄｅｎ）で覆われた。

【０３０９】

随意－ｃＤＮＡ合成に先立つ組織透過処理

プロテイナーゼＫを使用する透過処理のために、プロテイナーゼＫ（Ｑｉａｇｅｎ、Ｈｉｌｄｅｎ、Ｇｅｒｍａｎｙ）は、ＰＢＳ中に１μｇ／ｍｌまで希釈された。前記溶液はウェルに添加され、スライドは室温で５分間インキュベートされ、続いて１０分間にわたり８０℃まで徐々に増加させた。スライドは、逆転写反応前にＰＢＳ中で短時間で洗浄された。

【０３１０】

代わりに、マイクロ波を使用する透過処理のために、組織付着後スライドは５０ｍｌの０．２×ＳＳＣ（Ｓｉｇｍａ－Ａｌｄｒｉｃｈ）を含有するガラス瓶の底に置かれ、マイクロ波オーブンにおいて８００Ｗで１分間加熱された。マイクロ波処理の直後、スライドは紙ティシューの上に置かれ、不必要な大気曝露から保護されたチャンバー内で３０分間乾燥された。乾燥後、ｃＤＮＡ合成が開始される前に、スライドは短時間水（リボヌクレアーゼ／デオキシリボヌクレアーゼなし）に浸され、最後に遠心分離により遠心乾燥された。

【０３１１】
ｃＤＮＡ合成
逆転写反応のために、プラチナＴａｑ付のＳｕｐｅｒＳｃｒｉｐｔＩＩＩ　Ｏｎｅ－Ｓｔｅｐ　ＲＴ－ＰＣＲ　Ｓｙｓｔｅｍ（Ｌｉｆｅ　Ｔｅｃｈｎｏｌｏｇｉｅｓ／Ｉｎｖｉｔｒｏｇｅｎ、Ｃａｒｌｓｂａｄ、ＣＡ、ＵＳＡ）が使用された。逆転写反応は、最終容積５０μｌで１×反応混合物、１×ＢＳＡ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ、Ｉｐｓｗｉｃｈ、ＭＡ、ＵＳＡ）及び２μｌのＳｕｐｅｒＳｃｒｉｐｔＩＩＩ　ＲＴ／Ｐｌａｔｉｎｕｍ　Ｔａｑ混合物を含有していた。この溶液は組織切片に適用する前に５０℃まで加熱され、反応は５０℃で３０分間実施された。逆転写溶液はそれに続いてウェルから取り除かれ、スライドは２時間風乾させておいた。

【０３１２】
組織視覚化
ｃＤＮＡ合成後、核染色及びマーカープローブの枠プローブ（アレイ上の組織試料の方向付けを可能にするためにアレイ基板に付着しているプローブ）へのハイブリダイゼーションは同時に実行された。濃度３００ｎＭのＤＡＰＩ及びＰＢＳ中濃度１７０ｎＭのマーカープローブを有する溶液が調製された。この溶液はウェルに添加され、スライドは室温で５分間インキュベートされ、続いてＰＢＳ中で短時間洗浄され、遠心乾燥させた。

【０３１３】
代わりに、マーカープローブは、組織をアレイ上に置くのに先立って枠プローブにハイブリダイズされた。次に、マーカープローブはハイブリダイゼーション溶液（４×ＳＳＣ、０．１％ＳＤＳ）中１７０ｎＭまで希釈された。この溶液はチップに適用する前に５０℃まで加熱され、ハイブリダイゼーションは５０℃、３０分間、３００ｒｐｍで実施された。ハイブリダイゼーション後、スライドは、２×ＳＳＣ、０．１％ＳＤＳ、５０℃、１０分間、３００ｒｐｍで、０．２×ＳＳＣ、１分間、３００ｒｐｍで、及び０．１×ＳＳＣ、１分間、３００ｒｐｍで洗浄された。その場合、ｃＤＮＡ合成後の染色液は、ＰＢＳ中３００ｎＭまで希釈された核ＤＡＰＩ染色を含有しているだけであった。前記溶液はウェルに適用され、スライドは室温で５分間インキュベートされ、続いてＰＢＳ中で短時間洗浄し、遠心乾燥させた。

【０３１４】
切片はＺｅｉｓｓ　Ａｘｉｏ　Ｉｍａｇｅｒ　Ｚ２を用いて顕微鏡的に調べられ、ＭｅｔａＳｙｓｔｅｍｓソフトウェアを用いて処理された。

【０３１５】
組織除去
組織切片は、ＲＮｅａｓｙ　ＦＦＰＥキットからのＰＫＤバッファー（両方ともＱｉａｇｅｎ社製）中に１．２５μｇ／μｌまで希釈されたプロテイナーゼＫを使用して、３００ｒｐｍで３秒間、次に静止させて６秒間の間隔を開けて混合させて、５６℃で３０分間消化された。スライドはそれに続いて２×ＳＳＣ、０．１％ＳＤＳ、５０℃、１０分間、３００ｒｐｍで、０．２×ＳＳＣ、１分間、３００ｒｐｍで、及び０．１×ＳＳＣ、１分間、３００ｒｐｍで洗浄された。

【０３１６】
プローブ放出
シリコンガスケット付の１６ウェルハイブリダイゼーションカセット（Ａｒｒａｙｉｔ）は３７℃まで予熱され、Ｎｉｍｂｌｅｇｅｎスライドに装着させた。濃度未知の溶解バッファー（Ｔａｋａｒａ）、０．１Ｕ／μｌ　ＵＳＥＲ酵素（ＮＥＢ）及び０．１μｇ／μｌのＢＳＡからなる、３７℃まで予熱した容積５０μｌの切断混合物が表面固定化ｃＤＮＡを含有するウェルのそれぞれに添加された。泡の除去後、スライドは密封され、サーモミキサーコンフォート内、３７℃で３０分間インキュベートされ、３００ｒｐｍで３秒間と間に静止６秒間をおくサイクル振盪させた。インキュベーション後、４５μｌの切断混合物は使用されたウェルのそれぞれから収集され、０．２ｍｌのＰＣＲ管に入れられた（図２４）。

【０３１７】
ライブラリー調製
エキソヌクレアーゼ処理
前記溶液を氷上で２分間冷却後、エキソヌクレアーゼＩ（ＮＥＢ）が添加され、伸長していないｃＤＮＡプローブを取り除き、最終容積４６．２μｌ及び最終濃度０．５２Ｕ／μｌにした。管は熱サイクラー（Ａｐｐｌｉｅｄ　Ｂｉｏｓｙｓｔｅｍｓ）において３７℃で３０分間インキュベートされ、続いてエキソヌクレアーゼは８０℃で２５分間不活化された。
【０３１８】
ターミナルトランスフェラーゼによるｄＡテーリング
エキソヌクレアーゼステップ後、４５μｌのポリＡテーリング混合物は、製造業者の使用説明書に従って、ＴｄＴバッファー（Ｔａｋａｒａ）、３ｍＭのｄＡＴＰ（Ｔａｋａｒａ）及びマニュファクチャラーズＴｄＴ酵素混合物（ＴｄＴ及びリボヌクレアーゼＨ）（Ｔａｋａｒａ）から成るが、試料のそれぞれに添加された。混合物は熱サイクラーにおいて３７℃で１５分間インキュベートされ、続いてＴｄＴは７０℃で１０分間不活化された。
【０３１９】
第二鎖合成及びＰＣＲ増幅
ｄＡテーリング後、２３μｌのＰＣＲマスター混合物は、試料あたり４個の新しい０．２ｍｌ　ＰＣＲ管に入れ、それぞれの管に２μｌ試料が鋳型として添加された。最終ＰＣＲは、１×Ｅｘ　Ｔａｑバッファー（Ｔａｋａｒａ）、２００μＭのそれぞれのｄＮＴＰ（Ｔａｋａｒａ）、６００ｎＭのＡ＿プライマー（ＭＷＧ）、６００ｎＭのＢ＿ｄＴ２０ＶＮ＿プライマー（ＭＷＧ）及び０．０２５Ｕ／μｌのＥｘ　Ｔａｑポリメラーゼ（Ｔａｋａｒａ）から成っていた（表２）。第二ｃＤＮＡ鎖は、熱サイクラーにおいて９５℃で３分間、５０℃で２分間及び７２℃で３分間の１サイクルを実行することにより作製された。次に、試料は、９５℃で３０秒間、６７℃で１分間及び７２℃で３分間の２０サイクル（ライブラリー調製のため）又は３０サイクル（ｃＤＮＡの存在を確認するため）を実行することにより増幅され、続いて７２℃で１０分間最終伸長させた。
【０３２０】
ライブラリー精製
増幅後、４つのＰＣＲ（１００μｌ）は５００μｌの結合バッファー（Ｑｉａｇｅｎ）と混合され、Ｑｉａｑｕｉｃｋ　ＰＣＲ精製カラム（Ｑｉａｇｅｎ）に入れられ、増幅されたｃＤＮＡを膜に結合させるために１７９００×ｇで１分間回転させた。次に、膜はエタノールを含有する洗浄バッファー（Ｑｉａｇｅｎ）を用いて洗浄され、最後に５０μｌの１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５に溶出させた。
【０３２１】
精製され濃縮された試料は、ＭＢＳロボット（Ｍａｇｎｅｔｉｃ　Ｂｉｏｓｏｌｕｔｉｏｎｓ）を用いてＣＡ－精製（カルボン酸にコンジュゲートされた超常磁性ビーズによる精製）によりさらに精製され濃縮された。１５０～２００ｂｐ未満の断片を取り除くために最終ＰＥＧ濃度１０％が使用された。増幅されたｃＤＮＡは１０分間ＣＡビーズ（Ｉｎｖｉｔｒｏｇｅｎ）に結合させておき、次に１５μｌの１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５に溶出させた。
【０３２２】
ライブラリー品質解析
３０サイクル増幅された試料は、増幅されたｃＤＮＡライブラリーの存在を確認するためにＡｇｉｌｅｎｔバイオアナライザー（Ａｇｉｌｅｎｔ）を用いて解析され、ＤＮＡ高感度キット又はＤＮＡ　１０００キットが、材料の量に応じて使用された。
【０３２３】
シーケンシングライブラリー調製
ライブラリーインデックス化

２０サイクル増幅された試料をさらに使用して、シーケンシングライブラリーを調製した。インデックスＰＣＲマスター混合物は試料ごとに調製され、２３μｌが６個の０．２ｍｌ管に入れられた。２μｌの増幅され精製されたｃＤＮＡが６つのＰＣＲのそれぞれに鋳型として添加され、１×Ｐｈｕｓｉｏｎマスター混合物（Ｆｅｒｍｅｎｔａｓ）、５００ｎＭのＩｎＰＥ１．０（Ｉｌｌｕｍｉｎａ）、５００ｎＭのＩｎｄｅｘ　１－１２（Ｉｌｌｕｍｉｎａ）及び０．４ｎＭのＩｎＰＥ２．０（Ｉｌｌｕｍｉｎａ）を含有するＰＣＲを作製した。試料は、熱サイクラーにおいて、９８℃で３０秒間、６５℃で３０秒間及び７２℃で１分間の１８サイクルで増幅され、続いて７２℃で５分間最終伸長させた。

【０３２４】

シーケンシングライブラリー精製

増幅後、６つのＰＣＲ（１５０μｌ）は７５０μｌの結合バッファーと混合され、Ｑｉａｑｕｉｃｋ　ＰＣＲ精製カラムに入れられ、増幅されたｃＤＮＡを膜に結合させるために１７９００×ｇで１分間回転させた（大きな試料容積（９００μｌ）のせいで、試料は２つ（それぞれ４５０μｌ）に分割され２つの別々のステップで結合された）。次に、膜はエタノールを含有する洗浄バッファーを用いて洗浄され、最後に５０μｌの１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５に溶出させた。

【０３２５】

精製され濃縮された試料は、ＭＢＳロボットを用いてＣＡ－精製によりさらに精製され濃縮された。３００～３５０ｂｐ未満の断片を取り除くために最終ＰＥＧ濃度７．８％が使用された。増幅されたｃＤＮＡは１０分間ＣＡビーズに結合させておき、次に１５μｌの１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５に溶出させた。試料は、完成ライブラリーの存在及びサイズを確認するためにＡｇｉｌｅｎｔバイオアナライザーを用いて解析され、ＤＮＡ高感度キット又はＤＮＡ　１０００キットが、材料の量に応じて製造業者の使用説明書に従って使用された（図２５）。

【０３２６】

シーケンシング

前記ライブラリーは、製造業者の使用説明書に従って、望ましいデータ処理能力に応じて、Ｉｌｌｕｍｉｎａ　Ｈｉｓｅｑ２０００又はＭｉｓｅｑ上でシーケンシングされた。場合によって読取り２では、カスタムシーケンシングプライマーＢ＿ｒ２を使用して、２０Ｔのホモポリマーストレッチを通じたシーケンシングを回避した。

【０３２７】

データ解析

読取り１は５’端部で４２塩基刈り取られた。リード２は５’端部で２５塩基刈り取られた（場合によって、カスタムプライマーが使用された場合には、読取り２から塩基は刈り取られなかった）。次に、前記読取りはリピート遮蔽マスマスキュラス（Ｍｕｓ　ｍｕｓｃｕｌｕｓ）９ゲノムアセンブリーヘボウタイ（ｂｏｗｔｉｅ）を用いてマッピングされ、出力はＳＡＭファイルフォーマットにフォーマットされた。マッピングされた読取りは抜き出され、ＵＣＳＣ　ｒｅｆＧｅｎｅ遺伝子アノテーションを用いて推定された。インデックスは「インデックスファインダー」（インデックス回収のための自製ソフトウェア）を用いて回収された。次に、すべての捕捉された転写物及びチップ上のそのそれぞれのインデックス位置に関する情報を含有するモンゴＤＢデータベースが作成された。

【０３２８】

ｍａｔｌａｂ実施はデータベースと結び付けられ、データの空間視覚化及び解析が可能になった（図２６）。

【０３２９】

場合によって、データ視覚化は、正確な整列化のために蛍光標識された枠プローブを使用し、空間トランスクリプトームデータ抽出を可能にする顕微鏡画像を重ね合わされた。

【０３３０】

実施例９

ＭｕｔＹシステム切断及びＴｄＴを介する増幅を用いて３’から５’高プローブ密度ア

レイ及びＦＦＰＥ組織を使用する空間トランスクリプトーム

アレイ調製

予め製作された高密度マイクロアレイチップはＲｏｃｈｅ　Ｎｉｍｂｌｅｇｅｎ（Ｍａｄｉｓｏｎ、ＷＩ、ＵＳＡ）に発注した。それぞれの使用された捕捉プローブアレイは７２ｋのフィーチャーを含有しており、そのうちの６６０２２が、独自のＩＤタグ相補的配列を含有していた。それぞれのフィーチャーは１６×１６μｍのサイズであった。捕捉プローブは、３つの追加の塩基がプローブの上部（Ｐ’）ジェネラルハンドルに付加されてプローブを長バージョンのＰ’、ＬＰ’にする以外は、自製プリントされた３’から５’アレイのために使用されたプローブと同じように３’から５’に構成されていた（表２）。それぞれのアレイは、アレイ視覚化中に方向付けを助ける蛍光プローブのハイブリダイゼーションを可能にするジェネリック３０ｂｐ配列を担持するプローブの枠も取り付けられていた。

【０３３１】

５’から３’配向捕捉プローブの合成

高密度アレイ上での５’から３’配向捕捉プローブの合成は、伸長及びライゲーションステップが５５℃で１５分間、続いて７２℃で１５分間実行されたこと以外は、自製プリントされたアレイの場合と同じように実行された。Ａハンドルプローブ（表２）は、下に説明されるＭｕｔＹ酵素システムを通じたプローブのその後の放出を可能にするＡ／Ｇミスマッチを含んでいた。Ｐプローブは、表面上のさらに長いプローブに適合するようにさらに長いＬＰバージョンで置き換えられた。

【０３３２】

ホルマリン固定パラフィン包埋組織の調製及び脱パラフィン

これは、自製プロトコールにおいて上で説明された通りに実行された。

【０３３３】

ｃＤＮＡ合成及び染色

ｃＤＮＡ合成及び染色は、ビオチン標識ｄＣＴＰ及びｄＡＴＰが４つの定まったｄＮＴＰ（それぞれがビオチン標識したものの２５倍で存在していた）と共にｃＤＮＡ合成に添加されたこと以外は、５’から３’配向高密度Ｎｉｍｂｌｅｇｅｎアレイについてのプロトコールの通りに実行された。

【０３３４】

組織除去

組織除去は、実施例８において説明される５’から３’配向高密度Ｎｉｍｂｌｅｇｅｎアレイについてのプロトコールと同じように実行された。

【０３３５】

ＭｕｔＹによるプローブ切断

シリコンガスケット（ＡｒｒａｙＩＴ）付の１６ウェルインキュベーションチャンバーは３７℃まで予熱され、Ｃｏｄｅｌｉｎｋスライドに装着された。３７℃まで予熱され、１×エンドヌクレアーゼＶＩＩＩバッファー（ＮＥＢ）、１０Ｕ／μｌのＭｕｔＹ（Ｔｒｅｖｉｇｅｎ）、１０Ｕ／μｌのエンドヌクレアーゼＶＩＩＩ（ＮＥＢ）、０．１μｇ／μｌのＢＳＡから成る容積５０μｌの切断混合物が、表面固定化ｃＤＮＡを含有するウェルのそれぞれに添加された。泡を除去後、スライドは密封され、サーモミキサーコンフォートにおいて３７℃で３０分間インキュベートされ、３００ｒｐｍで３秒間と間に６秒間の静止でサイクル振盪した。インキュベーション後、プレートシーラーは取り除かれ、４０μｌの切断混合物は使用されたウェルのそれぞれから収集されて、ＰＣＲプレートに置かれた。

【０３３６】

ライブラリー調製

ビオチン－ストレプトアビジン媒介ライブラリー精製

非伸長ｃＤＮＡプローブを取り除きバッファーを交換するために、ビオチン標識ｃＤＮＡをストレプトアビジン被覆Ｃ１ビーズ（Ｉｎｖｉｔｒｏｇｅｎ）に結合させビーズを０

．１Ｍ　ＮａＯＨ（新たに作製される）で洗浄することにより試料は精製された。精製はＭＢＳロボット（Ｍａｇｎｅｔｉｃ　Ｂｉｏｓｏｌｕｔｉｏｎｓ）を用いて実行され、ビオチン標識ｃＤＮＡはＣ１ビーズに１０分間結合させておき、次に、ビーズ－水溶液を８０℃まで加熱してビオチンーストレプトアビジン結合を切断することにより２０μｌの水中に溶出させた。

【０３３７】

ターミナルトランスフェラーゼによるｄＡテーリング

精製ステップ後、製造業者の使用説明書に従って、１８μｌのそれぞれの試料は新しい０．２ｍｌのＰＣＲ管に入れられ、２２μｌのポリＡテーリングマスター混合物と混合されて、溶解バッファー（Ｔａｋａｒａ、Ｃｅｌｌａｍｐ全トランスクリプトーム増幅キット）、ＴｄＴバッファー（Ｔａｋａｒａ）、１．５ｍＭ　ｄＡＴＰ（Ｔａｋａｒａ）及びＴｄＴ酵素混合物（ＴｄＴ及びリボヌクレアーゼＨ）（Ｔａｋａｒａ）から成る４０μｌの反応混合物とした。混合物は熱サイクラーにおいて３７℃で１５分間インキュベートされ、続いてＴｄＴは７０℃で１０分間不活化された。

【０３３８】

第二鎖合成及びＰＣＲ増幅

ｄＡテーリング後、２３μｌのＰＣＲマスター混合物は、試料あたり４個の新しい０．２ｍｌ　ＰＣＲ管に入れ、それぞれの管に２μｌ試料が鋳型として添加された。最終ＰＣＲは、１×Ｅｘ　Ｔａｑバッファー（Ｔａｋａｒａ）、２００μＭのそれぞれのｄＮＴＰ（Ｔａｋａｒａ）、６００ｎＭのＡ＿プライマー（ＭＷＧ）、６００ｎＭのＢ＿ｄＴ２０ＶＮ＿プライマー（ＭＷＧ）及び０．０２５Ｕ／μｌのＥｘ　Ｔａｑポリメラーゼ（Ｔａｋａｒａ）から成っていた。第二ｃＤＮＡ鎖は、熱サイクラーにおいて９５℃で３分間、５０℃で２分間及び７２℃で３分間の１サイクルを実行することにより作製された。次に、試料は、９５℃で３０秒間、６７℃で１分間及び７２℃で３分間の２０サイクル（ライブラリー調製のため）又は３０サイクル（ｃＤＮＡの存在を確認するため）を実行することにより増幅され、続いて７２℃で１０分間最終伸長させた。

【０３３９】

ライブラリー精製

増幅後、４つのＰＣＲ（１００μｌ）は５００μｌの結合バッファー（Ｑｉａｇｅｎ）と混合され、Ｑｉａｑｕｉｃｋ　ＰＣＲ精製カラム（Ｑｉａｇｅｎ）に入れられ、増幅されたｃＤＮＡを膜に結合させるために１７９００×ｇで１分間回転させた。次に、膜はエタノールを含有する洗浄バッファー（Ｑｉａｇｅｎ）を用いて洗浄され、最後に５０μｌの１０ｍＭ　Ｔｒｉｓ－ＨＣｌ、ｐＨ８．５に溶出させた。

【０３４０】

精製され濃縮された試料は、ＭＢＳロボット（Ｍａｇｎｅｔｉｃ　Ｂｉｏｓｏｌｕｔｉｏｎｓ）を用いてＣＡ－精製（カルボン酸にコンジュゲートされた超常磁性ビーズによる精製）によりさらに精製され濃縮された。１５０～２００ｂｐ未満の断片を取り除くために最終ＰＥＧ濃度１０％が使用された。増幅されたｃＤＮＡは１０分間ＣＡビーズ（Ｉｎｖｉｔｒｏｇｅｎ）に結合させておき、次に１５μｌの１０ｍＭ　Ｔｒｉｓ－ＨＣｌ、ｐＨ８．５に溶出させた。

【０３４１】

二次ＰＣＲ増幅

最終ＰＣＲは、１×ＥＸ　Ｔａｑバッファー（Ｔａｋａｒａ）、２００μＭのそれぞれのｄＮＴＰ（Ｔａｋａｒａ）、６００ｎＭのＡ＿プライマー（ＭＷＧ）、６００ｎＭのＢ＿プライマー（ＭＷＧ）及び０．０２５Ｕ／μｌのＥｘ　Ｔａｑポリメラーゼ（Ｔａｋａｒａ）から成っていた。試料は３分間で９５℃まで加熱し、次に、９５℃で３０秒間、６５℃で１分間及び７２℃で３分間の１０サイクルを実行することにより増幅され、続いて７２℃で１０分間最終伸長させた。

【０３４２】

二次ライブラリー精製

増幅後、４つのＰＣＲ（１００μｌ）は５００μｌの結合バッファー（Ｑｉａｇｅｎ）と混合され、Ｑｉａｑｕｉｃｋ　ＰＣＲ精製カラム（Ｑｉａｇｅｎ）に入れられ、増幅されたｃＤＮＡを膜に結合させるために１７９００×ｇで１分間回転させた。次に、膜はエタノールを含有する洗浄バッファー（Ｑｉａｇｅｎ）を用いて洗浄され、最後に５０μｌの１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５に溶出させた。

【０３４３】

精製され濃縮された試料は、ＭＢＳロボット（Ｍａｇｎｅｔｉｃ　Ｂｉｏｓｏｌｕｔｉｏｎｓ）を用いてＣＡ－精製（カルボン酸にコンジュゲートされた超常磁性ビーズによる精製）によりさらに精製され濃縮された。１５０～２００ｂｐ未満の断片を取り除くために最終ＰＥＧ濃度１０％が使用された。増幅されたｃＤＮＡは１０分間ＣＡビーズ（Ｉｎｖｉｔｒｏｇｅｎ）に結合させておき、次に１５μｌの１０ｍＭ　Ｔｒｉｓ－ＨＣｌ、ｐＨ８．５に溶出させた。

【０３４４】

シーケンシングライブラリー調製

ライブラリーインデックス化

２０サイクル増幅された試料をさらに使用して、シーケンシングライブラリーを調製した。インデックスＰＣＲマスター混合物は試料ごとに調製され、２３μｌが６個の０．２ｍｌ管に入れられた。２μｌの増幅され精製されたｃＤＮＡが６つのＰＣＲのそれぞれに鋳型として添加され、１×Ｐｈｕｓｉｏｎマスター混合物（Ｆｅｒｍｅｎｔａｓ）、５００ｎＭのＩｎＰＥ１．０（Ｉｌｌｕｍｉｎａ）、５００ｎＭのＩｎｄｅｘ　１－１２（Ｉｌｌｕｍｉｎａ）及び０．４ｎＭのＩｎＰＥ２．０（Ｉｌｌｕｍｉｎａ）を含有するＰＣＲを作製した。試料は、熱サイクラーにおいて、９８℃で３０秒間、６５℃で３０秒間及び７２℃で１分間の１８サイクルで増幅され、続いて７２℃で５分間最終伸長させた。

【０３４５】

シーケンシングライブラリー精製

増幅後、試料はＭＢＳロボットを用いてＣＡ精製により精製され濃縮された。３００～３５０ｂｐ未満の断片を取り除くために最終ＰＥＧ濃度７．８％が使用された。増幅されたｃＤＮＡを１０分間、ＣＡビーズに結合させておき、次に、１５μｌの１０ｍＭ　Ｔｒｉｓ－ＨＣｌ、ｐＨ８．５に溶出させた。

【０３４６】

１０μｌの増幅され精製された試料は、Ｃａｌｉｐｅｒ　ＸＴチップ上に置かれ、４８０ｂｐから７２０ｂｐまでの断片はＣａｌｉｐｅｒ　ＸＴ（Ｃａｌｉｐｅｒ）で切り出された。試料は、完成ライブラリーの存在及びサイズを確認するためにＡｇｉｌｅｎｔバイオアナライザーを用いて解析され、ＤＮＡ高感度キットが使用された。

【０３４７】

シーケンシング及びデータ解析

シーケンシング及びバイオインフォマティックは、実施例８において説明された５’から３’配向高密度Ｎｉｍｂｌｅｇｅｎアレイについてのプロトコールと同じように実行された。しかし、データ解析では、転写物のマッピングでは読取り１は使用されなかった。特異的Ｏｌｆｒ転写物は、Ｍａｔｌａｂ視覚化ツールを使用して分類することができた（図２７）。

【０３４８】

実施例１０

プロテイナーゼＫ又はマイクロ波を通じた透過処理を用い、ＵＳＥＲシステム切断及びＴｄＴを介した増幅を用いる、５’から３’配向プローブとホルマリン固定凍結（ＦＦ－凍結）組織を有する自製プリントされた４１－タグマイクロアレイを使用する空間トランスクリプトーム

アレイ調製

前記の通りであるが、実施例８の５’から３’配向高密度アレイにおけるプローブと同じ組成を有する４１の独自のＩＤタグプローブのパターンで自製アレイはプリントされた

（図２８）。

【０３４９】

他のすべてのステップは、実施例８において説明されたプロトコールと同じように実行された。

【０３５０】

実施例１１

ｃＤＮＡ合成ステップを実施するための代替方法

上記のチップ上のｃＤＮＡ合成は、鋳型切替えプライマーをｃＤＮＡ合成反応に添加することにより第二鎖を作製する鋳型切替えと組み合わせることもできる（表２）。第二増幅ドメインは、逆転写酵素により第一ｃＤＮＡ鎖の３’端部にカップリングされる終端塩基に前記ドメインを結合させることにより導入され、第二鎖の合成をプライミングする。ライブラリーは、アレイ表面からの二本鎖複合体の放出の直後に容易に増幅させることができる。

【０３５１】

実施例１２

ＵＳＥＲシステム切断及びＴｄＴ－テーリング又は転座特異的プライマーを介した増幅を用いた、５’から３’配向プローブ及び断片化されたポリＡテールｇＤＮＡを有する自製プリントされた４１タグマイクロアレイを使用する空間ゲノミクス

アレイ調製

前記の通りであるが、実施例８の５’から３’配向高密度におけるプローブと同じ組成を有する４１の独自のＩＤタグプローブのパターンでＣｏｄｅｌｉｎｋスライド（Ｓｕｒｍｏｄｉｃｓ）を使用して自製アレイはプリントされた。

【０３５２】

細胞からの全ＤＮＡ調製

ＤＮＡ断片化

ゲノムＤＮＡ（ｇＤＮＡ）は、Ａ４３１及びＵ２ＯＳ株化細胞から、製造業者の使用説明書に従ってＤＮｅａｓｙキット（Ｑｉａｇｅｎ）により抽出された。ＤＮＡは製造業者の使用説明書に従ってＣｏｖａｒｉｓ超音波処理器（Ｃｏｖａｒｉｓ）上で５００ｂｐまで断片化された。

【０３５３】

試料は、ＭＢＳロボット（Ｍａｇｎｅｔｉｃ　Ｂｉｏｓｏｌｕｔｉｏｎｓ）を用いるＣＡ精製（カルボン酸にコンジュゲートされた超常磁性ビーズによる精製）により精製され濃縮された。１５０～２００ｂｐ未満の断片を取り除くために最終ＰＥＧ濃度１０％が使用された。断片化されたＤＮＡは１０分間ＣＡビース（Ｉｎｖｉｔｒｏｇｅｎ）に結合させておき、次に１５μｌの１０ｍＭ　Ｔｒｉｓ－ＨＣｌ、ｐＨ８．５に溶出させた。

【０３５４】

随意対照－異なる株化細胞のスパイク

Ａ４３１　ＤＮＡのＵ２ＯＳ　ＤＮＡへのスパイクを通じて、Ａ４３１　ＤＮＡの１％、１０％又は５０％のスパイクからなど、異なるレベルの捕捉感度を測定することができる。

【０３５５】

ターミナルトランスフェラーゼによるｄＡテーリング

４５μｌポリＡテーリング混合物（製造業者の使用説明書によれば、ＴｄＴバッファー（Ｔａｋａｒａ）、３ｍＭ　ｄＡＴＰ（Ｔａｋａｒａ）及びＴｄＴ酵素混合物（ＴｄＴ及びリボヌクレアーゼＨ）（Ｔａｋａｒａ）から成る）は、０．５μｇの断片化されたＤＮＡに添加された。混合物は熱サイクラーにおいて３７℃で３０分間インキュベートされ、続いてＴｄＴは８０℃で２０分間不活化された。次に、ｄＡテール断片は製造業者の使用説明書に従って、Ｑｉａｑｕｉｃｋ（Ｑｉａｇｅｎ）カラムを通じて浄化され、濃度は製造業者の使用説明書に従って、Ｑｕｂｉｔシステム（Ｉｎｖｉｔｒｏｇｅｎ）を使用して測定された。

【０３５６】
オンチップ実験
ハイブリダイゼーション、第二鎖合成及び切断反応は、１６ウェルシリコンガスケット（Ａｒｒａｙｉｔ、Ｓｕｎｎｙｖａｌｅ、ＣＡ、ＵＳＡ）のチップ上で実施された。蒸発を防ぐために、カセットはプレートシーラー（ｉｎ　Ｖｉｔｒｏ　ＡＢ、Ｓｔｏｃｋｈｏｌｍ、Ｓｗｅｄｅｎ）で覆われた。
【０３５７】
ハイブリダイゼーション
１１７ｎｇのＤＮＡは、１×ＮＥＢバッファー（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）及び１×ＢＳＡから成る総容積４５μｌ中で予め暖められたアレイ（５０℃）上のウェルに置かれた。混合物はＭＴＰブロックを備えたサーモミキサーコンフォート（Ｅｐｐｅｎｄｏｒｆ）において５０℃で３０分間、３００ｒｐｍ振盪でインキュベートされた。
【０３５８】
第二鎖合成
ハイブリダイゼーション混合物を取り除かずに、１×ＮＥＢバッファー、１．５μｌのクレノーポリメラーゼ及び３．７５μｌのｄＮＴＰ（それぞれ２ｍＭ）から成る１５μｌのクレノー伸長反応混合物がウェルに添加された。反応混合物はサーモミキサーコンフォート（Ｅｐｐｅｎｄｏｒｆ）において３７℃で３０分間、振盪せずにインキュベートされた。
【０３５９】
スライドはそれに続いて、２×ＳＳＣ、０．１％ＳＤＳ、５０℃、１０分間、３００ｒｐｍで、０．２×ＳＳＣ、１分間、３００ｒｐｍで、及び０．１×ＳＳＣ、１分間、３００ｒｐｍで洗浄された。
【０３６０】
プローブ放出
１．８ｍＭ　$MgCl_2$を有する１×ＦａｓｔＳｔａｒｔ高忠実度反応バッファー（Ｒｏｃｈｅ）、２００μＭ　ｄＮＴＰ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）、１×ＢＳＡ及び０．１Ｕ／１μｌ　ＵＳＥＲ酵素（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）を含有する容積５０μｌの混合物を３７℃まで加熱し、それぞれのウェルに添加し、混ぜながら（３００ｒｐｍで３秒間、静止して６秒間）（サーモミキサーコンフォート；Ｅｐｐｅｎｄｏｒｆ）３７℃で３０分間インキュベートした。次に、放出されたＤＮＡを含有する反応混合物は、ピペットを用いてウェルから回収された。
【０３６１】
ライブラリー調製
増幅反応
増幅は、７．５μｌの放出された試料、１μｌのそれぞれのプライマー及び０．５μｌの酵素から成る１０μｌの反応において実行された（Ｒｏｃｈｅ、ＦａｓｔＳｔａｒｔ　ＨｉＦｉ　ＰＣＲシステム）。反応は、９４℃で２分間、１サイクルの９４℃で１５秒間、５５℃で２分間、７２℃で２分間、３０サイクルの９４℃で１５秒間、６５℃で３０秒間、７２℃で９０秒間及び最終伸長７２℃で５分間として循環された。
【０３６２】
シーケンシングのためのライブラリーの調製では、２つのプライマーは、表面プローブＡハンドル及び特異的転座プライマー（Ａ４３１では）又はＢハンドルに連結された特異的ＳＮＰプライマーのいずれかから成っていた（表２）。
【０３６３】
ライブラリー精製
精製され濃縮された試料は、ＭＢＳロボット（Ｍａｇｎｅｔｉｃ　Ｂｉｏｓｏｌｕｔｉｏｎｓ）を用いてＣＡ－精製（カルボン酸にコンジュゲートされた超常磁性ビーズによる精製）によりさらに精製され濃縮された。１５０～２００ｂｐ未満の断片を取り除くため

に最終ＰＥＧ濃度１０％が使用された。増幅されたＤＮＡは１０分間ＣＡビーズ（Ｉｎｖｉｔｒｏｇｅｎ）に結合させておき、次に１５μｌの１０ｍＭ　Ｔｒｉｓ－ＨＣｌ、ｐＨ８．５に溶出させた。

【０３６４】

ライブラリー品質解析

試料は、増幅されたＤＮＡライブラリーの存在を確認するためにＡｇｉｌｅｎｔバイオアナライザー（Ａｇｉｌｅｎｔ）を用いて解析され、ＤＮＡ高感度キット又はＤＮＡ　１０００キットが、材料の量に応じて使用された。

【０３６５】

ライブラリーインデックス化

２０サイクル増幅された試料をさらに使用して、シーケンシングライブラリーを調製した。インデックスＰＣＲマスター混合物は試料ごとに調製され、２３μｌが６個の０．２ｍｌ管に入れられた。２μｌの増幅され精製されたｃＤＮＡが６つのＰＣＲのそれぞれに鋳型として添加され、１×Ｐｈｕｓｉｏｎマスター混合物（Ｆｅｒｍｅｎｔａｓ）、５００ｎＭのＩｎＰＥ１．０（Ｉｌｌｕｍｉｎａ）、５００ｎＭのＩｎｄｅｘ　１－１２（Ｉｌｌｕｍｉｎａ）及び０．４ｎＭのＩｎＰＥ２．０（Ｉｌｌｕｍｉｎａ）を含有するＰＣＲを作製した。試料は、熱サイクラーにおいて、９８℃で３０秒間、６５℃で３０秒間及び７２℃で１分間の１８サイクルで増幅され、続いて７２℃で５分間最終伸長させた。

【０３６６】

シーケンシングライブラリー精製

精製され濃縮された試料は、ＭＢＳロボットを用いてＣＡ－精製によりさらに精製され濃縮された。３００～３５０ｂｐ未満の断片を取り除くために最終ＰＥＧ濃度７．８％が使用された。増幅されたＤＮＡは１０分間ＣＡビーズに結合させておき、次に１５μｌの１０ｍＭ　Ｔｒｉｓ－Ｃｌ、ｐＨ８．５に溶出させた。試料は、完成されたライブラリーの存在及びサイズを確認するためにＡｇｉｌｅｎｔバイオアナライザーを用いて解析され、ＤＮＡ高感度キット又はＤＮＡ　１０００キットは製造業者の使用説明書に従って、材料の量に応じて使用された（図２９）。

【０３６７】

シーケンシング

シーケンシングは、実施例８において説明された５’から３’配向高密度Ｎｉｍｂｌｅｇｅｎアレイについてのプロトコールと同じように実行された。

【０３６８】

ＤＮＡ解析

ＤＮＡ解析は、整列配置されたＩＤ捕捉プローブの捕捉感度を決定するために実行された。読取り２は、転座又はＳＮＰプライマーのいずれかのその含有量に基づいて分類された。次に、これらの読取りは、読取り１に含有されるそのＩＤあたりで分類された。

【０３６９】

随意対照－株化細胞特異的転座の直接増幅

これは、ＰＣＲにより直接スパイクされた株化細胞の捕捉感度を測定するために使用された。Ａ４３１転座のためのフォワード及びリバースプライマー（表２）を使用して、第二鎖コピーされ放出された物質における転座を試みその存在を検出した（図３０）。

【０３７０】

【表２】

表2.空間トランスクリプトーム及び空間ゲノミクスのために使用されるオリゴ

実施例8

アレイ遊離3'端部アレイプローブを有するNimblegen 5'から3'アレイ

	5'から3'
プローブ1 (配列番号50)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCCGATATGATTGCCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ2 (配列番号51)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATGAGCCGGGTTCATCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ3 (配列番号52)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAGGCACTCTGTTGGGATTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ4 (配列番号53)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATGATTAGTCGCCATTCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ5 (配列番号54)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTACTTGAGGGTAGATGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ6 (配列番号55)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATGGCCAATACTGTTATCTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ7 (配列番号56)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCGCTACCCTGATTCGACCTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ8 (配列番号57)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCCACTTTCGCCGTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ9 (配列番号58)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCAACTTTGAGCAAGATTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ10 (配列番号59)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCAATTCGGAATTCCGGTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ11 (配列番号60)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGCCCAAGGTAATACATTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ12 (配列番号61)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGCATTTCCTATTCGAGTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ13 (配列番号62)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGCTAAATCTAACCGCCTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ14 (配列番号63)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAATTAAATTCTGATGGTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ15 (配列番号64)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCATTACATAGGTGCTAAGTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ16 (配列番号65)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATTGACTTGCGCTCGCACTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ17 (配列番号66)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATAGTATCTCCCAAGTTCTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ18 (配列番号67)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCGCCTGTAATCCGCATTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ19 (配列番号68)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGCCACTCTTTAGGTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ20 (配列番号69)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTATGCAAGTGATTGGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ21 (配列番号70)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAAGCCACGTTTATACGTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ22 (配列番号71)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTACTCCTGATTGCTGTATAACTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ23 (配列番号72)	UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGCGCATCTATCCTCTATTTTTTTTTTTTTTTTTTTTTTTTTTTVN

プローブ24 (配列番号73) UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCACGCGTAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN
プローブ25 (配列番号74) UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCGACTAAGTATGTAGCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN

枠プローブ
レイアウト1 (配列番号75) AAATTTCGTCTGCTATCGCGCTTCTGTACC

蛍光マーカープローブ
PS_1 (配列番号76) GGTACAGAAGCGCGATAGCAG - Cy3

第二鎖合成及び第一PCR増幅ハンドル
A_プライマー(配列番号77) ACACTCTTTCCCTACACGACGCTCTTCCGATCT
B_dt20VN_プライマー
(配列番号78) AGACGTGTGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTTVN

カスタムシークエンスプライマー
B_r2 (配列番号79) TCA GAC GTG TGC TCT TCC GAT CTT TTT TTT TTT TTT TTT TTT T

実施例9
遊離5'端部アレイプローブを有するNimblegen 3'から5'アレイ
5'から3'
プローブ1 (配列番号80) GCGTTCAGAGTGGCAGTCGAGATCACGCGGCAATCATATCGGACAGATCGGAAGAGCGTAGTGTAG
プローブ2 (配列番号81) GCGTTCAGAGTGGCAGTCGAGATCACAAGATGAACCCGGCTCATAGATCGGAAGAGCGTAGTGTAG
プローブ3 (配列番号82) GCGTTCAGAGTGGCAGTCGAGATCACTCCCAACAGAGTGCCTCAAGATCGGAAGAGCGTAGTGTAG
プローブ4 (配列番号83) GCGTTCAGAGTGGCAGTCGAGATCACCGAATGGCGACTAATCATAGATCGGAAGAGCGTAGTGTAG
プローブ5 (配列番号84) GCGTTCAGAGTGGCAGTCGAGATCACAAACATCTACCCTCAAGTAGATCGGAAGAGCGTAGTGTAG
プローブ6 (配列番号85) GCGTTCAGAGTGGCAGTCGAGATCACGATAACAGTATTGGCCATAGATCGGAAGAGCGTAGTGTAG
プローブ7 (配列番号86) GCGTTCAGAGTGGCAGTCGAGATCACGGTCGAATCAGGGTAGCGAGATCGGAAGAGCGTAGTGTAG
プローブ8 (配列番号87) GCGTTCAGAGTGGCAGTCGAGATCACACTACGGCGAAAGTGGGCAGATCGGAAGAGCGTAGTGTAG
プローブ9 (配列番号88) GCGTTCAGAGTGGCAGTCGAGATCACATCTTGCTCAAAGTTGCTAGATCGGAAGAGCGTAGTGTAG
プローブ10 (配列番号89) GCGTTCAGAGTGGCAGTCGAGATCACCCGGAATTCCGAATTGGCAGATCGGAAGAGCGTAGTGTAG
プローブ11 (配列番号90) GCGTTCAGAGTGGCAGTCGAGATCACATGTATTACCTTGGGCGAAGATCGGAAGAGCGTAGTGTAG
プローブ12 (配列番号91) GCGTTCAGAGTGGCAGTCGAGATCACCTCGAATAGGAAATGCGAAGATCGGAAGAGCGTAGTGTAG

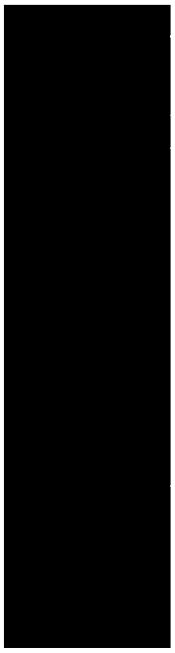
プローブ13 (配列番号92)	GCGTTCAGAGTGGCAGTCGAGATCACGGCGGTTAGATTTAGCAAAGATCGGAAGAGCGTAGTGTAG
プローブ14 (配列番号93)	GCGTTCAGAGTGGCAGTCGAGATCACCCATCAGAATTTAATTCCAGATCGGAAGAGCGTAGTGTAG
プローブ15 (配列番号94)	GCGTTCAGAGTGGCAGTCGAGATCACCTTAGCACCTATGTAATGAGATCGGAAGAGCGTAGTGTAG
プローブ16 (配列番号95)	GCGTTCAGAGTGGCAGTCGAGATCACGTGCGAGCGCAAGTCAATAGATCGGAAGAGCGTAGTGTAG
プローブ17 (配列番号96)	GCGTTCAGAGTGGCAGTCGAGATCACGAACTTGGGAGATACTATAGATCGGAAGAGCGTAGTGTAG
プローブ18 (配列番号97)	GCGTTCAGAGTGGCAGTCGAGATCACTGCGGATTACAGGCGCACAGATCGGAAGAGCGTAGTGTAG
プローブ19 (配列番号98)	GCGTTCAGAGTGGCAGTCGAGATCACCTACCTAAAGAGTGGCGCAGATCGGAAGAGCGTAGTGTAG
プローブ20 (配列番号99)	GCGTTCAGAGTGGCAGTCGAGATCACAAGCCAATCACTTGCATAAGATCGGAAGAGCGTAGTGTAG
プローブ21 (配列番号100)	GCGTTCAGAGTGGCAGTCGAGATCACCGTATAAACGTGGCTTGGAGATCGGAAGAGCGTAGTGTAG
プローブ22 (配列番号101)	GCGTTCAGAGTGGCAGTCGAGATCACGTTATACAGCAATCAGGTAGATCGGAAGAGCGTAGTGTAG
プローブ23 (配列番号102)	GCGTTCAGAGTGGCAGTCGAGATCACTAGAGGATAGATGCGCTGAGATCGGAAGAGCGTAGTGTAG
プローブ24 (配列番号103)	GCGTTCAGAGTGGCAGTCGAGATCACACTAGTCCTACGCGTGGAAGATCGGAAGAGCGTAGTGTAG
プローブ25 (配列番号104)	GCGTTCAGAGTGGCAGTCGAGATCACGCGCTACATACTTAGTCGAGATCGGAAGAGCGTAGTGTAG
枠プローブ	
レイアウト1 (配列番号105)	AAATTTCGTCTGCTATCGCGCTTCTGTACC
捕捉プローブ	
LP_ポリ-dTVN (配列番号106)	GTGATCTCGACTGCCACTCTGAATTTTTTTTTTTTTTTTTTTTTTVN
増幅ハンドルプローブ	
A-ハンドル(配列番号107)	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
第二鎖合成及び第一PCR増幅ハンドル	
A_プライマー(配列番号108)	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
B_dt20VN_プライマー (配列番号109)	AGACGTGTGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTVN
第二PCR	
A_プライマー(配列番号110)	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
B_プライマー(配列番号111)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

実施例11
鋳型切替え
鋳型切替え_長B
(配列番号112) GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATrGrGrG

実施例12
空間ゲノミクス
A_プライマー(配列番号113) ACACTCTTTCCCTACACGACGCTCTTCCGATCT
B_A431_Chr2+2_FW_A
(配列番号114) AGACGTGTGCTCTTCCGATCTTGGCTGCCTGAGGCAATG
B_A431_Chr2+2_RE_A
(配列番号115) AGACGTGTGCTCTTCCGATCTCTCGCTAACAAGCAGAGAGAAC
B_A431_Chr3+7_FW_B
(配列番号116) AGACGTGTGCTCTTCCGATCTTGAGAACAAGGGGGAAGAG
B_A431_Chr3+7_RE_B
(配列番号117) AGACGTGTGCTCTTCCGATCTCGGTGAAACAAGCAGGTAAC
B_NT_1_FW (配列番号118) AGACGTGTGCTCTTCCGATCTCATTCCCACACTCATCACAC
B_NT_1_RE (配列番号119) AGACGTGTGCTCTTCCGATCTTCACACTGGAGAAAGACCC
B_NT_2_FW (配列番号120) AGACGTGTGCTCTTCCGATCTGGGGTTCAGAGTGATTTTTCAG
B_NT_2_RE (配列番号121) AGACGTGTGCTCTTCCGATCTTCCGTTTTCTTTCAGTGCC

【図５】

Figure 5



【図８】

Figure 8



【図９】

Figure 9



【図１０】

Figure 10



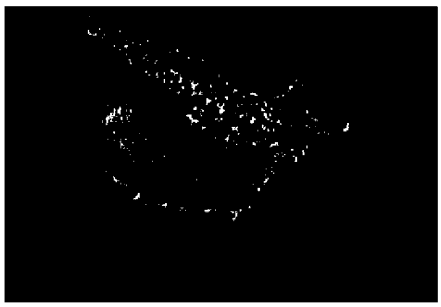
【図１２】

Figure 12



【図１１】

Figure 11



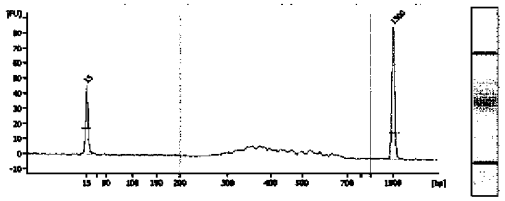
【図２３】

Figure 23



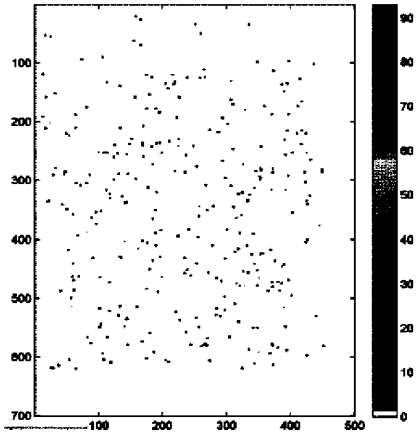
【図２５】

Figure 25



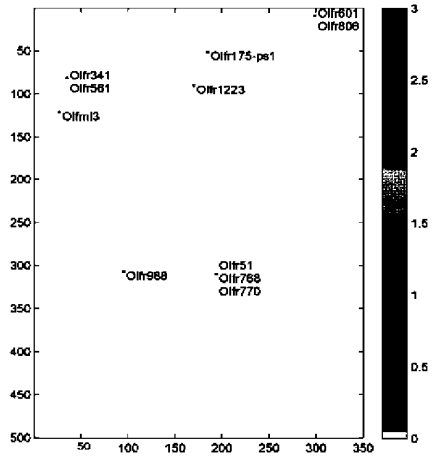
【図２６】

Figure 26



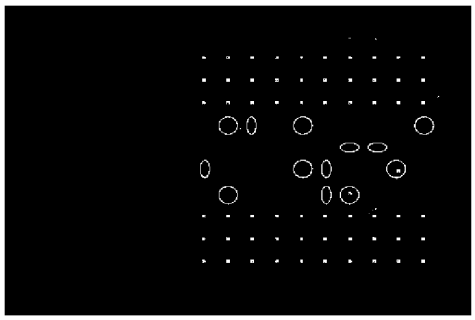
【図２７】

Figure 27

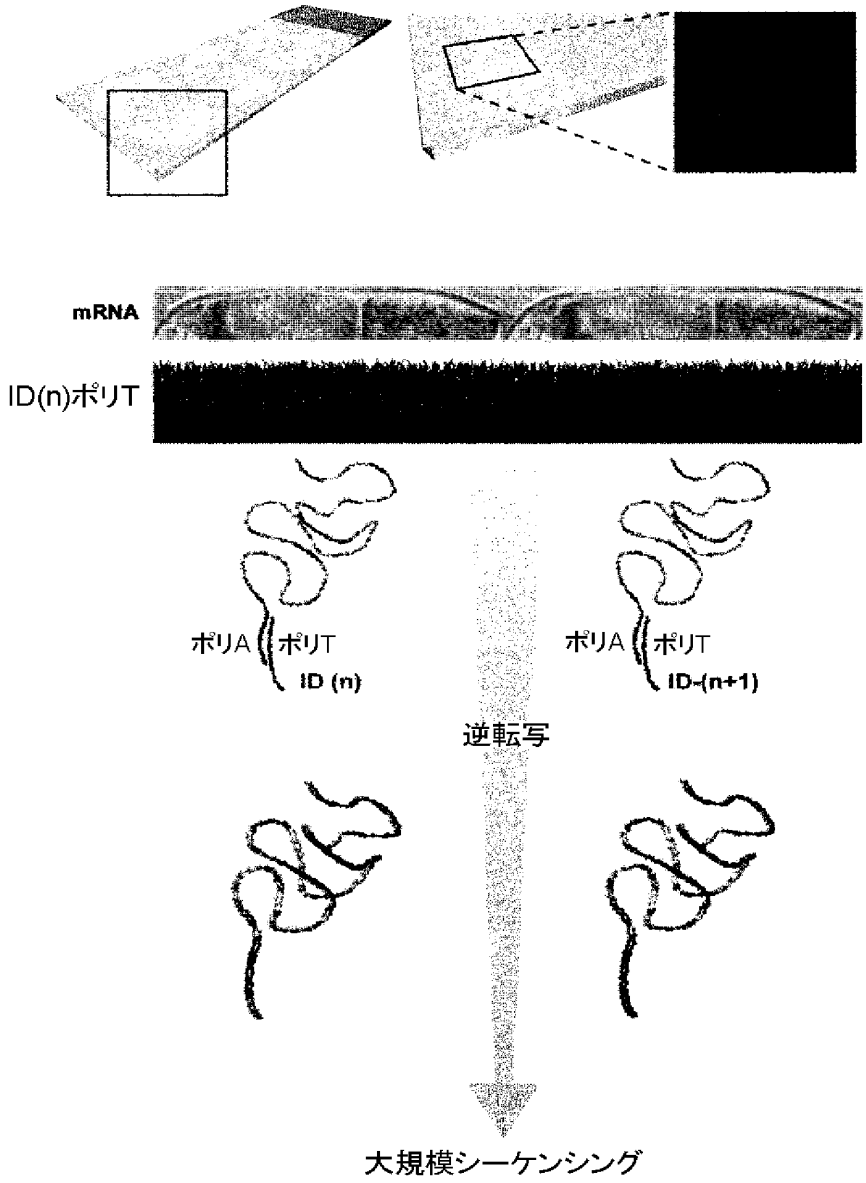


【図３１】

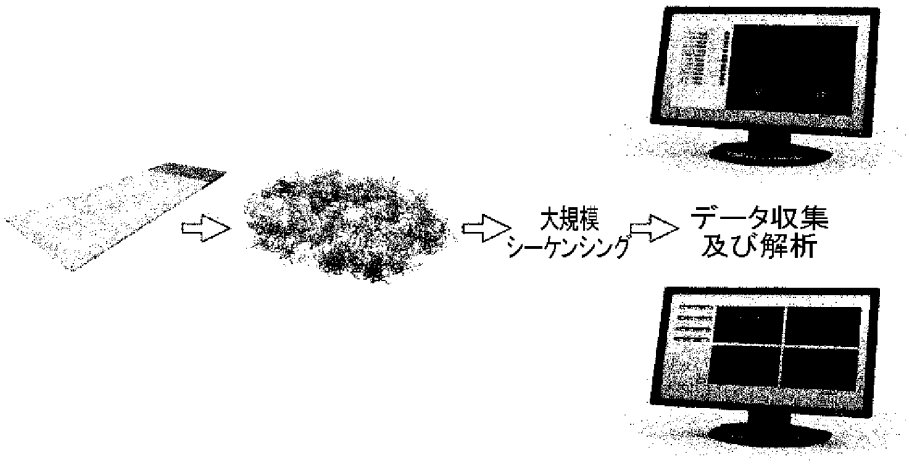
Figure 31



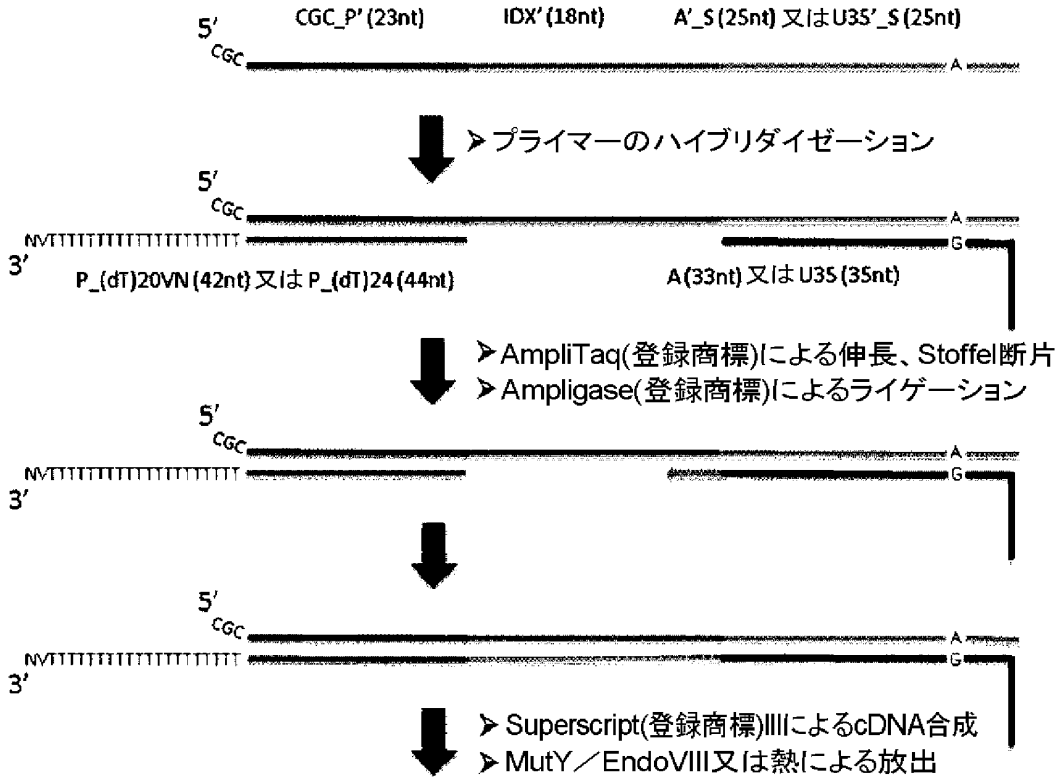
【図１】



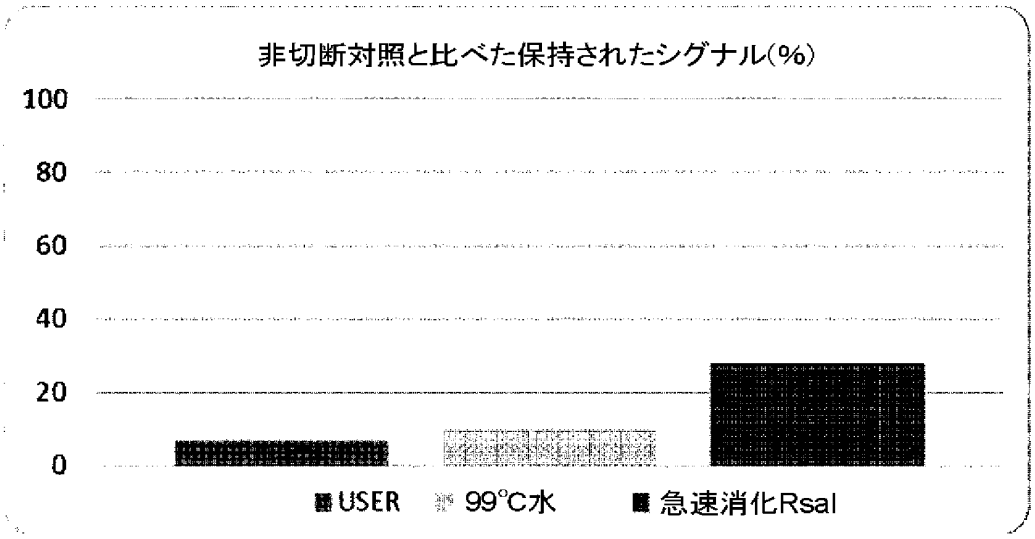
【図２】



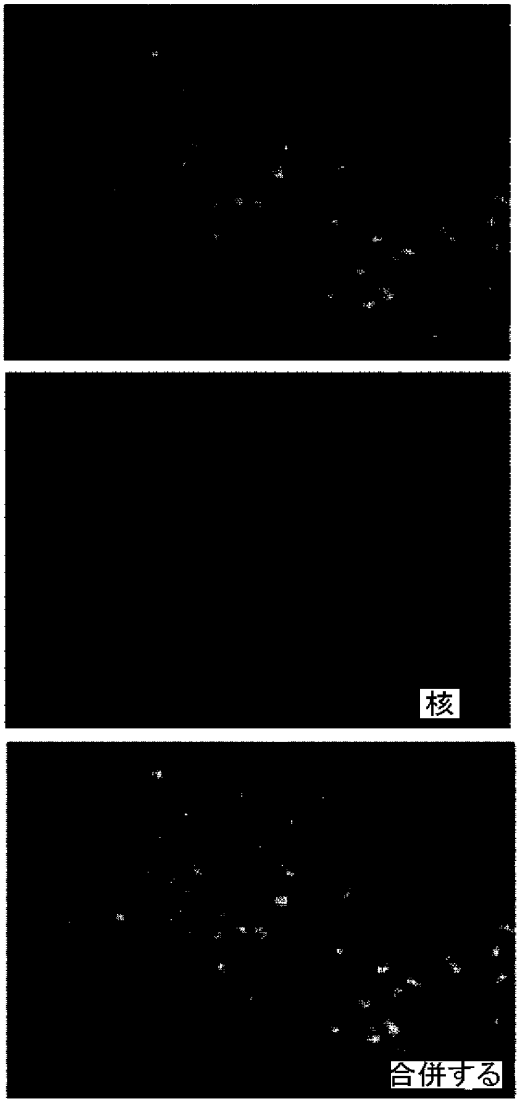
【図３】


5′
CGC_P′(23nt)
IDX′(18nt)
A′_S(25nt)又はU35′_S(25nt)
CGC
A
➢プライマーのハイブリダイゼーション
5′
CGC
A
NVTTTTTTTTTTTTTTTTTT
G
3′
P_(dT)20VN(42nt)又はP_(dT)24(44nt)
A(33nt)又はU35(35nt)
➢AmpliTaq(登録商標)による伸長、Stoffel断片
➢Ampligase(登録商標)によるライゲーション
5′
CGC
A
NVTTTTTTTTTTTTTTTTTT
G
3′
5′
CGC
A
NVTTTTTTTTTTTTTTTTTT
G
3′
➢Superscript(登録商標)IIIによるcDNA合成
➢MutY／EndoVIII又は熱による放出

【図４】

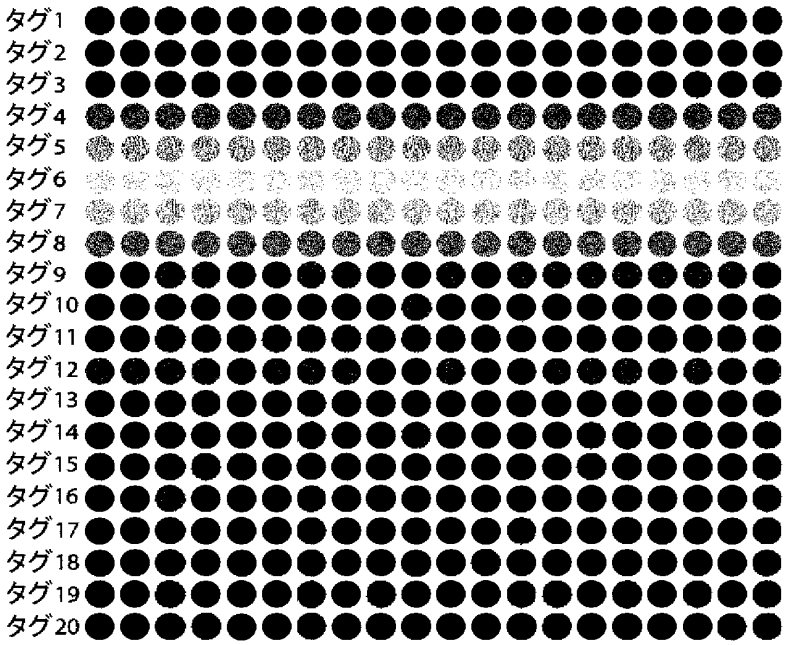


【図６】

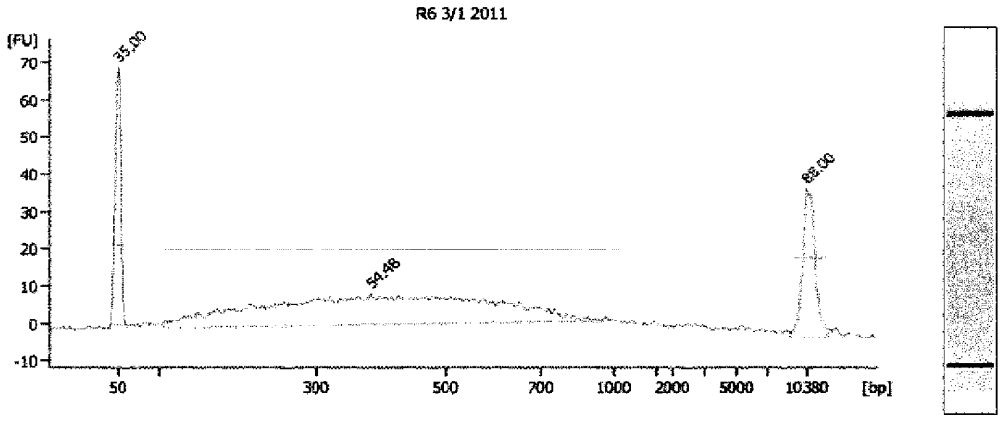


【図７】

レーン7blast結果	
E値	1,00E-06
バーコード	ヒット
タグ1	2165
タグ2	1223
タグ3	1338
タグ4	3605
タグ5	763
タグ6	1083
タグ7	1175
タグ8	1350
タグ9	616
タグ10	710
タグ11	1602
タグ12	1359
タグ13	1404
タグ14	1124
タグ15	1077
タグ16	1238
タグ17	1122
タグ18	1547
タグ19	1401
タグ20	1354



【図１３】



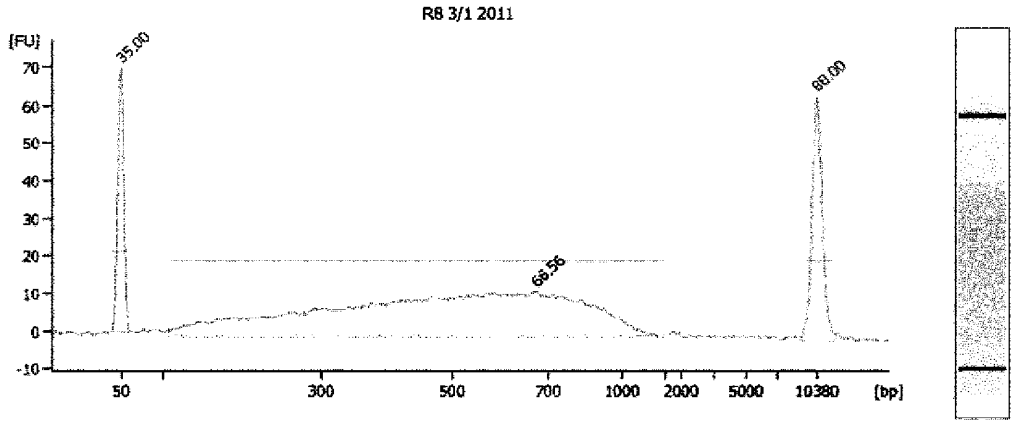
試料3の全結果 ： **R6 3/1 2011**

見出されたピークの数： 1

試料3のピーク表： **R6 3/1 2011**

ピーク		サイズ[bp]	濃度 [ng/µl]	モル濃度[nmol/l]	観測
1	◀	50	8.30	251.5	下マーカー
2		386	22.87	89.8	
3	▶	10,380	4.20	0.6	上マーカー

【図１４】



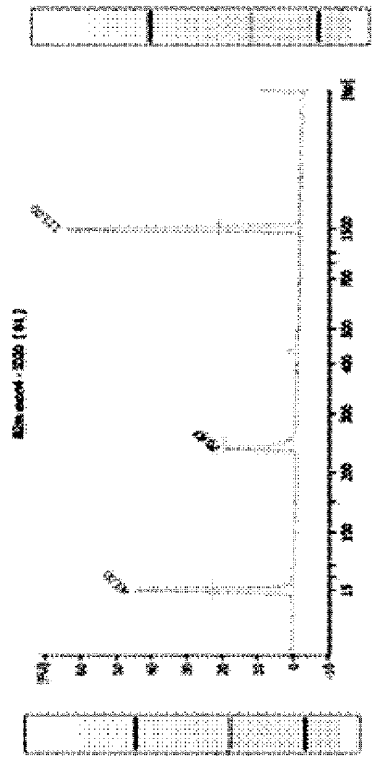
試料4の全結果 ： **R8 3/1 2011**

見出されたピークの数： 1

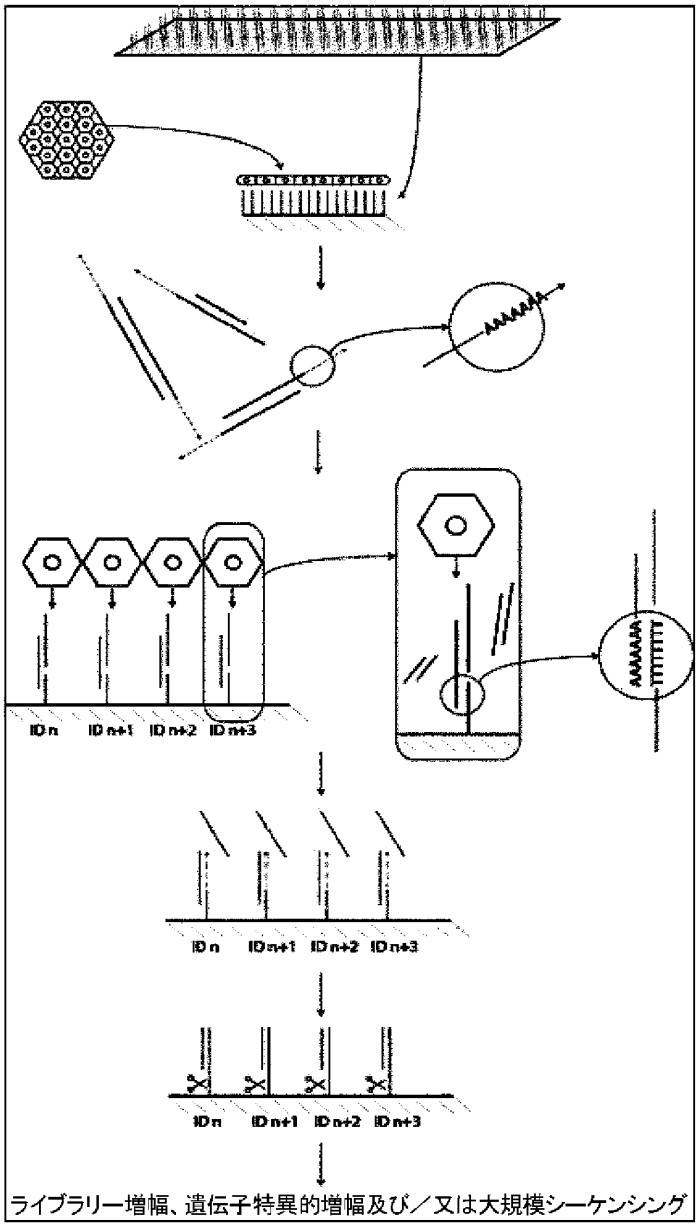
試料4のピーク表 ： **R8 3/1 2011**

ピーク		サイズ[bp]	濃度[ng/µl]	モル濃度[nmol/l]	観測
1	◀	50	8.30	251.5	下マーカー
2		676	21.53	48.2	
3	▶	10,380	4.20	0.6	上マーカー

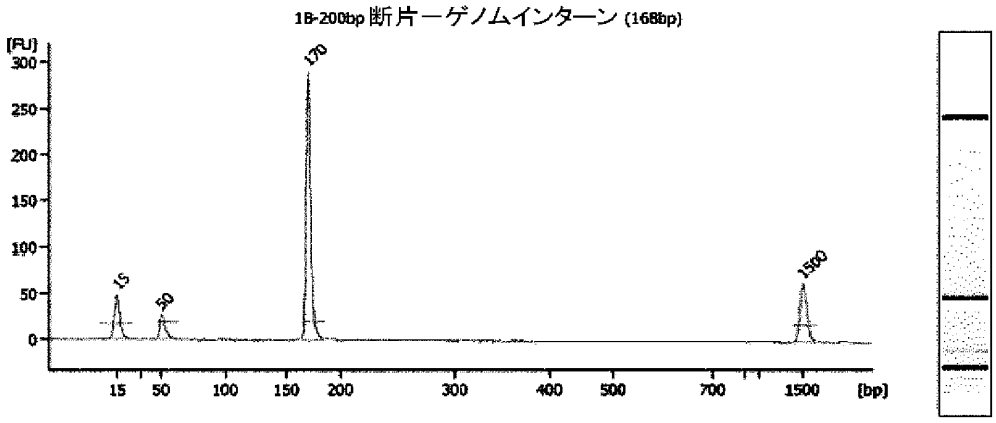
【図１５】



【図１６】


ID n
ID n+1
ID n+2
ID n+3
ID n
ID n+1
ID n+2
ID n+3
ID n
ID n+1
ID n+2
ID n+3
ライブラリー増幅、遺伝子特異的増幅及び／又は大規模シーケンシング

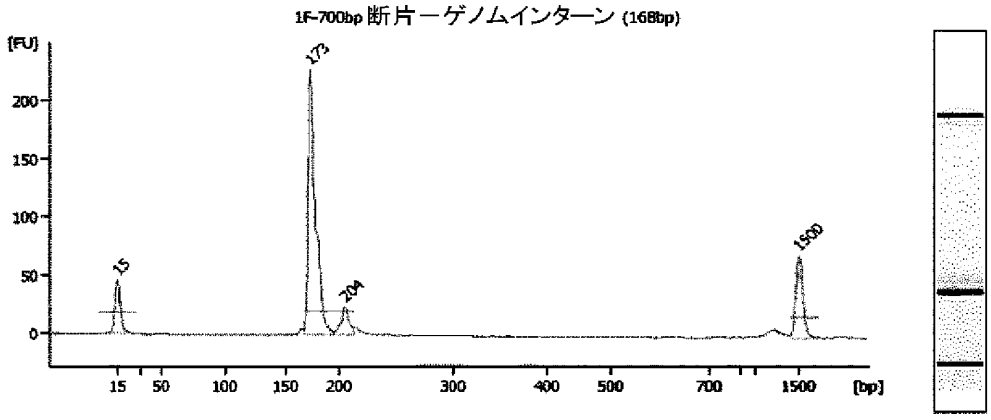
【図１７】



試料3の全結果 : 1B-200bp 断片ーゲノムインターン (168bp)

見出されたピークの数 2

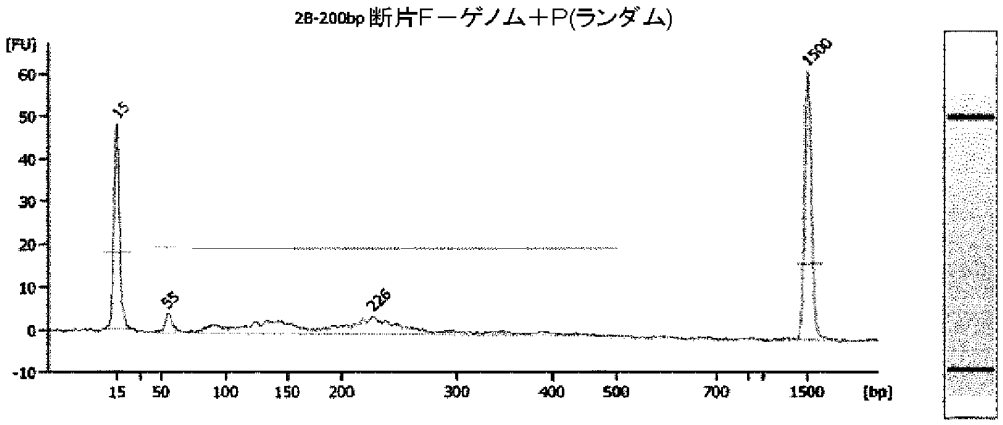
【図１８】



試料7の全結果 : 1F-700bp 断片－ゲノムインターン (168bp)

見出されたピークの数 2

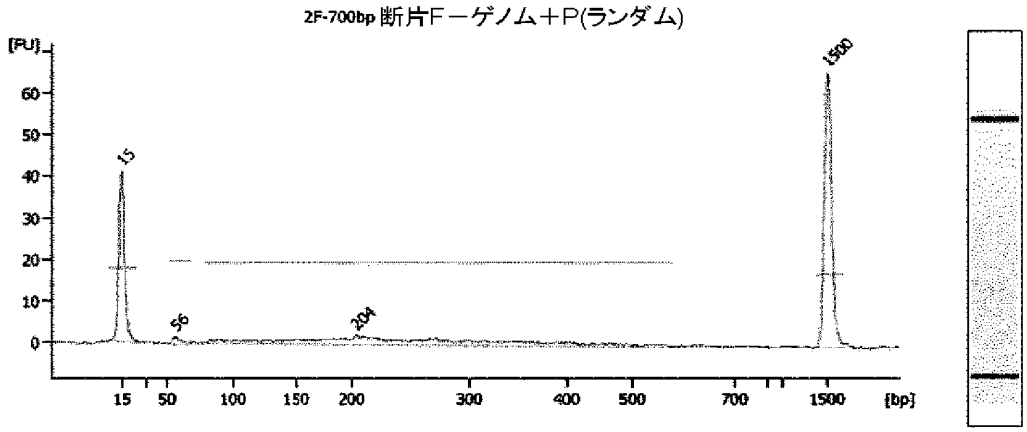
【図１９】



試料4の全結果 : 2B-200bp断片Ｆ－ゲノム＋P(ランダム)

見出されたピークの数 2

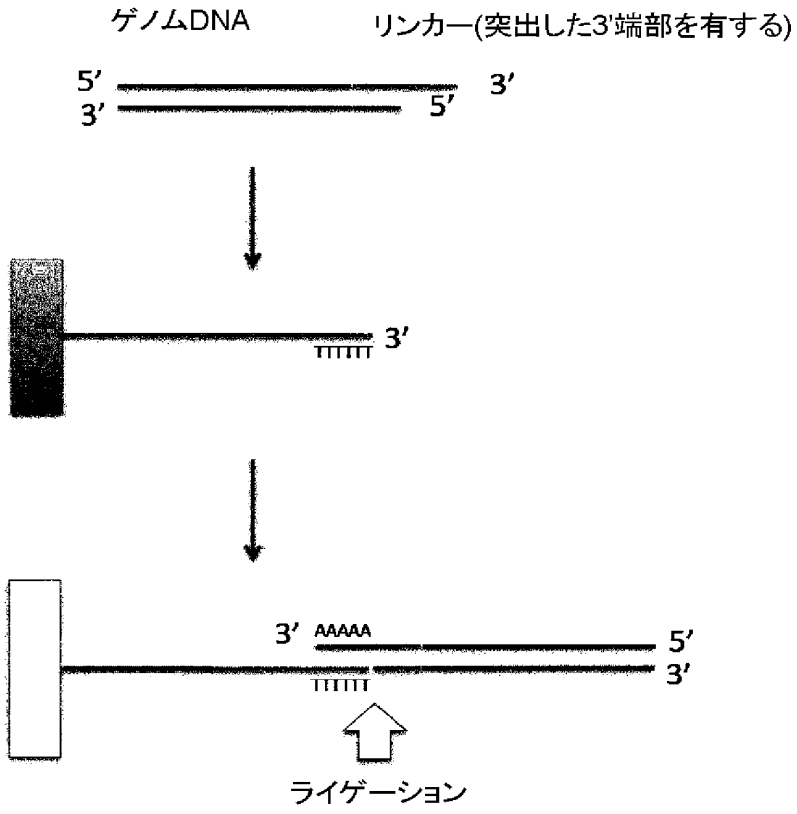
【図２０】



試料8の全結果 : 2F-700bp断片Ｆ－ゲノム＋P(ランダム)

見出されたピークの数 2

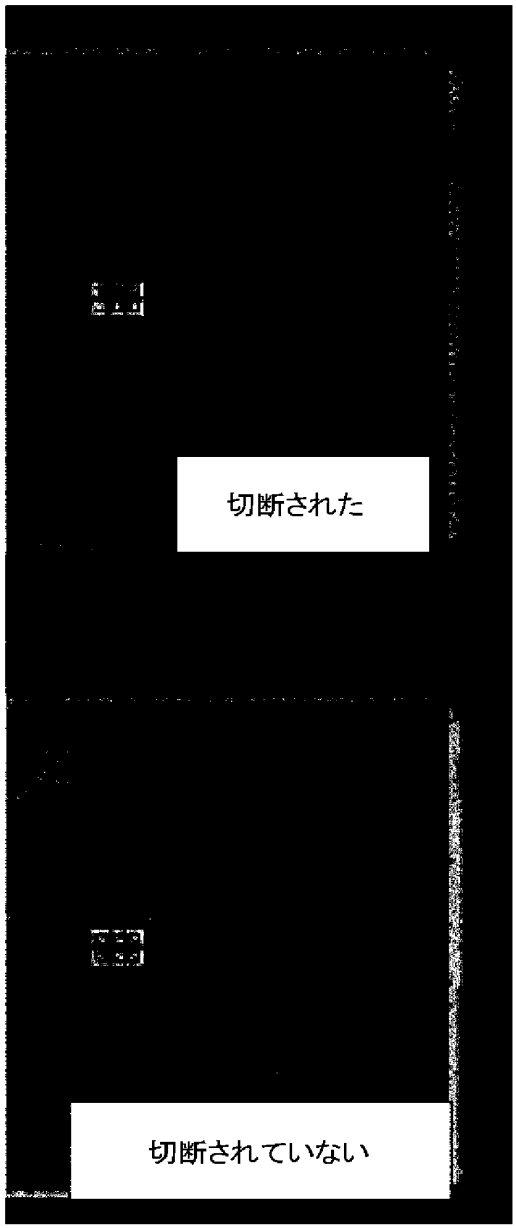
【図２１】



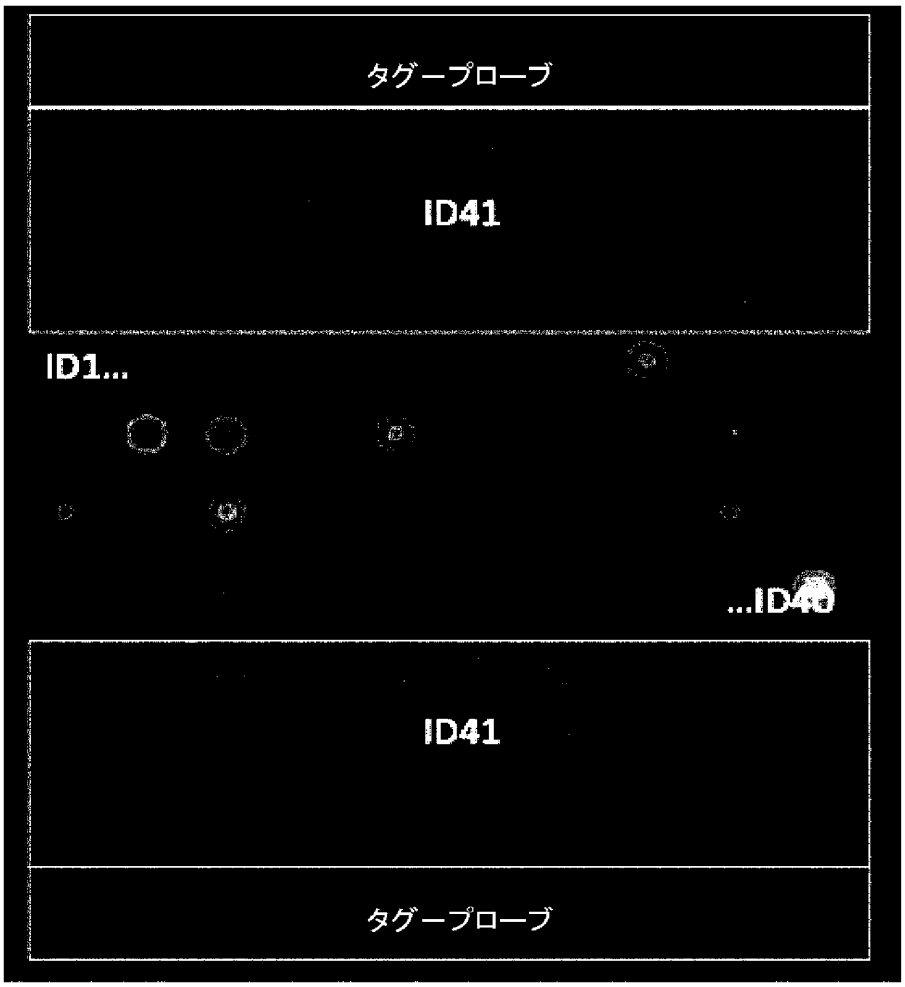
【図２２】



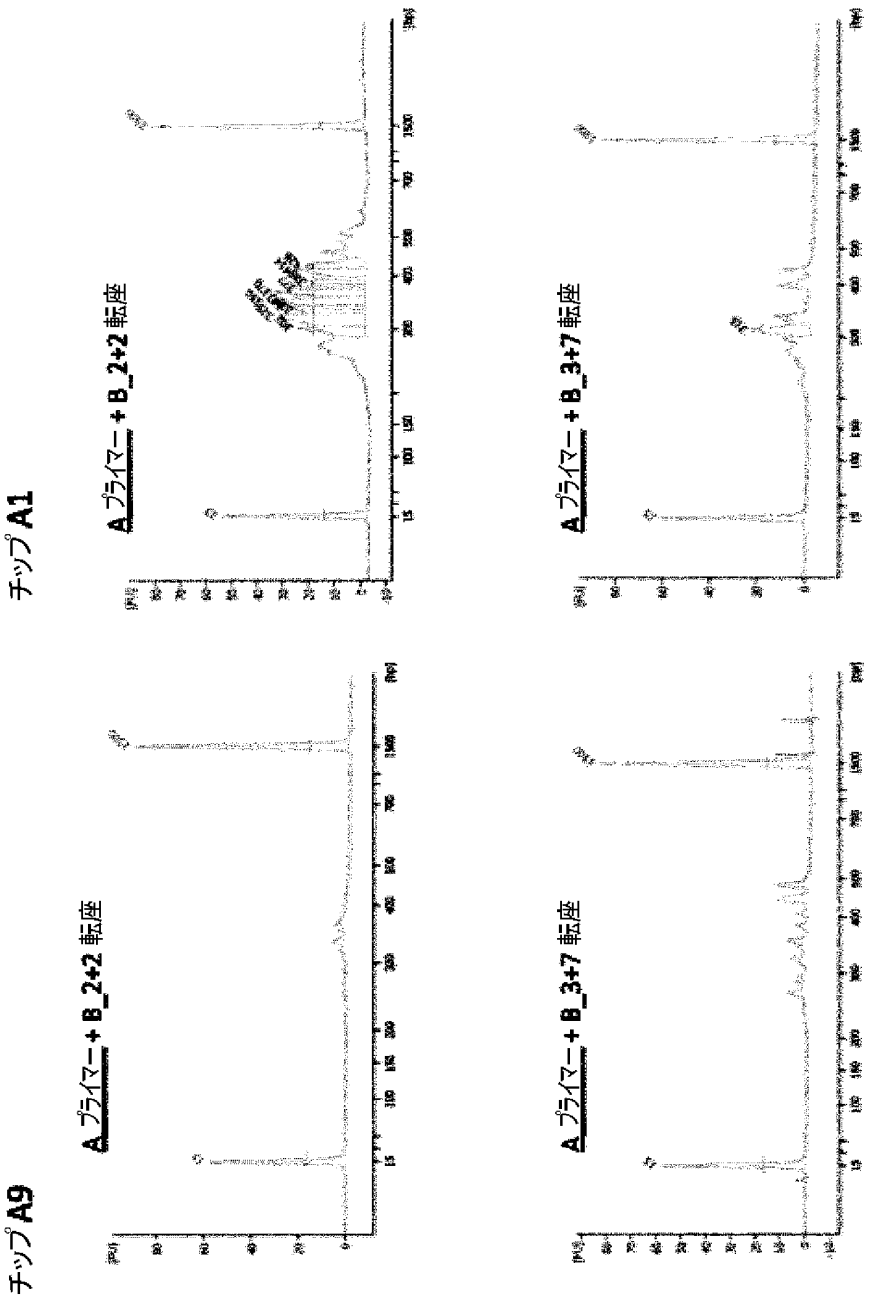
【図２４】



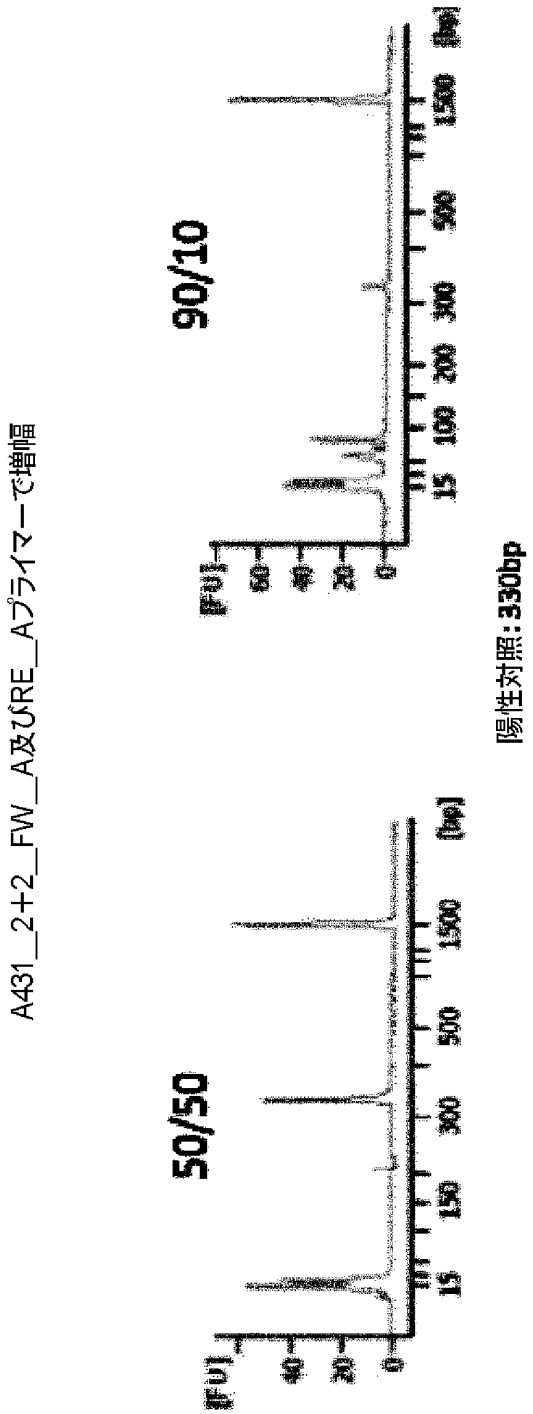
【図２８】



【図２９】


チップA1
Aプライマー＋B_2+2転座
Aプライマー＋B_3+7転座
チップA9
Aプライマー＋B_2+2転座
Aプライマー＋B_3+7転座

【図３０】



【配列表】
2014513523000001.app

【国際調査報告】

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056823

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"Human Genome U95Av2", INTERNET CITATION, 2 October 2002 (2002-10-02), XP002215481, Retrieved from the Internet: URL:http://www.affymetrix.com [retrieved on 2002-10-02] abstract -----	37-41
X	"GeneChip Human Genome U133 Set", INTERNET CITATION, 26 February 2003 (2003-02-26), XP002232760, Retrieved from the Internet: URL:http://www.affymetrix.com/support/technical/datasheets/hgu133_datasheet.pdf [retrieved on 2003-02-26] abstract ----- -/--	37-41

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 June 2012	12/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Barz, Wolfgang

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056823

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CONSTANTINE L ET AL: "Use of genechip high-density oligonucleotide arrays for gene expression monitoring", LIFE SCIENCE NEWS, AMERSHAM LIFE SCIENCE, US, 1 January 1998 (1998-01-01), pages 11-14, XP002964122, ISSN: 0969-0190 abstract	37-41
A	US 2006/211001 A1 (YU JINGWEI [US] ET AL) 21 September 2006 (2006-09-21) abstract; examples 1-3 claims 1,5-6,13-14	1-43
A	US 2003/040035 A1 (SLAMON DENNIS J [US] ET AL) 27 February 2003 (2003-02-27) abstract; figures 1-4 claims 1-19	1-43
A	WO 2007/030373 A2 (ST JUDE CHILDRENS RES HOSPITAL [US]; CURRAN THOMAS [US]; RICE DENNIS S) 15 March 2007 (2007-03-15) abstract; claims 1-7	1-43
A	EGUILUZ C ET AL: "Multitissue array review: A chronological description of tissue array techniques, applications and procedures", PATHOLOGY RESEARCH AND PRACTICE, GUSTAV FISCHER, STUTTGART, DE, vol. 202, no. 8, 10 August 2006 (2006-08-10), pages 561-568, XP028031005, ISSN: 0344-0338, DOI: 10.1016/J.PRP.2006.04.003 [retrieved on 2006-08-10] the whole document	1-43
A	SIEVERTZON MARIA ET AL: "Transcriptome analysis in primary neural stem cells using a tag cDNA amplification method", BMC NEUROSCIENCE, BIOMED CENTRAL, LONDON, GB, vol. 6, no. 1, 15 April 2005 (2005-04-15), page 28, XP021002918, ISSN: 1471-2202, DOI: 10.1186/1471-2202-6-28 the whole document	1-43

-/--

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056823

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WANG D ET AL: "Single cell analysis: the new frontier in 'omics'", TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 28, no. 6, 1 June 2010 (2010-06-01), pages 281-290, XP027059906, ISSN: 0167-7799 [retrieved on 2010-05-21] cited in the application the whole document	1-43
A	SCHENA M ET AL: "QUANTITATIVE MONITORING OF GENE EXPRESSION PATTERNS WITH A COMPLEMENTARY DNAMICROARRAY", SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, WASHINGTON, DC; US, vol. 270, no. 5235, 20 October 1995 (1995-10-20), pages 467-470, XP000644675, ISSN: 0036-8075, DOI: 10.1126/SCIENCE.270.5235.467 the whole document	1-43
A	WILLI-MONNERAT ET AL: "Comprehensive spatiotemporal transcriptomic analyses of the ganglionic eminences demonstrate the uniqueness of its caudal subdivision", MOLECULAR AND CELLULAR NEUROSCIENCES, SAN DIEGO, US, vol. 37, no. 4, 26 January 2008 (2008-01-26), pages 845-856, XP022576911, ISSN: 1044-7431 abstract; figures 1-3	1-43

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/056823

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2006211001 A1	21-09-2006	NONE	
US 2003040035 A1	27-02-2003	US 2003040035 A1	27-02-2003
		US 2004171101 A1	02-09-2004
WO 2007030373 A2	15-03-2007	NONE	

Form PCT/ISA/210 (patent family annex) (April 2005)

フロントページの続き

(51)Int.Cl.	F I		テーマコード（参考）
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	Ｇ０１Ｎ 33/566		

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(72)発明者 フライセン， ヨナス
スウェーデン， エスイー－１１４ ２６ ストックホルム， ブラゲヴァーゲン ５
(72)発明者 スタール， パトリック
スウェーデン， エスイー－１１８ ２６ ストックホルム， ティー３：１， ファットブルストラッパン １８
(72)発明者 ルンデベルグ， ヨアキム
スウェーデン， エスイー－１８１ ３４ リディンゴ， トルスヴィクスヴァン ２０

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【要約の続き】
らＤＮＡ分子を生成させるステップであって、伸長された又はライゲートされたＤＮＡ分子が位置ドメインによりタグ付けされる、ステップと、（ｄ）場合によってタグ付けされたＤＮＡの相補鎖を生成し、及び／又は場合によってタグ付けされたＤＮＡを増幅するステップと、（ｅ）タグ付けされたＤＮＡ分子及び／又はその相補体若しくはアンプリコンの少なくとも一部をアレイの表面から放出するステップであって、一部が位置ドメイン又はその相補体を含む、ステップと、（ｆ）放出されたＤＮＡ分子の配列を直接的に又は間接的に解析するステップと、を含む方法に関する。
【選択図】図１

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METHOD AND PRODUCT FOR LOCALISED OR SPATIAL DETECTION OF NUCLEIC ACID IN A TISSUE SAMPLE

Inventor(s): フライセン， ヨナス, ; スタール， パトリック, ; ルンデベルグ， ヨアキム

Applicant(s): スペイシャル トランスクリプトミクス アーベー SPATIAL TRANSCRIPTOMICS AB

Classification: - international: ***C12N15/09; C12Q1/68; G01N33/53; G01N33/566; G01N37/00***

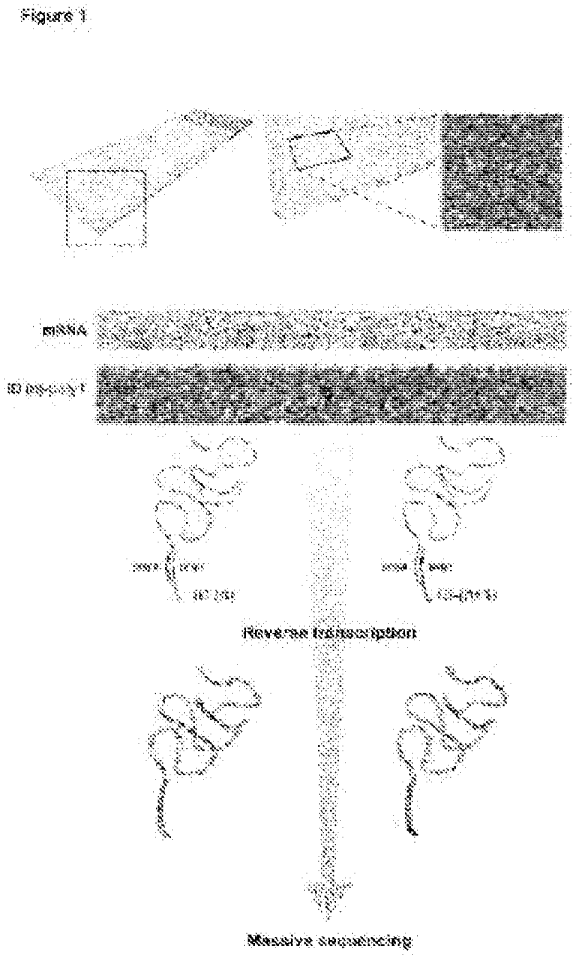
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The present invention relates to methods and products for the localised or spatial detection of nucleic acid in a tissue sample and in particular to a method for localised detection of nucleic acid in a tissue sample comprising: (a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3': (i) a positional domain that corresponds to the position of the capture probe on the array, and (ii) a capture domain; (b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing nucleic acid of the tissue sample to hybridise to the capture domain in said capture probes; (c) generating DNA molecules from the captured nucleic acid molecules using said capture probes as extension or ligation primers, wherein said extended or ligated

DNA molecules are tagged by virtue of the positional domain; (d) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying said tagged DNA; (e) releasing at least part of the tagged DNA molecules and/or their complements or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof; and (f) directly or indirectly analysing the sequence of the released DNA molecules.

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Description: JP2014513523 (A) — 2014-06-05

METHOD AND PRODUCT FOR LOCALISED OR SPATIAL DETECTION OF NUCLEIC ACID IN A TISSUE SAMPLE

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A high quality text as facsimile in your desired language may be available amongst the following family members:

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Method and product for localised or spatial detection of nucleic acid in a tissue sample

The present invention relates generally to the localised or spatial detection of nucleic acid in a tissue sample. The nucleic acid may be RNA or DNA. Thus, the present invention provides methods for detecting and/or analysing RNA, e.g. RNA transcripts or genomic DNA, so as to obtain spatial information about the localisation, distribution or expression of genes, or indeed about the localisation or distribution of any genomic variation (not necessarily in a gene) in a tissue sample, for example in an individual cell. The present invention thus enables spatial genomics and spatial transcriptomics.

More particularly, the present invention relates to a method for determining and/or analysing a transcriptome or genome and especially the global

transcriptome or genome, of a tissue sample. In particular the method relates to a quantitative and/or qualitative method for analysing the distribution, location or expression of genomic sequences in a tissue sample wherein the spatial expression or distribution or location pattern within the tissue sample is retained. Thus, the new method provides a process for performing "spatial transcriptomics" or "spatial genomics", which enables the user to determine simultaneously the expression pattern, or the location/distribution pattern of the genes

expressed or genes or genomic loci present in a tissue sample.

The invention is particularly based on array technology coupled with high throughput DNA sequencing technologies, which allows the nucleic acid molecule (e.g. RNA or DNA molecules) in the tissue sample, particularly mRNA or DNA, to be captured and labelled with a positional tag. This step is followed by synthesis of DNA molecules which are sequenced and analysed to determine which genes are expressed in any and all parts of the tissue sample. Advantageously, the individual, separate and specific transcriptome of each cell in the tissue sample may be obtained at the same time. Hence, the methods of the invention may be said to provide highly parallel comprehensive transcriptome signatures from individual cells within a tissue sample without losing spatial information within said investigated tissue sample. The invention also provides an array for performing the method of the invention and methods for making the arrays of the invention.

The human body comprises over 100 trillion cells and is organized into more than 250 different organs and tissues. The development and organization of complex organs, such as the brain, are far from understood and there is a need to dissect the expression of genes expressed in such tissues using quantitative methods to investigate and determine the genes that control the development and function of such tissues. The organs are in themselves a mixture of differentiated cells that enable all bodily functions, such as nutrient transport, defence etc. to be coordinated and maintained. Consequently, cell function is dependent on the position of the cell within a particular tissue structure and the interactions it shares with other cells within that tissue, both directly and indirectly. Hence, there is a need to disentangle how these interactions influence each cell within a tissue at the transcriptional level.

Recent findings by deep RNA sequencing have demonstrated that a majority of the transcripts can be detected in a human cell line and that a large fraction (75%) of the human protein-coding genes are expressed in most tissues. Similarly, a detailed study of 1 % of the human genome showed that chromosomes are ubiquitously transcribed and that the majority of all bases are included in primary transcripts. The transcription machinery can therefore be described as promiscuous at a global level.

It is well-known that transcripts are merely a proxy for protein abundance, because the rates of RNA translation, degradation etc will influence the amount of protein produced from any one transcript. In this respect, a recent antibody-based analysis of human organs and tissues suggests that tissue specificity is

achieved by precise regulation of protein levels in space and time, and that different tissues in the body acquire their unique characteristics by controlling not which proteins are expressed but how much of each is produced.

However, in subsequent global studies transcriptome and proteome correlations have been compared demonstrating that the majority of all genes were shown to be expressed. Interestingly, there was shown to be a high correlation between changes in RNA and protein levels for individual gene products which is indicative of the biological usefulness of studying the transcriptome in individual cells in the context of the functional role of proteins.

Indeed, analysis of the histology and expression pattern in tissues is a cornerstone in biomedical research and diagnostics. Histology, utilizing different staining techniques, first established the basic structural organization of healthy organs and the changes that take place in common pathologies more than a century ago. Developments in this field resulted in the possibility of studying protein distribution by immunohistochemistry and gene expression by in situ hybridization.

However, the parallel development of increasingly advanced histological and gene expression techniques has resulted in the separation of imaging and transcriptome analysis and, until the methods of the present invention, there has not been any feasible method available for global transcriptome analysis with spatial resolution.

As an alternative, or in addition, to in situ techniques, methods have developed for the in vitro analysis of proteins and nucleic acids, i.e. by extracting molecules from whole tissue samples, single cell types, or even single cells, and quantifying specific molecules in said extracts, e.g. by ELISA, qPCR etc.

Recent developments in the analysis of gene expression have resulted in the possibility of assessing the complete transcriptome of tissues using microarrays or RNA sequencing, and such developments have been instrumental in our understanding of biological processes and for diagnostics. However, transcriptome analysis typically is performed on mRNA extracted from whole tissues (or even whole organisms), and methods for collecting smaller tissue areas or individual cells for transcriptome analysis are typically labour intensive, costly and have low precision.

Hence, the majority of gene expression studies based on microarrays or next generation sequencing of RNA use a representative sample containing many cells. Thus the results represent the average expression

levels of the investigated genes. The separation of cells that are phenotypically different has been used in some cases together with the global gene expression platforms (Tang F et al, Nat Protoc. 2010; 5: 516-35; Wang D & Bodovitz S, Trends Biotechnol. 2010; 28:281-90) and resulted in very precise information about cell-to-cell variations. However, high throughput methods to study transcriptional activity with high resolution in intact tissues have not, until now, been available.

Thus, existing techniques for the analysis of gene expression patterns provide spatial transcriptional information only for one or a handful of genes at a time or offer transcriptional information for all of the genes in a sample at the cost of losing positional information. Hence, it is evident that methods to determine simultaneously, separately and specifically the transcriptome of each cell in a sample are required, i.e. to enable global gene expression analysis in tissue samples that yields transcriptomic information with spatial resolution, and the present invention addresses this need.

The novel approach of the methods and products of the present invention utilizes now well established array and sequencing technology to yield

transcriptional information for all of the genes in a sample, whilst retaining the positional information for each transcript. It will be evident to the person of skill in the art that this represents a milestone in the life sciences. The new technology opens a new field of so-called "spatial transcriptomics", which is likely to have profound consequences for our understanding of tissue development and tissue and cellular function in all multicellular organisms. It will be apparent that such techniques will be particularly useful in our understanding of the cause and progress of disease states and in developing effective treatments for such diseases, e.g. cancer. The methods of the invention will also find uses in the diagnosis of numerous medical conditions.

Whilst initially conceived with the aim of transcriptome analysis in mind, as described in detail below, the principles and methods of the present invention may be applied also to the analysis of DNA and hence for genomic analyses also ("spatial genomics"). Accordingly, at its broadest the invention pertains to the detection and/or analysis of nucleic acid in general.

Array technology, particularly microarrays, arose from research at Stanford

University where small amounts of DNA oligonucleotides were successfully attached to a glass surface in an ordered arrangement, a so-called "array",

and used it to monitor the transcription of 45 genes (Schena M et al, Science. 1995; 270: 368-9, 371).

Since then, researchers around the world have published more than 30,000 papers using microarray technology. Multiple types of microarray have been developed for various applications, e.g. to detect single nucleotide polymorphisms (SNPs) or to genotype or re-sequence mutant genomes, and an important use of microarray technology has been for the investigation of gene expression. Indeed, the gene expression microarray was created as a means to analyze the level of expressed genetic material in a particular sample, with the real gain being the possibility to compare expression levels of many genes simultaneously. Several commercial microarray platforms are available for these types of experiments but it has also been possible to create custom made gene expression arrays. Whilst the use of microarrays in gene expression studies is now

commonplace, it is evident that new and more comprehensive so-called "next- generation DNA sequencing" (NGS) technologies are starting to replace DNA microarrays for many applications, e.g. in-depth transcriptome analysis.

The development of NGS technologies for ultra-fast genome sequencing represents a milestone in the life sciences (Petterson E et al, Genomics. 2009; 93: 105-1 1). These new technologies have dramatically decreased the cost of DNA sequencing and enabled the determination of the genome of higher organisms at an unprecedented rate, including those of specific individuals (Wade CM et al Science. 2009; 326: 865-7; Rubin J et al, Nature 2010; 464: 587-91). The new advances in high-throughput genomics have reshaped the biological research landscape and in addition to complete characterization of genomes it is possible also to study the full transcriptome in a digital and quantitative fashion. The bioinformatics tools to visualize and integrate these comprehensive sets of data have also been significantly improved during recent years.

However, it has surprisingly been found that a unique combination of histological, microarray and NGS techniques can yield comprehensive

transcriptional or genomic information from multiple cells in a tissue sample which information is characterised by a two-dimensional spatial resolution. Thus, at one extreme the methods of the present invention can be used to analyse the expression of a single gene in a single cell in a sample, whilst retaining the cell within its context in the tissue sample. At the other extreme, and in a preferred aspect of the invention, the methods can be used to determine the expression of every gene in

each and every cell, or substantially all cells, in a sample

simultaneously, i.e. the global spatial expression pattern of a tissue sample. It will be apparent that the methods of the invention also enable intermediate analyses to be performed.

In its simplest form, the invention may be illustrated by the following summary. The invention requires reverse transcription (RT) primers, which comprise also unique positional tags (domains), to be arrayed on an object substrate, e.g. a glass slide, to generate an "array". The unique positional tags correspond to the location of the RT primers on the array (the features of the array). Thin tissue sections are placed onto the array and a reverse transcription reaction is performed in the tissue section on the object slide. The RT primers, to which the RNA in the tissue sample binds (or hybridizes), are extended using the bound RNA as a template to obtain cDNA, which is therefore bound to the surface of the array. As consequence of the unique positional tags in the RT primers, each cDNA strand carries information about the position of the template RNA in the tissue section. The tissue section may be visualised or imaged, e.g. stained and photographed, before or after the cDNA synthesis step to enable the positional tag in the cDNA molecule to be correlated with a position within the tissue sample. The cDNA is sequenced, which results in a transcriptome with exact positional information. A schematic of the process is shown in Figure 1. The sequence data can then be matched to a position in the tissue sample, which enables the visualization, e.g. using a computer, of the sequence data together with the tissue section, for instance to display the expression pattern of any gene of interest across the tissue (Figure 2). Similarly, it would be possible to mark different areas of the tissue section on the computer screen and obtain information on differentially expressed genes between any selected areas of interest. It will be evident that the methods of the invention result in data that is in stark contrast to the data obtained using current methods to study mRNA populations. For example, methods based on in situ hybridization provide only relative information of single mRNA transcripts. Thus, the methods of the present invention have clear advantages over current in situ technologies. The global gene expression information obtainable from the methods of the invention also allows co-expression information and quantitative estimates of transcript abundance. It will be evident that this is a generally applicable strategy available for the analysis of any tissue in any species, e.g. animal, plant, fungus.

As noted above, and described in more detail below, it will be evident that this basic methodology could readily be extended to the analysis of genomic DNA, e.g. to

identify cells within a tissue sample that comprise one or more specific mutations. For instance, the genomic DNA may be fragmented and allowed to hybridise to primers (equivalent to the RT primers described above), which are capable of capturing the fragmented DNA (e.g. an adapter with a sequence that is complementary to the primer may be ligated to the fragmented DNA or the fragmented DNA may be extended e.g. using an enzyme to incorporate additional nucleotides at the end of the sequence, e.g. a poly-A tail, to generate a sequence that is complementary to the primer) and priming the synthesis of complementary strands to the capture molecules. The remaining steps of the analysis may be as described above. Hence, the specific embodiments of the invention described below in the context of transcriptome analysis may also be employed in methods of analysing genomic DNA, where appropriate.

It will be seen from the above explanation that there is an immense value in coupling positional information to transcriptome or genome information. For instance, it enables global gene expression mapping at high resolution, which will find utility in numerous applications, including e.g. cancer research and diagnostics.

Furthermore, it is evident that the methods described herein differ significantly from the previously described methods for analysis of the global transcriptome of a tissue sample and these differences result in numerous advantages. The present invention is predicated on the surprising discovery that the use of tissue sections does not interfere with synthesis of DNA (e.g. cDNA) primed by primers (e.g. reverse transcription primers) that are coupled to the surface of an array.

Thus, in its first and broadest aspect, the present invention provides a method for localised detection of nucleic acid in a tissue sample comprising:

(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be

correlated with a position in the tissue sample and allowing nucleic acid of the tissue sample to hybridise to the capture domain in said capture probes;

(c) generating DNA molecules from the captured nucleic acid molecules using said capture probes as extension or ligation primers, wherein said extended or ligated DNA molecules are tagged by virtue of the positional domain;

(d) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying said tagged DNA; (e) releasing at least part of the tagged DNA molecules and/or their complements or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof ;

(f) directly or indirectly analysing the sequence of the released DNA molecules.

The methods of the invention represent a significant advance over other methods for spatial transcriptomics known in the art. For example the methods described herein result in a global and spatial profile of all transcripts in the tissue sample. Moreover, the expression of every gene can be quantified for each position or feature on the array, which enables a multiplicity of analyses to be performed based on data from a single assay. Thus, the methods of the present invention make it possible to detect and/or quantify the spatial expression of all genes in single tissue sample. Moreover, as the abundance of the transcripts is not visualised directly, e.g. by fluorescence, akin to a standard microarray, it is possible to measure the expression of genes in a single sample simultaneously even wherein said transcripts are present at vastly different concentrations in the same sample.

Accordingly, in a second and more particular aspect, the present invention can be seen to provide a method for determining and/or analysing a transcriptome of a tissue sample comprising:

(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a reverse transcriptase (RT) primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing RNA of the tissue sample to hybridise to the capture domain in said capture probes;

(c) generating cDNA molecules from the captured RNA molecules using said capture probes as RT primers, and optionally amplifying said cDNA molecules; (d) releasing at least part of the cDNA molecules and/or optionally their amplicons from the surface of the array, wherein said released molecule may be a first strand and/or second strand cDNA molecule or an amplicon thereof and wherein said part includes the positional domain or a complement thereof;

(e) directly or indirectly analysing the sequence of the released molecules.

As described in more detail below, any method of nucleic acid analysis may be used in the analysis step. Typically this may involve sequencing, but it is not necessary to perform an actual sequence determination. For example sequence- specific methods of analysis may be used. For example a sequence-specific amplification reaction may be performed, for example using primers which are specific for the positional domain and/or for a specific target sequence, e.g. a particular target DNA to be detected (i.e. corresponding to a particular cDNA/RNA or gene etc.). An exemplary analysis method is a sequence-specific PCR reaction.

The sequence analysis information obtained in step (e) may be used to obtain spatial information as to the RNA in the sample. In other words the sequence analysis information may provide information as to the location of the RNA in the sample. This spatial information may be derived from the nature of the sequence analysis information determined, for example it may reveal the presence of a particular RNA which may itself be spatially informative in the context of the tissue sample used, and/or the spatial information (e.g. spatial localisation) may be derived from the position of the tissue sample on the array, coupled with the sequencing information. Thus, the method may involve simply correlating the sequence analysis information to a position in the tissue sample e.g. by virtue of the positional tag and its correlation to a position in the tissue sample. However, as described above, spatial information may conveniently be obtained by correlating the sequence analysis data to an image of the tissue sample and this represents one preferred embodiment of the invention. Accordingly, in a preferred embodiment the method also includes a step of:

(f) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue

sample is imaged before or after step (c).

In its broadest sense, the method of the invention may be used for localised detection of a nucleic acid in a tissue sample. Thus, in one embodiment, the method of the invention may be used for determining and/or analysing all of the transcriptome or genome of a tissue sample e.g. the global transcriptome of a tissue sample. However, the method is not limited to this and encompasses determining and/or analysing all or part of the transcriptome or genome. Thus, the method may involve determining and/or analysing a part or subset of the

transcriptome or genome, e.g. a transcriptome corresponding to a subset of genes, e.g. a set of particular genes, for example related to a particular disease or condition, tissue type etc.

Viewed from another aspect, the method steps set out above can be seen as providing a method of obtaining a spatially defined transcriptome or genome, and in particular the spatially defined global transcriptome or genome, of a tissue sample.

Alternatively viewed, the method of the invention may be seen as a method for localised or spatial detection of nucleic acid, whether DNA or RNA in a tissue sample, or for localised or spatial determination and/or analysis of nucleic acid (DNA or RNA) in a tissue sample. In particular, the method may be used for the localised or spatial detection or determination and/or analysis of gene expression or genomic variation in a tissue sample. The localised/spatial

detection/determination/analysis means that the RNA or DNA may be localised to its native position or location within a cell or tissue in the tissue sample. Thus for example, the RNA or DNA may be localised to a cell or group of cells, or type of cells in the sample, or to particular regions of areas within a tissue sample. The native location or position of the RNA or DNA (or in other words, the location or position of the RNA or DNA in the tissue sample), e.g. an expressed gene or genomic locus, may be determined.

The invention can also be seen to provide an array for use in the methods of the invention comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a reverse transcriptase (RT) primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position

of the capture probe on the array, and

(ii) a capture domain to capture RNA of a tissue sample that is contacted with said array.

In a related aspect, the present invention also provides use of an array, comprising a substrate on which multiple species of capture probe are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a reverse transcriptase (RT) primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array; and

(ii) a capture domain;

to capture RNA of a tissue sample that is contacted with said array.

Preferably, said use is for determining and/or analysing a transcriptome and in particular the global transcriptome, of a tissue sample and further comprises steps of:

(a) generating cDNA molecules from the captured RNA molecules using said capture probes as RT primers and optionally amplifying said cDNA molecules;

(b) releasing at least part of the cDNA molecules and/or optionally their amplicons from the surface of the array, wherein said released molecule may be a first strand and/or second strand cDNA molecule or an amplicon thereof and wherein said part includes the positional domain or a complement thereof;

(c) directly or indirectly analysing the sequence of the released molecules; and optionally

(d) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (a).

It will be seen therefore that the array of the present invention may be used to capture RNA, e.g. mRNA of a tissue sample that is contacted with said array. The array may also be used for determining and/or analysing a partial or global transcriptome of a tissue sample or for obtaining a spatially defined partial or global transcriptome of a tissue sample. The methods of the invention may thus be considered as methods of quantifying the spatial expression of one or more genes in a tissue sample. Expressed another way, the methods of the present invention may be used to detect

the spatial expression of one or more genes in a tissue sample. In yet another way, the methods of the present invention may be used to determine simultaneously the expression of one or more genes at one or more positions within a tissue sample. Still further, the methods may be seen as methods for partial or global transcriptome analysis of a tissue sample with two-dimensional spatial resolution.

The RNA may be any RNA molecule which may occur in a cell. Thus it may be mRNA, tRNA, rRNA, viral RNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), small interfering RNA (siRNA), piwi-interacting RNA (piRNA), ribozymal RNA, antisense RNA or non-coding RNA. Preferably however it is mRNA.

Step (c) in the method above (corresponding to step (a) in the preferred statement of use set out above) of generating cDNA from the captured RNA will be seen as relating to the synthesis of the cDNA. This will involve a step of reverse transcription of the captured RNA, extending the capture probe, which functions as the RT primer, using the captured RNA as template. Such a step generates so- called first strand cDNA. As will be described in more detail below, second strand cDNA synthesis may optionally take place on the array, or it may take place in a separate step, after release of first strand cDNA from the array. As also described in more detail below, in certain embodiments second strand synthesis may occur in the first step of amplification of a released first strand cDNA molecule.

Arrays for use in the context of nucleic acid analysis in general, and DNA analysis in particular, are discussed and described below. Specific details and embodiments described herein in relation to arrays and capture probes for use in the context of RNA, apply equally (where appropriate) to all such arrays, including those for use with DNA.

As used herein the term "multiple" means two or more, or at least two, e.g. 3, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 400, 500, 1000, 2000,

5000, 10,000, or more etc. Thus for example, the number of capture probes may be any integer in any range between any two of the aforementioned numbers. It will be appreciated however that it is envisaged that conventional-type arrays with many hundreds, thousands, tens of thousands, hundreds of thousands or even millions of capture probes may be used.

Thus, the methods outlined herein utilise high density nucleic acid arrays comprising "capture probes" for capturing and labelling transcripts from all of the single cells within a tissue sample e.g. a thin tissue sample

slice, or "section". The tissue samples or sections for analysis are produced in a highly parallelized fashion, such that the spatial information in the section is retained. The captured RNA (preferably mRNA) molecules for each cell, or "transcriptomes", are transcribed into cDNA and the resultant cDNA molecules are analyzed, for example by high throughput sequencing. The resultant data may be correlated to images of the original tissue samples e.g. sections through so-called barcode sequences (or ID tags, defined herein as positional domains) incorporated into the arrayed nucleic acid probes.

High density nucleic acid arrays or microarrays are a core component of the spatial transcriptome labelling method described herein. A microarray is a multiplex technology used in molecular biology. A typical microarray consists of an arrayed series of microscopic spots of oligonucleotides (hundreds of thousands of spots, generally tens of thousands, can be incorporated on a single array). The distinct position of each nucleic acid (oligonucleotide) spot (each species of

oligonucleotide/nucleic acid molecule) is known as a "feature" (and hence in the methods set out above each species of capture probe may be viewed as a specific feature of the array; each feature occupies a distinct position on the array), and typically each separate feature contains in the region of picomoles (10<~12> moles) of a specific DNA sequence (a "species"), which are known as "probes" (or

"reporters"). Typically, these can be a short section of a gene or other nucleic acid element to which a cDNA or cRNA sample (or "target") can hybridize under high-stringency hybridization conditions. However, as described below, the probes of the present invention differ from the probes of standard microarrays.

In gene expression microarrays, probe-target hybridization is usually detected and quantified by detection of visual signal, e.g. a fluorophore, silver ion, or chemiluminescence-label, which has been incorporated into all of the targets.

The intensity of the visual signal correlates to the relative abundance of each target nucleic acid in the sample. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel.

In standard microarrays, the probes are attached to a solid surface or substrate by a covalent bond to a chemical matrix, e.g. epoxy-silane, amino-silane, lysine, polyacrylamide etc. The substrate typically is a glass, plastic or silicon chip or slide, although other microarray platforms are known, e.g. microscopic beads.

The probes may be attached to the array of the invention by any suitable means. In a preferred embodiment the probes are immobilized to the substrate of the array by chemical immobilization. This may be an interaction between the substrate (support material) and the probe based on a chemical reaction. Such a chemical reaction typically does not rely on the input of energy via heat or light, but can be enhanced by either applying heat, e.g. a certain optimal temperature for a chemical reaction, or light of certain wavelength. For example, a chemical immobilization may take place between functional groups on the substrate and corresponding functional elements on the probes. Such corresponding functional elements in the probes may either be an inherent chemical group of the probe, e.g. a hydroxyl group or be additionally introduced. An example of such a functional group is an amine group. Typically, the probe to be immobilized comprises a functional amine group or is chemically modified in order to comprise a functional amine group. Means and methods for such a chemical modification are well known.

The localization of said functional group within the probe to be immobilized may be used in order to control and shape the binding behaviour and/or orientation of the probe, e.g. the functional group may be placed at the 5' or 3' end of the probe or within sequence of the probe. A typical substrate for a probe to be immobilized comprises moieties which are capable of binding to such probes, e.g. to amine- functionalized nucleic acids. Examples of such substrates are carboxy, aldehyde or epoxy substrates. Such materials are known to the person skilled in the art.

Functional groups, which impart a connecting reaction between probes which are chemically reactive by the introduction of an amine group, and array substrates are known to the person skilled in the art.

Alternative substrates on which probes may be immobilized may have to be chemically activated, e.g. by the activation of functional groups, available on the array substrate. The term "activated substrate" relates to a material in which interacting or reactive chemical functional groups were established or enabled by chemical modification procedures as known to the person skilled in the art. For example, a substrate comprising carboxyl groups has to be activated before use. Furthermore, there are substrates available that contain functional groups that can react with specific moieties already present in the nucleic acid probes.

Alternatively, the probes may be synthesized directly on the substrate.

Suitable methods for such an approach are known to the person skilled in the art. Examples are manufacture techniques developed by Agilent Inc., Affymetrix Inc., Roche Nimblegen Inc. or Flexgen BV. Typically, lasers and a set of mirrors that specifically activate the spots where nucleotide additions are to take place are used. Such an approach may provide, for example, spot sizes (i.e. features) of around 30 μm or larger.

The substrate therefore may be any suitable substrate known to the person skilled in the art. The substrate may have any suitable form or format, e.g. it may be flat, curved, e.g. convexly or concavely curved towards the area where the interaction between the tissue sample and the substrate takes place. Particularly preferred is the where the substrate is a flat, i.e. planar, chip or slide.

Typically, the substrate is a solid support and thereby allows for an accurate and traceable positioning of the probes on the substrate. An example of a substrate is a solid material or a substrate comprising functional chemical groups, e.g. amine groups or amine-functionalized groups. A substrate envisaged by the present invention is a non-porous substrate. Preferred non-porous substrates are glass, silicon, poly-L-lysine coated material, nitrocellulose, polystyrene, cyclic olefin copolymers (COCs), cyclic olefin polymers (COPs), polypropylene, polyethylene and polycarbonate.

Any suitable material known to the person skilled in the art may be used. Typically, glass or polystyrene is used. Polystyrene is a hydrophobic material suitable for binding negatively charged macromolecules because it normally contains few hydrophilic groups. For nucleic acids immobilized on glass slides, it is furthermore known that by increasing the hydrophobicity of the glass surface the nucleic acid immobilization may be increased. Such an enhancement may permit a relatively more densely packed formation. In addition to a coating or surface treatment with poly-L-lysine, the substrate, in particular glass, may be treated by silanation, e.g. with epoxy-silane or amino-silane or by silynation or by a treatment with polyacrylamide.

A number of standard arrays are commercially available and both the number and size of the features may be varied. In the present invention, the arrangement of the features may be altered to correspond to the size and/or density of the cells present in different tissues or organisms. For instance, animal cells typically have a cross-section in the region of 1-100μm, whereas the cross-section of plant cells typically may range from 1-10000μm. Hence, Nimblegen® arrays, which are available with up to 2.1 million features, or 4.2 million features, and feature sizes of 13 micrometers, may be preferred for tissue samples from an animal or fungus,

whereas other formats, e.g. with 8x130k features, may be sufficient for plant tissue samples. Commercial arrays are also available or known for use in the context of sequence analysis and in particular in the context of NGS technologies. Such arrays may also be used as the array surface in the context of the present invention e.g. an Illumina bead array. In addition to commercially available arrays, which can themselves be customized, it is possible to make custom or non-standard "in-house" arrays and methods for generating arrays are well-established. The methods of the invention may utilise both standard and nonstandard arrays that comprise probes as defined below.

The probes on a microarray may be immobilized, i.e. attached or bound, to the array preferably via the 5' or 3' end, depending on the chemical matrix of the array. Typically, for commercially available arrays, the probes are attached via a 3' linkage, thereby leaving a free 5' end. However, arrays comprising probes attached to the substrate via a 5' linkage, thereby leaving a free 3' end, are available and may be synthesized using standard techniques that are well known in the art and are described elsewhere herein.

The covalent linkage used to couple a nucleic acid probe to an array substrate may be viewed as both a direct and indirect linkage, in that the although the probe is attached by a "direct" covalent bond, there may be a chemical moiety or linker separating the "first" nucleotide of the nucleic acid probe from the, e.g. glass or silicon, substrate i.e. an indirect linkage. For the purposes of the present invention probes that are immobilized to the substrate by a covalent bond and/or chemical linker are generally seen to be immobilized or attached directly to the substrate.

As will be described in more detail below, the capture probes of the invention may be immobilized on, or interact with, the array directly or indirectly. Thus the capture probes need not bind directly to the array, but may interact indirectly, for example by binding to a molecule which itself binds directly or indirectly to the array (e.g. the capture probe may interact with (e.g. bind or hybridize to) a binding partner for the capture probe, i.e. a surface probe, which is itself bound to the array directly or indirectly). Generally speaking, however, the capture probe will be, directly or indirectly (by one or more intermediaries), bound to, or immobilized on, the array.

The use, method and array of the invention may comprise probes that are immobilized via their 5' or 3' end. However, when the capture probe is immobilized directly to the array substrate, it may be immobilized only such that the 3' end of the capture probe is free to be extended, e.g. it is immobilized by its 5' end. The

capture probe may be immobilized indirectly, such that it has a free, i.e. extendible, 3' end.

By extended or extendible 3' end, it is meant that further nucleotides may be added to the most 3' nucleotide of the nucleic acid molecule, e.g. capture probe, to extend the length of the nucleic acid molecule, i.e. the standard polymerization reaction utilized to extend nucleic acid molecules, e.g. templated polymerization catalyzed by a polymerase.

Thus, in one embodiment, the array comprises probes that are immobilized directly via their 3' end, so-called surface probes, which are defined below. Each species of surface probe comprises a region of complementarity to each species of capture probe, such that the capture probe may hybridize to the surface probe, resulting in the capture probe comprising a free extendible 3' end. In a preferred aspect of the invention, when the array comprises surface probes, the capture probes are synthesized in situ on the array.

The array probes may be made up of ribonucleotides and/or

deoxyribonucleotides as well as synthetic nucleotide residues that are capable of participating in Watson-Crick type or analogous base pair interactions. Thus, the nucleic acid domain may be DNA or RNA or any modification thereof e.g. PNA or other derivatives containing non-nucleotide backbones. However, in the context of transcriptome analysis the capture domain of the capture probe must capable of priming a reverse transcription reaction to generate cDNA that is complementary to the captured RNA molecules. As described below in more detail, in the context of genome analysis, the capture domain of the capture probe must be capable of binding to the DNA fragments, which may comprise binding to a binding domain that has been added to the fragmented DNA. In some embodiments, the capture domain of the capture probe may prime a DNA extension (polymerase) reaction to generate DNA that is complementary to the captured DNA molecules. In other embodiments, the capture domain may template a ligation reaction between the captured DNA molecules and a surface probe that is directly or indirectly immobilised on the substrate. In yet other embodiments, the capture domain may be ligated to one strand of the captured DNA molecules.

In a preferred embodiment of the invention at least the capture domain of the capture probe comprises or consists of deoxyribonucleotides (dNTPs). In a particularly preferred embodiment the whole of the capture probe comprises or consists of deoxyribonucleotides.

In a preferred embodiment of the invention the capture probes are immobilized on the substrate of the array directly, i.e. by their 5' end, resulting in a free extendible 3' end.

The capture probes of the invention comprise at least two domains, a capture domain and a positional domain (or a feature identification tag or domain; the positional domain may alternatively be defined as an identification (ID) domain or tag, or as a positional tag). The capture probe may further comprise a universal domain as defined further below. Where the capture probe is indirectly attached to the array surface via hybridization to a surface probe, the capture probe requires a sequence (e.g. a portion or domain) which is complementary to the surface probe. Such a complementary sequence may be complementary to a

positional/identification domain and/or a universal domain on the surface probe. In other words the positional domain and/or universal domain may constitute the region or portion of the probe which is complementary to the surface probe.

However, the capture probe may also comprise an additional domain (or region, portion or sequence) which is complementary to the surface probe. For ease of synthesis, as described in more detail below, such a surface probe-complementary region may be provided as part, or as an extension of the capture domain (such a part or extension not itself being used for, or capable of, binding to the target nucleic acid, e.g. RNA).

The capture domain is typically located at the 3' end of the capture probe and comprises a free 3' end that can be extended, e.g. by template dependent polymerization. The capture domain comprises a nucleotide sequence that is capable of hybridizing to nucleic acid, e.g. RNA (preferably mRNA) present in the cells of the tissue sample contact with the array.

Advantageously, the capture domain may be selected or designed to bind (or put more generally may be capable of binding) selectively or specifically to the particular nucleic acid, e.g. RNA it is desired to detect or analyse. For example the capture domain may be selected or designed for the selective capture of mRNA. As is well known in the art, this may be on the basis of hybridisation to the poly-A tail of mRNA. Thus, in a preferred embodiment the capture domain comprises a poly-T DNA oligonucleotide, i.e. a series of consecutive deoxythymidine residues linked by phosphodiester bonds, which is capable of hybridizing to the poly-A tail of mRNA. Alternatively, the capture domain may comprise nucleotides which are functionally or structurally analogous to poly-T i.e., are capable of binding selectively to poly-A, for example a poly-U

oligonucleotide or an oligonucleotide comprised of

deoxythymidine analogues, wherein said oligonucleotide retains the functional property of binding to poly-A. In a particularly preferred embodiment the capture domain, or more particularly the poly-T element of the capture domain, comprises at least 10 nucleotides, preferably at least 11 , 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleotides. In a further embodiment, the capture domain, or more particularly the poly-T element of the capture domain comprises at least 25, 30 or 35 nucleotides.

Random sequences may also be used in the capture of nucleic acid, as is known in the art, e.g. random hexamers or similar sequences, and hence such random sequences may be used to form all or a part of the capture domain. For example, random sequences may be used in conjunction with poly-T (or poly-T analogue etc.) sequences. Thus where a capture domain comprises a poly-T(or a "poly-T-like") oligonucleotide, it may also comprise a random oligonucleotide sequence. This may for example be located 5' or 3' of the poly-T sequence, e.g. at the 3' end of the capture probe, but the positioning of such a random sequence is not critical. Such a construct may facilitate the capturing of the initial part of the poly-A of mRNA. Alternatively, the capture domain may be an entirely random sequence. Degenerate capture domains may also be used, according to principles known in the art.

The capture domain may be capable of binding selectively to a desired subtype or subset of nucleic acid, e.g. RNA, for example a particular type of RNA such mRNA or rRNA etc. as listed above, or to a particular subset of a given type of RNA, for example, a particular mRNA species e.g. corresponding to a particular gene or group of genes. Such a capture probe may be selected or designed based on sequence of the RNA it is desired to capture. Thus it may be a sequence- specific capture probe, specific for a particular RNA target or group of targets (target group etc). Thus, it may be based on a particular gene sequence or particular motif sequence or common/conserved sequence etc., according to principles well known in the art.

In embodiments where the capture probe is immobilized on the substrate of the array indirectly, e.g. via hybridization to a surface probe, the capture domain may further comprise an upstream sequence (5' to the sequence that hybridizes to the nucleic acid, e.g. RNA of the tissue sample) that is capable of hybridizing to 5' end of the surface probe. Alone, the capture domain of the capture probe may be seen as a capture domain oligonucleotide, which may be used in the synthesis of

the capture probe in embodiments where the capture probe is immobilized on the array indirectly.

The positional domain (feature identification domain or tag) of the capture probe is located directly or indirectly upstream, i.e. closer to the 5' end of the capture probe nucleic acid molecule, of the capture domain. Preferably the positional domain is directly adjacent to the capture domain, i.e. there is no intermediate sequence between the capture domain and the positional domain. In some embodiments the positional domain forms the 5' end of the capture probe, which may be immobilized directly or indirectly on the substrate of the array.

As discussed above, each feature (distinct position) of the array comprises a spot of a species of nucleic acid probe, wherein the positional domain at each feature is unique. Thus, a "species" of capture probe is defined with reference to its positional domain; a single species of capture probe will have the same positional domain. However, it is not required that each member of a species of capture probe has the same sequence in its entirety. In particular, since the capture domain may be or may comprise a random or degenerate sequence, the capture domains of individual probes within a species may vary. Accordingly, in some embodiments where the capture domains of the capture probes are the same, each feature comprises a single probe sequence. However in other embodiments where the capture probe varies, members of a species of probe will not have the exact same sequence, although the sequence of the positional domain of each member in the species will be the same. What is required is that each feature or position of the array carries a capture probe of a single species (specifically each feature or position carries a capture probe which has an identical positional tag, i.e. there is a single positional domain at each feature or position). Each species has a different positional domain which identifies the species. However, each member of a species, may in some cases, as described in more detail herein, have a different capture domain, as the capture domain may be random or degenerate or may have a random or degenerate component. This means that within a given feature, or position, the capture domain of the probes may differ.

Thus in some, but not necessarily in all embodiments, the nucleotide sequence of any one probe molecule immobilized at a particular feature is the same as the other probe molecules immobilized at the same feature, but the nucleotide sequence of the probes at each feature is different, distinct or distinguishable from the probes immobilized at every other feature. Preferably each feature comprises a different species of probe. However, in some embodiments it may be advantageous for a group of features to comprise the same species of probe, i.e. effectively to produce a

feature covering an area of the array that is greater than a single feature, e.g. to lower the resolution of the array. In other embodiments of the array, the nucleotide sequence of the positional domain of any one probe molecule immobilized at a particular feature may be the same as the other probe molecules immobilized at the same feature but the capture domain may vary. The capture domain may nonetheless be designed to capture the same type of molecule, e.g. mRNA in general.

The positional domain (or tag) of the capture probe comprises the sequence which is unique to each feature and acts as a positional or spatial marker (the identification tag). In this way each region or domain of the tissue sample, e.g. each cell in the tissue, will be identifiable by spatial resolution across the array linking the nucleic acid, e.g. RNA (e.g. the transcripts) from a certain cell to a unique positional domain sequence in the capture probe. By virtue of the positional domain a capture probe in the array may be correlated to a position in the tissue sample, for example it may be correlated to a cell in the sample. Thus, the positional domain of the capture domain may be seen as a nucleic acid tag (identification tag).

Any suitable sequence may be used as the positional domain in the capture probes of the invention. By a suitable sequence, it is meant that the positional domain should not interfere with (i.e. inhibit or distort) the interaction between the RNA of the tissue sample and the capture domain of the capture probe. For example, the positional domain should be designed such that nucleic acid molecules in the tissue sample do not hybridize specifically to the positional domain. Preferably, the nucleic acid sequence of the positional domain of the capture probes has less than 80% sequence identity to the nucleic acid sequences in the tissue sample. Preferably, the positional domain of the capture probe has less than 70%, 60%, 50% or less than 40% sequence identity across a substantial part of the nucleic acids molecules in the tissue sample. Sequence identity may be determined by any appropriate method known in the art, e.g. the using BLAST alignment algorithm.

In a preferred embodiment the positional domain of each species of capture probe contains a unique barcode sequence. The barcode sequences may be generated using random sequence generation. The randomly generated sequences may be followed by stringent filtering by mapping to the genomes of all common reference species and with pre-set Tm intervals, GC content and a defined distance of difference to the other barcode sequences to ensure that the barcode sequences will not interfere with the capture of the nucleic acid, e.g. RNA from the tissue

sample and will be distinguishable from each other without difficulty. As mentioned above, and in a preferred embodiment, the capture probe comprises also a universal domain (or linker domain or tag). The universal domain of the capture probe is located directly or indirectly upstream, i.e. closer to the 5' end of the capture probe nucleic acid molecule, of the positional domain. Preferably the universal domain is directly adjacent to the positional domain, i.e. there is no intermediate sequence between the positional domain and the universal domain. In embodiments where the capture probe comprises a universal domain, the domain will form the 5' end of the capture probe, which may be immobilized directly or indirectly on the substrate of the array.

The universal domain may be utilized in a number of ways in the methods and uses of the invention. For example, the methods of the invention comprise a step of releasing (e.g. removing) at least part of the synthesised (i.e. extended or ligated) nucleic acid, e.g. cDNA molecules from the surface of the array. As described elsewhere herein, this may be achieved in a number of ways, of which one comprises cleaving the nucleic acid, e.g. cDNA molecule from the surface of the array. Thus, the universal domain may itself comprise a cleavage domain, i.e. a sequence that can be cleaved specifically, either chemically or preferably enzymatically.

Thus, the cleavage domain may comprise a sequence that is recognised by one or more enzymes capable of cleaving a nucleic acid molecule, i.e. capable of breaking the phosphodiester linkage between two or more nucleotides. For instance, the cleavage domain may comprise a restriction endonuclease (restriction enzyme) recognition sequence. Restriction enzymes cut double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites and suitable enzymes are well known in the art. For example, it is particularly advantageous to use rare-cutting restriction enzymes, i.e. enzymes with a long recognition site (at least 8 base pairs in length), to reduce the possibility of cleaving elsewhere in the nucleic acid, e.g. cDNA molecule. In this respect, it will be seen that removing or releasing at least part of the nucleic acid, e.g. cDNA molecule requires releasing a part comprising the positional domain of the nucleic acid, e.g. cDNA and all of the sequence downstream of the domain, i.e. all of the sequence that is 3' to the positional domain. Hence, cleavage of the nucleic acid, e.g. cDNA molecule should take place 5' to the positional domain. By way of example, the cleavage domain may comprise a poly-U sequence which may be cleaved by a mixture of Uracil DNA glycosylase (UDG) and the DNA glycosylase-lyase Endonuclease VIII, commercially known as the USER™ enzyme.

A further example of a cleavage domain can be utilised in embodiments where the capture probe is immobilized to the array substrate indirectly, i.e. via a surface probe. The cleavage domain may comprise one or more mismatch nucleotides, i.e. when the complementary parts of the surface probe and the capture probe are not 100% complementary. Such a mismatch is recognised, e.g. by the MutY and T7 endonuclease I enzymes, which results in cleavage of the nucleic acid molecule at the position of the mismatch.

In some embodiments of the invention, the positional domain of the capture probe comprises a cleavage domain, wherein the said cleavage domain is located at the 5' end of the positional domain.

The universal domain may comprise also an amplification domain. This may be in addition to, or instead of, a cleavage domain. In some embodiments of the invention, as described elsewhere herein, it may be advantageous to amplify the nucleic acid, e.g. cDNA molecules, for example after they have been released (e.g. removed or cleaved) from the array substrate. It will be appreciated however, that the initial cycle of amplification, or indeed any or all further cycles of amplification may also take place in situ on the array. The amplification domain comprises a distinct sequence to which an amplification primer may hybridize. The amplification domain of the universal domain of the capture probe is preferably identical for each species of capture probe. Hence a single amplification reaction will be sufficient to amplify all of the nucleic acid, e.g. cDNA molecules (which may or may not be released from the array substrate prior to amplification).

Any suitable sequence may be used as the amplification domain in the capture probes of the invention. By a suitable sequence, it is meant that the amplification domain should not interfere with (i.e. inhibit or distort) the interaction between the nucleic acid, e.g. RNA of the tissue sample and the capture domain of the capture probe. Furthermore, the amplification domain should comprise a sequence that is not the same or substantially the same as any sequence in the nucleic acid, e.g. RNA of the tissue sample, such that the primer used in the amplification reaction can hybridized only to the amplification domain under the amplification conditions of the reaction. For example, the amplification domain should be designed such that nucleic acid molecules in the tissue sample do not hybridize specifically to the amplification domain or the complementary sequence of the amplification domain. Preferably, the nucleic acid sequence of the amplification domain of the capture probes and the complement thereof has less than 80% sequence identity to the nucleic acid sequences in the tissue

sample. Preferably, the positional domain of the capture probe has less than 70%, 60%, 50% or less than 40% sequence identity across a substantial part of the nucleic acid molecules in the tissue sample. Sequence identity may be determined by any appropriate method known in the art, e.g. the using BLAST alignment algorithm.

Thus, alone, the universal domain of the capture probe may be seen as a universal domain oligonucleotide, which may be used in the synthesis of the capture probe in embodiments where the capture probe is immobilized on the array indirectly.

In one representative embodiment of the invention only the positional domain of each species of capture probe is unique. Hence, the capture domains and universal domains (if present) are in one embodiment the same for every species of capture probe for any particular array to ensure that the capture of the nucleic acid, e.g. RNA from the tissue sample is uniform across the array. However, as discussed above, in some embodiments the capture domains may differ by virtue of including random or degenerate sequences.

In embodiments where the capture probe is immobilized on the substrate of the array indirectly, e.g. via hybridisation to a surface probe, the capture probe may be synthesised on the array as described below.

The surface probes are immobilized on the substrate of the array directly by or at, e.g. their 3' end. Each species of surface probe is unique to each feature (distinct position) of the array and is partly complementary to the capture probe, defined above.

Hence the surface probe comprises at its 5' end a domain (complementary capture domain) that is complementary to a part of the capture domain that does not bind to the nucleic acid, e.g. RNA of the tissue sample. In other words, it comprises a domain that can hybridize to at least part of a capture domain oligonucleotide. The surface probe further comprises a domain (complementary positional domain or complementary feature identification domain) that is complementary to the positional domain of the capture probe. The complementary positional domain is located directly or indirectly downstream (i.e. at the 3' end) of the complementary capture domain, i.e. there may be an intermediary or linker sequence separating the complementary positional domain and the complementary capture domain. In embodiments where the capture probe is synthesized on the array surface, the surface probes of the array always comprise a domain

(complementary universal domain) at the 3' end of the

surface probe, i.e. directly or indirectly downstream of the positional domain, which is complementary to the universal domain of the capture probe. In other words, it comprises a domain that can hybridize to at least part of the universal domain oligonucleotide.

In some embodiments of the invention the sequence of the surface probe shows 100% complementarity or sequence identity to the positional and universal domains and to the part of the capture domain that does not bind to the nucleic acid, e.g. RNA of the tissue sample. In other embodiments the sequence of the surface probe may show less than 100% sequence identity to the domains of the capture probe, e.g. less than 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91 % or 90%. In a particularly preferred embodiment of the invention, the complementary universal domain shares less than 100% sequence identity to the universal domain of the capture probe.

In one embodiment of the invention, the capture probe is synthesized or generated on the substrate of the array. In a representative embodiment (see figure 3), the array comprises surface probes as defined above. Oligonucleotides that correspond to the capture domain and universal domain of the capture probe are contacted with the array and allowed to hybridize to the complementary domains of the surface probes. Excess oligonucleotides may be removed by washing the array under standard hybridization conditions. The resultant array comprises partially single stranded probes, wherein both the 5' and 3' ends of the surface probe are double stranded and the complementary positional domain is single stranded. The array may be treated with a polymerase enzyme to extend the 3' end of the universal domain oligonucleotide, in a template dependent manner, so as to synthesize the positional domain of the capture probe. The 3' end of the

synthesized positional domain is then ligated, e.g. using a ligase enzyme, to the 5' end of the capture domain oligonucleotide to generate the capture probe. It will be understood in this regard that the 5' end of the capture domain oligonucleotide is phosphorylated to enable ligation to take place. As each species of surface probe comprises a unique complementary positional domain, each species of capture probe will comprise a unique positional domain.

The term "hybridisation" or "hybridises" as used herein refers to the formation of a duplex between nucleotide sequences which are sufficiently complementary to form duplexes via Watson-Crick base pairing. Two nucleotide sequences are "complementary" to one another when those molecules share base pair organization homology. "Complementary" nucleotide sequences will combine with specificity to form a stable duplex under

appropriate hybridization conditions. For instance, two sequences are complementary when a section of a first sequence can bind to a section of a second sequence in an anti-parallel sense wherein the 3'- end of each sequence binds to the 5'-end of the other sequence and each A, T(U), G and C of one sequence is then aligned with a T(U), A, C and G, respectively, of the other sequence. RNA sequences can also include complementary G=U or U=G base pairs. Thus, two sequences need not have perfect homology to be

"complementary" under the invention. Usually two sequences are sufficiently complementary when at least about 90% (preferably at least about 95%) of the nucleotides share base pair organization over a defined length of the molecule. The domains of the capture and surface probes thus contain a region of complementarity. Furthermore the capture domain of the capture probe contains a region of complementarity for the nucleic acid, e.g. RNA (preferably mRNA) of the tissue sample.

The capture probe may also be synthesised on the array substrate using polymerase extension (similarly to as described above) and a terminal transferase enzyme to add a "tail" which may constitute the capture domain. This is described further in Example 7 below. The use of terminal transferases to add nucleotide sequences to the end of an oligonucleotide is known in the art, e.g. to introduce a homopolymeric tail e.g. a poly-T tail. Accordingly, in such a synthesis an oligonucleotide that corresponds to the universal domain of the capture probe may be contacted with the array and allowed to hybridize to the complementary domain of the surface probes. Excess oligonucleotides may be removed by washing the array under standard hybridization conditions. The resultant array comprises partially single stranded probes, wherein the 5' ends of the surface probes are double stranded and the complementary positional domain is single stranded. The array may be treated with a polymerase enzyme to extend the 3' end of the universal domain oligonucleotide, in a template dependent manner, so as to synthesize the positional domain of the capture probe. The capture domain, e.g. comprising a poly-T sequence may then be introduced using a terminal transferase to add a poly-T tail to generate the capture probe.

The typical array of, and for use in the methods of, the invention may contain multiple spots, or "features". A feature may be defined as an area or distinct position on the array substrate at which a single species of capture probe is immobilized. Hence each feature will comprise a multiplicity of probe molecules, of the same species. It will be understood in this context that whilst it is encompassed that each capture probe of the same species may have the same sequence, this need not

necessarily be the case. Each species of capture probe will have the same positional domain (i.e. each member of a species and hence each probe in a feature will be identically "tagged"), but the sequence of each member of the feature (species) may differ, because the sequence of a capture domain may differ. As described above, random or degenerate capture domains may be used. Thus the capture probes within a feature may comprise different random or degenerate sequences. The number and density of the features on the array will determine the resolution of the array, i.e. the level of detail at which the transcriptome or genome of the tissue sample can be analysed. Hence, a higher density of features will typically increase the resolution of the array.

As discussed above, the size and number of the features on the array of the invention will depend on the nature of the tissue sample and required resolution. Thus, if it is desirable to determine a transcriptome or genome only for regions of cells within a tissue sample (or the sample contains large cells) then the number and/or density of features on the array may be reduced (i.e. lower than the possible maximum number of features) and/or the size of the features may be increased (i.e. the area of each feature may be greater than the smallest possible feature), e.g. an array comprising few large features. Alternatively, if it is desirable to determine a transcriptome or genome of individual cells within a sample, it may be necessary to use the maximum number of features possible, which would necessitate using the smallest possible feature size, e.g. an array comprising many small features.

Whilst single cell resolution may be a preferred and advantageous feature of the present invention, it is not essential to achieve this, and resolution at the cell group level is also of interest, for example to detect or distinguish a particular cell type or tissue region, e.g. normal vs tumour cells. In representative embodiments of the invention, an array may contain at least 2, 5, 10, 50, 100, 500, 750, 1000, 1500, 3000, 5000, 10000, 20000, 40000, 50000, 75000, 100000, 150000, 200000, 300000, 400000, 500000, 750000, 800000, 1000000, 1200000, 1500000, 1750000, 2000000, 2100000, 3000000, 3500000, 4000000 or 4200000 features. Whilst 4200000 represents the maximum number of features presently available on a commercial array, it is envisaged that arrays with features in excess of this may be prepared and such arrays are of interest in the present invention. As noted above, feature size may be decreased and this may allow greater numbers of features to be accommodated within the same or a similar area. By way of example, these features may be comprised in an area of less than about 20cm<2>, 10cm<2>, 5cm<2>, 1cm<2>, 1 mm<2>, or 100μm<2>.

Thus, in some embodiments of the invention the area of each feature may be from about 1 μη<2>, 2 μη<2>, 3 μη<2>, 4 μη<2>, 5 μη<2>, 10 μη<2>, 12 μη<2>, 15 μη<2>, 20 μη<2>, 50 μη<2>, 75 μη<2>, 100 μη<2>, 150 μη<2>, 200 μη<2>, 250 μη<2>, 300 μη<2>, 400 μη<2>, or 500 μη<2>.

It will be evident that a tissue sample from any organism could be used in the methods of the invention, e.g. plant, animal or fungal. The array of the invention allows the capture of any nucleic acid, e.g. mRNA molecules, which are present in cells that are capable of transcription and/or translation. The arrays and methods of the invention are particularly suitable for isolating and analysing the transcriptome or genome of cells within a sample, wherein spatial resolution of the transcriptomes or genomes is desirable, e.g. where the cells are interconnected or in contact directly with adjacent cells. However, it will be apparent to a person of skill in the art that the methods of the invention may also be useful for the analysis of the transcriptome or genome of different cells or cell types within a sample even if said cells do not interact directly, e.g. a blood sample. In other words, the cells do not need to present in the context of a tissue and can be applied to the array as single cells (e.g. cells isolated from a non-fixed tissue). Such single cells, whilst not necessarily fixed to a certain position in a tissue, are nonetheless applied to a certain position on the array and can be individually identified. Thus, in the context of analysing cells that do not interact directly, or are not present in a tissue context, the spatial properties of the described methods may be applied to obtaining or retrieving unique or independent transcriptome or genome information from individual cells. The sample may thus be a harvested or biopsied tissue sample, or possibly a cultured sample. Representative samples include clinical samples e.g. whole blood or blood-derived products, blood cells, tissues, biopsies, or cultured tissues or cells etc. including cell suspensions. Artificial tissues may for example be prepared from cell suspension (including for example blood cells). Cells may be captured in a matrix (for example a gel matrix e.g. agar, agarose, etc) and may then be sectioned in a conventional way. Such procedures are known in the art in the context of immunohistochemistry (see e.g. Andersson et al 2006, J. Histochem. Cytochem. 54(12): 1413-23. Epub 2006 Sep 6).

The mode of tissue preparation and how the resulting sample is handled may effect the transcriptomic or genomic analysis of the methods of the invention. Moreover, various tissue samples will have different physical characteristics and it is well within the skill of a person in the art to perform the necessary manipulations to yield a tissue sample for use with the methods of the invention. However, it is evident from the disclosures herein that any method of sample

preparation may be used to obtain a tissue sample that is suitable for use in the methods of the invention. For instance any layer of cells with a thickness of approximately 1 cell or less may be used in the methods of the invention. In one embodiment, the thickness of the tissue sample may be less than 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 or 0.1 of the cross-section of a cell. However, since as noted above, the present invention is not limited to single cell resolution and hence it is not a requirement that the tissue sample has a thickness of one cell diameter or less; thicker tissue samples may if desired be used. For example cryostat sections may be used, which may be e.g. 10-20 μm thick.

The tissue sample may be prepared in any convenient or desired way and the invention is not restricted to any particular type of tissue preparation. Fresh, frozen, fixed or unfixed tissues may be used. Any desired convenient procedure may be used for fixing or embedding the tissue sample, as described and known in the art. Thus any known fixatives or embedding materials may be used.

As a first representative example of a tissue sample for use in the invention, the tissue may prepared by deep freezing at temperature suitable to maintain or preserve the integrity (i.e. the physical characteristics) of the tissue structure, e.g. less than -20°C and preferably less than -25, -30, -40, -50, -60, -70 or -80 °C. The frozen tissue sample may be sectioned, i.e. thinly sliced, onto the array surface by any suitable means. For example, the tissue sample may be prepared using a chilled microtome, a cryostat, set at a temperature suitable to maintain both the structural integrity of the tissue sample and the chemical properties of the nucleic acids in the sample, e.g. to less than -15°C and preferably less than -20 or -25°C. Thus, the sample should be treated so as to minimize the degeneration or degradation of the nucleic acid, e.g. RNA in the tissue. Such conditions are well- established in the art and the extent of any degradation may be monitored through nucleic acid extraction, e.g. total RNA extraction and subsequent quality analysis at various stages of the preparation of the tissue sample.

In a second representative example, the tissue may be prepared using standard methods of formalin-fixation and paraffin-embedding (FFPE), which are well-established in the art. Following fixation of the tissue sample and embedding in a paraffin or resin block, the tissue samples may sectioned, i.e. thinly sliced, onto the array. As noted above, other fixatives and/or embedding materials can be used.

It will be apparent that the tissue sample section will need to be treated to remove the embedding material e.g. to deparaffinize, i.e. to remove the paraffin or resin,

from the sample prior to carrying out the methods of the invention. This may be achieved by any suitable method and the removal of paraffin or resin or other material from tissue samples is well established in the art, e.g. by incubating the sample (on the surface of the array) in an appropriate solvent e.g. xylene, e.g. twice for 10 minutes, followed by an ethanol rinse, e.g. 99.5% ethanol for 2 minutes, 96% ethanol for 2 minutes, and 70% ethanol for 2 minutes.

It will be evident to the skilled person that the RNA in tissue sections prepared using methods of FFPE or other methods of fixing and embedding is more likely to be partially degraded than in the case of frozen tissue. However, without wishing to be bound by any particular theory, it is believed that this may be advantageous in the methods of the invention. For instance, if the RNA in the sample is partially degraded the average length of the RNA polynucleotides will be less and more randomized than a non-degraded sample. It is postulated therefore that partially degraded RNA would result in less bias in the various processing steps, described elsewhere herein, e.g. ligation of adaptors (amplification domains), amplification of the cDNA molecules and sequencing thereof.

Hence, in one embodiment of the invention the tissue sample, i.e. the section of the tissue sample contacted with the array, is prepared using FFPE or other methods of fixing and embedding. In other words the sample may be fixed, e.g. fixed and embedded. In an alternative embodiment of the invention the tissue sample is prepared by deep-freezing. In another embodiment a touch imprint of a tissue may be used, according to procedures known in the art. In other

embodiments an unfixed sample may be used.

The thickness of the tissue sample section for use in the methods of the invention may be dependent on the method used to prepare the sample and the physical characteristics of the tissue. Thus, any suitable section thickness may be used in the methods of the invention. In representative embodiments of the invention the thickness of the tissue sample section will be at least 0.1 μη, further preferably at least 0.2, 0.3, 0.4, 0.5, 0.7, 1.0, 1.5, 2, 3, 4, 5, 6, 7, 8, 9 or 10μτη. In other embodiments the thickness of the tissue sample section is at least 10, 12, 13 , 14, 15, 20, 30, 40 or 50μη. However, the thickness is not critical and these are representative values only. Thicker samples may be used if desired or convenient e.g. 70 or 100 μη or more. Typically, the thickness of the tissue sample section is between 1-100 μη, 1-50 μη , 1-30 μη, 1-25 μη, 1-20 μη, 1-15 μη, 1-10 μη, 2- 8μη, 3-7μη or 4-6μη, but as mentioned above thicker samples may be used.

On contact of the tissue sample section with the array, e.g. following removal of the embedding material e.g. deparrafinization, the nucleic acid, e.g. RNA molecules in the tissue sample will bind to the immobilized capture probes on the array. In some embodiments it may be advantageous to facilitate the hybridization of the nucleic acid, e.g. RNA molecules to the capture probes. Typically, facilitating the hybridization comprises modifying the conditions under which hybridization occurs. The primary conditions that can be modified are the time and temperature of the incubation of the tissue section on the array prior to the reverse transcription step, which is described elsewhere herein.

For instance, on contacting the tissue sample section with the array, the array may be incubated for at least 1 hour to allow the nucleic acid, e.g. RNA to hybridize to the capture probes. Preferably the array may be incubated for at least 2, 3, 5, 10, 12, 15, 20, 22 or 24 hours or until the tissue sample section has dried. The array incubation time is not critical and any convenient or desired time may be used. Typical array incubations may be up to 72 hours. Thus, the incubation may occur at any suitable temperature, for instance at room temperature, although in a preferred embodiment the tissue sample section is incubated on the array at a temperature of at least 25, 26, 27, 28, 29, 30, 31 , 32, 33, 34, 35, 36 or 37°C.

Incubation temperatures of up to 55°C are commonplace in the art. In a particularly preferred embodiment the tissue sample section is allowed to dry on the array at 37°C for 24 hours. Once the tissue sample section has dried the array may be stored at room temperature before performing the reverse transcription step. It will be understood that the if the tissue sample section is allowed to dry on the surface of the array, it will need to be rehydrated before further manipulation of the captured nucleic acid can be achieved, e.g. the step of reverse transcribing the captured RNA.

Hence, the method of the invention may comprise a further step of rehydrating the tissue sample after contacting the sample with the array.

In some embodiments it may be advantageous to block (e.g. mask or modify) the capture probes prior to contacting the tissue sample with the array, particularly when the nucleic acid in the tissue sample is subject to a process of modification prior to its capture on the array. Specifically, it may be advantageous to block or modify the free 3' end of the capture probe. In a particular embodiment, the nucleic acid in the tissue sample, e.g. fragmented genomic DNA, may be modified such that it can be captured by the capture probe. For instance, and as described in more detail

below, an adaptor sequence (comprising a binding domain capable of binding to the capture domain of the capture probe) may be added to the end of the nucleic acid, e.g. fragmented genomic DNA. This may be achieved by, e.g. ligation of an adaptor or extension of the nucleic acid, e.g. using an enzyme to incorporate additional nucleotides at the end of the sequence, e.g. a poly-A tail. It is necessary to block or modify the capture probes, particularly the free 3' end of the capture probe, prior to contacting the tissue sample with the array to avoid modification of the capture probes, e.g. to avoid the addition of a poly-A tail to the free 3' end of the capture probes. Preferably the incorporation of a blocking domain may be incorporated into the capture probe when it is synthesised. However, the blocking domain may be incorporated to the capture probe after its synthesis.

In some embodiments the capture probes may be blocked by any suitable and reversible means that would prevent modification of the capture domains during the process of modifying the nucleic acid of the tissue sample, which occurs after the tissue sample has been contacted with the array. In other words, the capture probes may be reversibly masked or modified such that the capture domain of the capture probe does not comprise a free 3' end, i.e. such that the 3' end is removed or modified, or made inaccessible so that the capture probe is not susceptible to the process which is used to modify the nucleic acid of the tissue sample, e.g. ligation or extension, or the additional nucleotides may be removed to reveal and/or restore the 3' end of the capture domain of the capture probe.

For example, blocking probes may be hybridised to the capture probes to mask the free 3' end of the capture domain, e.g. hairpin probes or partially double stranded probes, suitable examples of which are known in the art. The free 3' end of the capture domain may be blocked by chemical modification, e.g. addition of an azidomethyl group as a chemically reversible capping moiety such that the capture probes do not comprise a free 3' end. Suitable alternative capping moieties are well known in the art, e.g. the terminal nucleotide of the capture domain could be a reversible terminator nucleotide, which could be included in the capture probe during or after probe synthesis.

Alternatively or additionally, the capture domain of the capture probe could be modified so as to allow the removal of any modifications of the capture probe, e.g. additional nucleotides, that occur when the nucleic acid molecules of the tissue sample are modified. For instance, the capture probes may comprise an additional sequence downstream of the capture domain, i.e. 3' to capture domain, namely a blocking domain. This could be in the form of, e.g. a restriction

endonuclease recognition sequence or a sequence of nucleotides cleavable by specific enzyme activities, e.g. uracil. Following the modification of the nucleic acid of the tissue sample, the capture probes could be subjected to an enzymatic cleavage, which would allow the removal of the blocking domain and any of the additional nucleotides that are added to the 3' end of the capture probe during the

modification process. The removal of the blocking domain would reveal and/or restore the free 3' end of the capture domain of the capture probe. The blocking domain could be synthesised as part of the capture probe or could be added to the capture probe in situ (i.e. as a modification of an existing array), e.g. by ligation of the blocking domain.

The capture probes may be blocked using any combination of the blocking mechanisms described above.

Once the nucleic acid of the tissue sample, e.g. fragmented genomic DNA, has been modified to enable it to hybridise to the capture domain of the capture probe, the capture probe must be unblocked, e.g. by dissociation of the blocking oligonucleotide, removal of the capping moiety and/or blocking domain.

In order to correlate the sequence analysis or transcriptome or genome information obtained from each feature of the array with the region (i.e. an area or cell) of the tissue sample the tissue sample is oriented in relation to the features on the array. In other words, the tissue sample is placed on the array such that the position of a capture probe on the array may be correlated with a position in the tissue sample. Thus it may be identified where in the tissue sample the position of each species of capture probe (or each feature of the array) corresponds. In other words, it may be identified to which location in the tissue sample the position of each species of capture probe corresponds. This may be done by virtue of positional markers present on the array, as described below. Conveniently, but not necessarily, the tissue sample may be imaged following its contact with the array. This may be performed before or after the nucleic acid of the tissue sample is processed, e.g. before or after the cDNA generation step of the method, in particular the step of generating the first strand cDNA by reverse transcription. In a preferred embodiment the tissue sample is imaged prior to the release of the captured and synthesised (i.e. extended or ligated) DNA, e.g. cDNA, from the array. In a particularly preferred embodiment the tissue is imaged after the nucleic acid of the tissue sample has been processed, e.g. after the reverse transcription step, and any residual tissue is removed (e.g. washed) from the array prior to the

release of molecules, e.g. of the cDNA from the array. In some embodiments, the step of processing the captured nucleic acid, e.g. the reverse transcription step, may act to remove residual tissue from the array surface, e.g. when using tissue preparing by deep-freezing. In such a case, imaging of the tissue sample may take place prior to the processing step, e.g. the cDNA synthesis step. Generally speaking, imaging may take place at any time after contacting the tissue sample with the area, but before any step which degrades or removes the tissue sample. As noted above, this may depend on the tissue sample.

Advantageously, the array may comprise markers to facilitate the orientation of the tissue sample or the image thereof in relation to the features of the array. Any suitable means for marking the array may be used such that they are detectable when the tissue sample is imaged. For instance, a molecule, e.g. a fluorescent molecule, that generates a signal, preferably a visible signal, may be immobilized directly or indirectly on the surface of the array. Preferably, the array comprises at least two markers in distinct positions on the surface of the array, further preferably at least 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 30, 40, 50, 60, 70, 80, 90 or 100 markers. Conveniently several hundred or even several thousand markers may be used. The markers may be provided in a pattern, for example make up an outer edge of the array, e.g. an entire outer row of the features of an array. Other informative patterns may be used, e.g. lines sectioning the array. This may facilitate aligning an image of the tissue sample to an array, or indeed generally in correlating the features of the array to the tissue sample. Thus, the marker may be an immobilized molecule to which a signal giving molecule may interact to generate a signal. In a

representative example, the array may comprise a marker feature, e.g. a nucleic acid probe immobilized on the substrate of array, to which a labelled nucleic acid may hybridize. For instance, the labelled nucleic acid molecule, or marker nucleic acid, may be linked or coupled to a chemical moiety capable of fluorescing when subjected to light of a specific wavelength (or range of wavelengths), i.e. excited. Such a marker nucleic acid molecule may be contacted with the array before, contemporaneously with or after the tissue sample is stained in order to visualize or image the tissue sample. However, the marker must be detectable when the tissue sample is imaged. Thus, in a preferred embodiment the marker may be detected using the same imaging conditions used to visualize the tissue sample.

In a particularly preferred embodiment of the invention, the array comprises marker features to which a labelled, preferably fluorescently labelled, marker nucleic acid

molecule, e.g. oligonucleotide, is hybridized.

The step of imaging the tissue may use any convenient histological means known in the art, e.g. light, bright field, dark field, phase contrast, fluorescence, reflection, interference, confocal microscopy or a combination thereof. Typically the tissue sample is stained prior to visualization to provide contrast between the different regions, e.g. cells, of the tissue sample. The type of stain used will be dependent on the type of tissue and the region of the cells to be stained. Such staining protocols are known in the art. In some embodiments more than one stain may be used to visualize (image) different aspects of the tissue sample, e.g.

different regions of the tissue sample, specific cell structures (e.g. organelles) or different cell types. In other embodiments, the tissue sample may be visualized or imaged without staining the sample, e.g. if the tissue sample contains already pigments that provide sufficient contrast or if particular forms of microscopy are used.

In a preferred embodiment, the tissue sample is visualized or imaged using fluorescence microscopy.

The tissue sample, i.e. any residual tissue that remains in contact with the array substrate following the reverse transcription step and optionally imaging, if imaging is desired and was not carried out before reverse transcription, preferably is removed prior to the step of releasing the cDNA molecules from the array. Thus, the methods of the invention may comprise a step of washing the array. Removal of the residual tissue sample may be performed using any suitable means and will be dependent on the tissue sample. In the simplest embodiment, the array may be washed with water. The water may contain various additives, e.g. surfactants (e.g. detergents), enzymes etc to facilitate to removal of the tissue. In some

embodiments, the array is washed with a solution comprising a proteinase enzyme (and suitable buffer) e.g. proteinase K. In other embodiments, the solution may comprise also or alternatively cellulase, hemicelluase or chitinase enzymes, e.g. if the tissue sample is from a plant or fungal source. In further embodiments, the temperature of the solution used to wash the array may be, e.g. at least 30°C, preferably at least 35, 40, 45, 50 or 55°C. It will be evident that the wash solution should minimize the disruption of the immobilized nucleic acid molecules. For instance, in some embodiments the nucleic acid molecules may be immobilized on the substrate of the array indirectly, e.g. via hybridization of the capture probe and the RNA and/or the capture probe and the surface probe, thus the wash step should not interfere with the interaction

between the molecules immobilized on the array, i.e. should not cause the nucleic acid molecules to be denatured.

Following the step of contacting the array with a tissue sample, under conditions sufficient to allow hybridization to occur between the nucleic acid, e.g. RNA (preferably mRNA), of the tissue sample to the capture probes, the step of securing (acquiring) the hybridized nucleic acid takes place. Securing or acquiring the captured nucleic acid involves a covalent attachment of a complementary strand of the hybridized nucleic acid to the capture probe (i.e. via a nucleotide bond, a phosphodiester bond between juxtaposed 3'-hydroxyl and 5'-phosphate termini of two immediately adjacent nucleotides), thereby tagging or marking the captured nucleic acid with the positional domain specific to the feature on which the nucleic acid is captured.

In some embodiments, securing the hybridized nucleic acid, e.g. a single stranded nucleic acid, may involve extending the capture probe to produce a copy of the captured nucleic acid, e.g. generating cDNA from the captured (hybridized) RNA. It will be understood that this refers to the synthesis of a complementary strand of the hybridized nucleic acid, e.g. generating cDNA based on the captured RNA template (the RNA hybridized to the capture domain of the capture probe). Thus, in an initial step of extending the capture probe, e.g. the cDNA generation, the captured (hybridized) nucleic acid, e.g. RNA acts as a template for the extension, e.g. reverse transcription, step. In other embodiments, as described below, securing the hybridized nucleic acid, e.g. partially double stranded DNA, may involve covalently coupling the hybridized nucleic acid, e.g. fragmented DNA, to the capture probe, e.g. ligating to the capture probe the complementary strand of the nucleic acid hybridized to the capture probe, in a ligation reaction.

Reverse transcription concerns the step of synthesizing cDNA

(complementary or copy DNA) from RNA, preferably mRNA (messenger RNA), by reverse transcriptase. Thus cDNA can be considered to be a copy of the RNA present in a cell at the time at which the tissue sample was taken, i.e. it represents all or some of the genes that were expressed in said cell at the time of isolation.

The capture probe, specifically the capture domain of the capture probe, acts as a primer for producing the complementary strand of the nucleic acid hybridized to the capture probe, e.g. a primer for reverse transcription. Hence, the nucleic acid, e.g. cDNA, molecules generated by the extension reaction, e.g.

reverse transcription reaction, incorporate the sequence of the capture probe, i.e. the extension reaction, e.g. reverse transcription reaction, may be seen as a way of labelling indirectly the nucleic acid, e.g. transcripts, of the tissue sample that are in contact with each feature of the array. As mentioned above, each species of capture probe comprises a positional domain (feature identification tag) that represents a unique sequence for each feature of the array. Thus, all of the nucleic acid, e.g. cDNA, molecules synthesized at a specific feature will comprise the same nucleic acid "tag".

The nucleic acid, e.g. cDNA, molecules synthesized at each feature of the array may represent the genome of, or genes expressed from, the region or area of the tissue sample in contact with that feature, e.g. a tissue or cell type or group or sub-group thereof, and may further represent genes expressed under specific conditions, e.g. at a particular time, in a specific environment, at a stage of development or in response to stimulus etc. Hence, the cDNA at any single feature may represent the genes expressed in a single cell, or if the feature is in contact with the sample at a cell junction, the cDNA may represent the genes expressed in more than one cell. Similarly, if a single cell is in contact with multiple features, then each feature may represent a proportion of the genes expressed in said cell.

Similarly, in embodiments in which the captured nucleic acid is DNA, any single feature may be representative of the genome of a single cell or more than one cell. Alternatively, the genome of a single cell may be represented by multiple features.

The step of extending the capture probe, e.g. reverse transcription, may be performed using any suitable enzymes and protocol of which many exist in the art, as described in detail below. However, it will be evident that it is not necessary to provide a primer for the synthesis of the first nucleic acid, e.g. cDNA, strand because the capture domain of the capture probe acts as the primer, e.g. reverse transcription primer.

Preferably, in the context of the present invention the secured nucleic acid (i.e. the nucleic acid covalently attached to the capture probe), e.g. cDNA is treated to comprise double stranded DNA. However, in some embodiments, the captured DNA may already comprise double stranded DNA, e.g. where partially double stranded fragmented DNA is ligated to the capture probe. Treatment of the captured nucleic acid to produce double stranded DNA may be achieved in a single reaction to generate only a second DNA, e.g. cDNA, strand, i.e. to produce double stranded DNA molecules without increasing the number of double stranded DNA molecules, or in an amplification reaction

to generate multiple copies of the second strand, which may be in the form of single stranded DNA (e.g. linear amplification) or double stranded DNA, e.g. cDNA (e.g. exponential amplification).

The step of second strand DNA, e.g. cDNA, synthesis may take place in situ on the array, either as a discrete step of second strand synthesis, for example using random primers as described in more detail below, or in the initial step of an amplification reaction. Alternatively, the first strand DNA, e.g. cDNA (the strand comprising, i.e. incorporating, the capture probe) may be released from the array and second strand synthesis, whether as a discrete step or in an amplification reaction may occur subsequently, e.g. in a reaction carried out in solution.

Where second strand synthesis takes place on the array (i.e. in situ) the method may include an optional step of removing the captured nucleic acid, e.g. RNA before the second strand synthesis, for example using an RNA digesting enzyme (RNase) e.g. RNase H. Procedures for this are well known and described in the art. However, this is generally not necessary, and in most cases the RNA degrades naturally. Removal of the tissue sample from the array will generally remove the RNA from the array. RNase H can be used if desired to increase the robustness of RNA removal. For instance, in tissue samples that comprise large amounts of RNA, the step of generating the double stranded cDNA may yield a sufficient amount of cDNA that it may be sequenced directly (following release from the array). In this case, second strand cDNA synthesis may be achieved by any means known in the art and as described below. The second strand synthesis reaction may be performed on the array directly, i.e. whilst the cDNA is immobilized on the array, or preferably after the cDNA has been released from the array substrate, as described below.

In other embodiments it will be necessary to enhance, i.e. amplify, the amount of secured nucleic acid, e.g. synthesized cDNA to yield quantities that are sufficient for DNA sequencing. In this embodiment, the first strand of the secured nucleic acid, e.g. cDNA molecules, which comprise also the capture probe of the features of the array, acts as a template for the amplification reaction, e.g. a polymerase chain reaction. The first reaction product of the amplification will be a second strand of DNA, e.g. cDNA, which itself will act as a template for further cycles of the amplification reaction.

In either of the above described embodiments, the second strand of DNA, e.g. cDNA, will comprise a complement of the capture probe. If the capture probe comprises a universal domain, and particularly an amplification domain within the universal domain, then this may be used for the subsequent amplification of the

DNA, e.g. cDNA, e.g. the amplification reaction may comprise a primer with the same sequence as the amplification domain, i.e. a primer that is complementary (i.e. hybridizes) to the complement of the amplification domain. In view of the fact that the amplification domain is upstream of the positional domain of the capture probe (in the secured nucleic acid, e.g. the first cDNA strand), the complement of the positional domain will be incorporated in the second strand of the DNA, e.g. cDNA molecules.

In embodiments where the second strand of DNA, e.g. cDNA is generated in a single reaction, the second strand synthesis may be achieved by any suitable means. For instance, the first strand cDNA, preferably, but not necessarily, released from the array substrate, may be incubated with random primers, e.g. hexamer primers, and a DNA polymerase, preferably a strand displacement polymerase, e.g. klenow (exo), under conditions sufficient for templated DNA synthesis to occur. This process will yield double stranded cDNA molecules of varying lengths and is unlikely to yield full-length cDNA molecules, i.e. cDNA molecules that correspond to entire mRNA from which they were synthesized. The random primers will hybridise to the first strand cDNA molecules at a random position, i.e. within the sequence rather than at the end of the sequence.

If it is desirable to generate full-length DNA, e.g. cDNA, molecules, i.e. molecules that correspond to the whole of the captured nucleic acid, e.g. RNA molecule (if the nucleic acid, e.g. RNA, was partially degraded in the tissue sample then the captured nucleic acid, e.g. RNA, molecules will not be "full-length" transcripts or the same length as the initial fragments of genomic DNA), then the 3' end of the secured nucleic acid, e.g. first stand cDNA, molecules may be modified. For example, a linker or adaptor may be ligated to the 3' end of the cDNA molecules. This may be achieved using single stranded ligation enzymes such as T4 RNA ligase or Circligase™ (Epicentre Biotechnologies).

Alternatively, a helper probe (a partially double stranded DNA molecule capable of hybridising to the 3' end of the first strand cDNA molecule), may be ligated to the 3' end of the secured nucleic acid, e.g. first strand cDNA, molecule using a double stranded ligation enzyme such as T4 DNA ligase. Other enzymes appropriate for the ligation step are known in the art and include, e.g. Tth DNA ligase, Taq DNA ligase, Thermococcus sp. (strain 9°N) DNA ligase (9°N™ DNA ligase, New England Biolabs), and Ampligase™ (Epicentre Biotechnologies). The helper probe comprises also a specific sequence from which the second strand

DNA, e.g. cDNA, synthesis may be primed using a

primer that is complementary to the part of the helper probe that is ligated to the secured nucleic acid, e.g. first cDNA strand. A further alternative comprises the use of a terminal transferase active enzyme to incorporate a polynucleotide tail, e.g. a poly-A tail, at the 3' end of the secured nucleic acid, e.g. first strand of cDNA, molecules. The second strand synthesis may be primed using a poly-T primer, which may also comprise a specific amplification domain for further amplification. Other methods for generating "full- length" double stranded DNA, e.g. cDNA, molecules (or maximal length second strand synthesis) are well-established in the art.

In some embodiments, second strand synthesis may use a method of template switching, e.g. using the SMART™ technology from Clontech®. SMART (Switching Mechanism at 5' End of RNA Template) technology is well established in the art and is based that the discovery that reverse transcriptase enzymes, e.g. Superscript® II (Invitrogen), are capable of adding a few nucleotides at the 3' end of an extended cDNA molecule, i.e. to produce a DNA/RNA hybrid with a single stranded DNA overhang at the 3' end. The DNA overhang may provide a target sequence to which an oligonucleotide probe can hybridise to provide an additional template for further extension of the cDNA molecule. Advantageously, the oligonucleotide probe that hybridises to the cDNA overhang contains an

amplification domain sequence, the complement of which is incorporated into the synthesised first strand cDNA product. Primers containing the amplification domain sequence, which will hybridise to the complementary amplification domain sequence incorporated into the cDNA first strand, can be added to the reaction mix to prime second strand synthesis using a suitable polymerase enzyme and the cDNA first strand as a template. This method avoids the need to ligate adaptors to the 3' end of the cDNA first strand. Whilst template switching was originally developed for full-length mRNAs, which have a 5' cap structure, it has since been demonstrated to work equally well with truncated mRNAs without the cap structure. Thus, template switching may be used in the methods of the invention to generate full length and/or partial or truncated cDNA molecules. Thus, in a preferred embodiment of the invention, the second strand synthesis may utilise, or be achieved by, template switching. In a particularly preferred embodiment, the template switching reaction, i.e. the further extension of the cDNA first strand to incorporate the complementary amplification domain, is performed in situ (whilst the capture probe is still attached, directly or indirectly, to the array). Preferably, the second strand synthesis reaction is also performed in situ.

In embodiments where it may be necessary or

advantageous to enhance, enrich or amplify the DNA, e.g. cDNA molecules, amplification domains may be incorporated in the DNA, e.g. cDNA molecules. As discussed above, a first amplification domain may be incorporated into the secured nucleic acid molecules, e.g. the first strand of the cDNA molecules, when the capture probe comprises a universal domain comprising an amplification domain. In these embodiments, the second strand synthesis may incorporate a second amplification domain. For example, the primers used to generate the second strand cDNA, e.g. random hexamer primers, poly-T primer, the primer that is complementary to the helper probe, may comprise at their 5' end an amplification domain, i.e. a nucleotide sequence to which an amplification primer may hybridize. Thus, the resultant double stranded DNA may comprise an amplification domain at or towards each 5' end of the double stranded DNA, e.g. cDNA molecules. These amplification domains may be used as targets for primers used in an amplification reaction, e.g. PCR. Alternatively, the linker or adaptor which is ligated to the 3' end of the secured nucleic acid molecules, e.g. first strand cDNA molecules, may comprise a second universal domain comprising a second amplification domain. Similarly, a second amplification domain may be incorporated into the first strand cDNA molecules by template switching.

In embodiments where the capture probe does not comprise a universal domain, particularly comprising an amplification domain, the second strand of the cDNA molecules may be synthesised in accordance with the above description. The resultant double stranded DNA molecules may be modified to incorporate an amplification domain at the 5' end of the first DNA, e.g. cDNA strand (a first amplification domain) and, if not incorporated in the second strand DNA, e.g. cDNA synthesis step, at the 5' end of the second DNA, e.g. cDNA strand (a second amplification domain). Such amplification domains may be incorporated, e.g. by ligating double stranded adaptors to the ends of the DNA, e.g. cDNA molecules. Enzymes appropriate for the ligation step are known in the art and include, e.g. Tth DNA ligase, Taq DNA ligase, Thermococcus sp. (strain 9°N) DNA ligase (9°N™ DNA ligase, New England Biolabs), Ampligase™ (Epicentre Biotechnologies) and T4 DNA ligase. In a preferred embodiment the first and second amplification domains comprise different sequences.

From the above, it is therefore apparent that universal domains, which may comprise an amplification domain, may be added to the secured (i.e. extended or ligated) DNA molecules, for example to the cDNA molecules, or their complements (e.g. second strand) by various methods and techniques and combinations of such techniques known in the art e.g. by use of primers which

include such a domain, ligation of adaptors, use of terminal transferase enzymes and/or by template switching methods. As is clear from the discussion herein, such domains may be added before or after release of the DNA molecules from the array.

It will be apparent from the above description that all of the DNA, e.g. cDNA molecules from a single array that have been synthesized by the methods of the invention may all comprise the same first and second amplification domains.

Consequently, a single amplification reaction, e.g. PCR, may be sufficient to amplify all of the DNA, e.g. cDNA molecules. Thus in a preferred embodiment, the method of the invention may comprise a step of amplifying the DNA, e.g. cDNA molecules. In one embodiment the amplification step is performed after the release of the DNA, e.g. cDNA molecules from the substrate of the array. In other embodiments amplification may be performed on the array (i.e. in situ on the array). It is known in the art that amplification reactions may be carried out on arrays and on-chip thermocyclers exist for carrying out such reactions. Thus, in one embodiment arrays which are known in the art as sequencing platforms or for use in any form of sequence analysis (e.g. in or by next generation sequencing technologies) may be used as the basis of the arrays of the present invention (e.g. Illumina bead arrays etc.)

For the synthesis of the second strand of DNA, e.g. cDNA it is preferable to use a strand displacement polymerase (e.g. Φ29 DNA polymerase, Bst (exo<">) DNA polymerase, klenow (exo<">) DNA polymerase) if the cDNA released from the substrate of the array comprises a partially double stranded nucleic acid molecule. For instance, the released nucleic acids will be at least partially double stranded (e.g. DNA:DNA, DNA:RNA or DNA:DNA/RNA hybrid) in embodiments where the capture probe is immobilized indirectly on the substrate of the array via a surface probe and the step of releasing the DNA, e.g. cDNA molecules comprises a cleavage step. The strand displacement polymerase is necessary to ensure that the second cDNA strand synthesis incorporates the complement of the positional domain (feature identification domain) into the second DNA, e.g. cDNA strand.

It will be evident that the step of releasing at least part of the DNA, e.g. cDNA molecules or their amplicons from the surface or substrate of the array may be achieved using a number of methods. The primary aim of the release step is to yield molecules into which the positional domain of the capture probe (or its complement) is incorporated (or included), such that the DNA, e.g. cDNA molecules or their amplicons are "tagged" according to their feature (or position) on the

array. The release step thus removes DNA, e.g. cDNA molecules or amplicons thereof from the array, which DNA, e.g. cDNA molecules or amplicons include the positional domain or its complement (by virtue of it having been incorporated into the secured nucleic acid, e.g. the first strand cDNA by, e.g. extension of the capture probe, and optionally copied in the second strand DNA if second strand synthesis takes place on the array, or copied into amplicons if amplification takes place on the array). Hence, in order to yield sequence analysis data that can be correlated with the various regions in the tissue sample it is essential that the released molecules comprise the positional domain of the capture probe (or its complement).

Since the released molecule may be a first and/or second strand DNA, e.g. cDNA molecule or amplicon, and since the capture probe may be immobilised indirectly on the array, it will be understood that whilst the release step may comprise a step of cleaving a DNA, e.g. cDNA molecule from the array, the release step does not require a step of nucleic acid cleavage; a DNA, e.g. cDNA molecule or an amplicon may simply be released by denaturing a double-stranded molecule, for example releasing the second cDNA strand from the first cDNA strand, or releasing an amplicon from its template or releasing the first strand cDNA molecule (i.e. the extended capture probe) from a surface probe. Accordingly, a DNA, e.g. cDNA molecule may be released from the array by nucleic acid cleavage and/or by denaturation (e.g. by heating to denature a double-stranded molecule). Where amplification is carried out in situ on the array, this will of course encompass releasing amplicons by denaturation in the cycling reaction.

In some embodiments, the DNA, e.g. cDNA molecules are released by enzymatic cleavage of a cleavage domain, which may be located in the universal domain or positional domain of the capture probe. As mentioned above, the cleavage domain must be located upstream (at the 5' end) of the positional domain, such that the released DNA, e.g. cDNA molecules comprise the positional

(identification) domain. Suitable enzymes for nucleic acid cleavage include restriction endonucleases, e.g. RsaI. Other enzymes, e.g. a mixture of Uracil DNA glycosylase (UDG) and the DNA glycosylase-lyase Endonuclease VIII (USER™ enzyme) or a combination of the MutY and T7 endonuclease I enzymes, are preferred embodiments of the methods of the invention.

In an alternative embodiment, the DNA, e.g. cDNA molecules may be released from the surface or substrate of the array by physical means. For instance, in embodiments where the capture probe is indirectly

immobilized on the substrate of the array, e.g. via hybridization to the surface probe, it may be sufficient to disrupt the interaction between the nucleic acid molecules. Methods for disrupting the interaction between nucleic acid molecules, e.g. denaturing double stranded nucleic acid molecules, are well known in the art. A straightforward method for releasing the DNA, e.g. cDNA molecules (i.e. of stripping the array of the

synthesized DNA, e.g. cDNA molecules) is to use a solution that interferes with the hydrogen bonds of the double stranded molecules. In a preferred embodiment of the invention, the DNA, e.g. cDNA molecules may be released by applying heated water, e.g. water or buffer of at least 85°C, preferably at least 90, 91 , 92, 93, 94, 95, 96, 97, 98, 99°C. As an alternative or addition to the use of a temperature sufficient to disrupt the hydrogen bonding, the solution may comprise salts, surfactants etc. that may further destabilize the interaction between the nucleic acid molecules, resulting in the release of the DNA, e.g. cDNA molecules.

It will be understood that the application of a high temperature solution, e.g. 90-99°C water may be sufficient to disrupt a covalent bond used to immobilize the capture probe or surface probe to the array substrate. Hence, in a preferred embodiment, the DNA, e.g. cDNA molecules may be released by applying hot water to the array to disrupt covalently immobilized capture or surface probes.

It is implicit that the released DNA, e.g. cDNA molecules (the solution comprising the released DNA, e.g. cDNA molecules) are collected for further manipulation, e.g. second strand synthesis and/or amplification. Nevertheless, the method of the invention may be seen to comprise a step of collecting or recovering the released DNA, e.g. cDNA molecules. As noted above, in the context of in situ amplification the released molecules may include amplicons of the secured nucleic acid, e.g. cDNA.

In embodiments of methods of the invention, it may be desirable to remove any unextended or unligated capture probes. This may be, for example, after the step of releasing DNA molecules from the array. Any desired or convenient method may be used for such removal including, for example, use of an enzyme to degrade the unextended or unligated probes, e.g. exonuclease.

The DNA, e.g. cDNA molecules, or amplicons, that have been released from the array, which may have been modified as discussed above, are analysed to investigate (e.g. determine their sequence, although as noted above actual sequence determination is not required - any method of analysing the sequence may be used). Thus, any method of nucleic acid analysis

may be used. The step of sequence analysis may identify the positional domain and hence allow the analysed molecule to be localised to a position in the tissue sample. Similarly, the nature or identity of the analysed molecule may be determined. In this way the nucleic acid, e.g. RNA at given position in the array, and hence in the tissue sample may be determined. Hence the analysis step may include or use any method which identifies the analysed molecule (and hence the "target" molecule) and its positional domain. Generally such a method will be a sequence-specific method. For example, the method may use sequence-specific primers or probes, particularly primers or probes specific for the positional domain and/or for a specific nucleic acid molecule to be detected or analysed e.g. a DNA molecule corresponding to a nucleic acid, e.g. RNA or cDNA molecule to be detected. Typically in such a method sequence-specific amplification primers e.g. PCR primers may be used.

In some embodiments it may be desirable to analyse a subset or family of target related molecules, e.g. all of the sequences that encode a particular group of proteins which share sequence similarity and/or conserved domains, e.g. a family of receptors. Hence, the amplification and/or analysis methods described herein may use degenerate or gene family specific primers or probes that hybridise to a subset of the captured nucleic acids or nucleic acids derived therefrom, e.g. amplicons. In a particularly preferred embodiment, the amplification and/or analysis methods may utilise a universal primer (i.e. a primer common to all of the captured sequences) in combination with a degenerate or gene family specific primer specific for a subset of target molecules.

Thus in one embodiment, amplification-based, especially PCR-based methods of sequence analysis are used.

However, the steps of modifying and/or amplifying the released DNA, e.g. cDNA molecules may introduce additional components into the sample, e.g.

enzymes, primers, nucleotides etc. Hence, the methods of the invention may further comprise a step of purifying the sample comprising the released DNA, e.g. cDNA molecules or amplicons prior to the sequence analysis, e.g. to remove

oligonucleotide primers, nucleotides, salts etc that may interfere with the

sequencing reactions. Any suitable method of purifying the DNA, e.g. cDNA molecules may be used.

As noted above, sequence analysis of the released

DNA molecules may be direct or indirect. Thus the sequence analysis substrate (which may be viewed as the molecule which is subjected to the sequence analysis step or process) may directly be the molecule which is released from the array or it may be a molecule which is derived therefrom. Thus, for example in the context of sequence analysis step which involves a sequencing reaction, the sequencing template may be the molecule which is released from the array or it may be a molecule derived therefrom. For example, a first and/or second strand DNA, e.g. cDNA molecule released from the array may be directly subjected to sequence analysis (e.g.

sequencing), i.e. may directly take part in the sequence analysis reaction or process (e.g. the sequencing reaction or sequencing process, or be the molecule which is sequenced or otherwise identified). In the context of in situ amplification the released molecule may be an amplicon. Alternatively, the released molecule may be subjected to a step of second strand synthesis or amplification before sequence analysis (e.g. sequencing or identification by other means). The sequence analysis substrate (e.g. template) may thus be an amplicon or a second strand of a molecule which is directly released from the array.

Both strands of a double stranded molecule may be subjected to sequence analysis (e.g. sequenced) but the invention is not limited to this and single stranded molecules (e.g. cDNA) may be analysed (e.g. sequenced). For example various sequencing technologies may be used for single molecule sequencing, e.g. the Helicos or Pacbio technologies, or nanopore sequencing technologies which are being developed. Thus, in one embodiment the first strand of DNA, e.g. cDNA may be subjected to sequencing. The first strand DNA, e.g. cDNA may need to be modified at the 3' end to enable single molecule sequencing. This may be done by procedures analogous to those for handling the second DNA, e.g. cDNA strand. Such procedures are known in the art.

In a preferred aspect of the invention the sequence analysis will identify or reveal a portion of captured nucleic acid, e.g. RNA sequence and the sequence of the positional domain. The sequence of the positional domain (or tag) will identify the feature to which the nucleic acid, e.g. mRNA molecule was captured. The sequence of the captured nucleic acid, e.g. RNA molecule may be compared with a sequence database of the organism from which the sample originated to determine the gene to which it corresponds. By determining which region (e.g. cell) of the tissue sample was in contact with the feature, it is possible to determine which region of the tissue sample was expressing said gene (or contained the gene, e.g. in the

case of spatial genomics). This analysis may be achieved for all of the DNA, e.g. cDNA molecules generated by the methods of the invention, yielding a spatial transcriptome or genome of the tissue sample.

By way of a representative example, sequencing data may be analysed to sort the sequences into specific species of capture probe, i.e. according to the sequence of the positional domain. This may be achieved by, e.g. using the FastX toolkit FASTQ Barcode splitter tool to sort the sequences into individual files for the respective capture probe positional domain (tag) sequences. The sequences of each species, i.e. from each feature, may be analyzed to determine the identity of the transcripts. For instance, the sequences may be identified using e.g. Blastn software, to compare the sequences to one or more genome databases, preferably the database for the organism from which the tissue sample was obtained. The identity of the database sequence with the greatest similarity to the sequence generated by the methods of the invention will be assigned to said sequence. In general, only hits with a certainty of at least 1e<"6>, preferably 1e<"7>, 1e<"8>, or 1e<"9> will be considered to have been successfully identified.

It will be apparent that any nucleic acid sequencing method may be utilised in the methods of the invention. However, the so-called "next generation

sequencing" techniques will find particular utility in the present invention. High- throughput sequencing is particularly useful in the methods of the invention because it enables a large number of nucleic acids to be partially sequenced in a very short period of time. In view of the recent explosion in the number of fully or partially sequenced genomes, it is not essential to sequence the full length of the generated DNA, e.g. cDNA molecules to determine the gene to which each molecule corresponds. For example, the first 100 nucleotides from each end of the DNA, e.g. cDNA molecules should be sufficient to identify both the feature to which the nucleic acid, e.g. mRNA was captured (i.e. its location on the array) and the gene expressed. The sequence reaction from the "capture probe end" of the DNA, e.g. cDNA molecules yields the sequence of the positional domain and at least about 20 bases, preferably 30 or 40 bases of transcript specific sequence data. The sequence reaction from the "non-capture probe end" may yield at least about 70 bases, preferably 80, 90, or 100 bases of transcript specific sequence data.

As a representative example, the sequencing reaction may be based on reversible dye-terminators, such as used in the Illumina™ technology. For example, DNA molecules are first attached to primers on, e.g. a glass or silicon slide and amplified so that local clonal colonies

are formed (bridge amplification). Four types of ddNTPs are added, and non-incorporated nucleotides are washed away. Unlike pyrosequencing, the DNA can only be extended one nucleotide at a time. A camera takes images of the fluorescently labelled nucleotides then the dye along with the terminal 3' blocker is chemically removed from the DNA, allowing a next cycle. This may be repeated until the required sequence data is obtained. Using this technology, thousands of nucleic acids may be sequenced simultaneously on a single slide.

Other high-throughput sequencing techniques may be equally suitable for the methods of the invention, e.g. pyrosequencing. In this method the DNA is amplified inside water droplets in an oil solution (emulsion PCR), with each droplet containing a single DNA template attached to a single primer-coated bead that then forms a clonal colony. The sequencing machine contains many picolitre-volume wells each containing a single bead and sequencing enzymes. Pyrosequencing uses luciferase to generate light for detection of the individual nucleotides added to the nascent DNA and the combined data are used to generate sequence read-outs.

An example of a technology in development is based on the detection of hydrogen ions that are released during the polymerisation of DNA. A microwell containing a template DNA strand to be sequenced is flooded with a single type of nucleotide. If the introduced nucleotide is complementary to the leading template nucleotide it is incorporated into the growing complementary strand. This causes the release of a hydrogen ion that triggers a hypersensitive ion sensor, which indicates that a reaction has occurred. If homopolymer repeats are present in the template sequence multiple nucleotides will be incorporated in a single cycle. This leads to a corresponding number of released hydrogen ions and a proportionally higher electronic signal.

Thus, it is clear that future sequencing formats are slowly being made available, and with shorter run times as one of the main features of those platforms it will be evident that other sequencing technologies will be useful in the methods of the invention.

An essential feature of the present invention, as described above, is a step of securing a complementary strand of the captured nucleic acid molecules to the capture probe, e.g. reverse transcribing the captured RNA molecules. The reverse transcription reaction is well known in the art and in representative reverse transcription reactions, the reaction mixture includes a reverse transcriptase, dNTPs and a suitable buffer. The reaction mixture may comprise other

components, e.g. RNase inhibitor(s). The primers and template are the capture domain of the capture probe and the captured RNA molecules are described above. In the subject methods, each dNTP will typically be present in an amount ranging from about 10 to 5000 μM, usually from about 20 to 1000 μM. It will be evident that an equivalent reaction may be performed to generate a complementary strand of a captured DNA molecule, using an enzyme with DNA polymerase activity. Reactions of this type are well known in the art and are described in more detail below.

The desired reverse transcriptase activity may be provided by one or more distinct enzymes, wherein suitable examples are: M-MLV, MuLV, AMV, HIV, ArrayScript™, MultiScribe™, ThermoScript™, and Superscript® I, II, and III enzymes. The reverse transcriptase reaction may be carried out at any suitable temperature, which will be dependent on the properties of the enzyme. Typically, reverse transcriptase reactions are performed between 37-55°C, although temperatures outside of this range may also be appropriate. The reaction time may be as little as 1 , 2, 3, 4 or 5 minutes or as much as 48 hours. Typically the reaction will be carried out for between 5-120 minutes, preferably 5-60, 5-45 or 5-30 minutes or 1-10 or 1-5 minutes according to choice. The reaction time is not critical and any desired reaction time may be used.

As indicated above, certain embodiments of the methods include an amplification step, where the copy number of generated DNA, e.g. cDNA molecules is increased, e.g., in order to enrich the sample to obtain a better representation of the nucleic acids, e.g. transcripts captured from the tissue sample. The

amplification may be linear or exponential, as desired, where representative amplification protocols of interest include, but are not limited to: polymerase chain reaction (PCR); isothermal amplification, etc.

The polymerase chain reaction (PCR) is well known in the art, being described in U.S. Pat. Nos.: 4,683,202; 4,683,195; 4,800, 159; 4,965,188 and 5,512,462, the disclosures of which are herein incorporated by reference. In representative PCR amplification reactions, the reaction mixture that includes the above released DNA, e.g. cDNA molecules from the array, which are combined with one or more primers that are employed in the primer extension reaction, e.g., the PCR primers that hybridize to the first and/or second amplification domains (such as forward and reverse primers employed in geometric (or exponential) amplification or a single primer employed in a linear amplification). The

oligonucleotide primers with which the released DNA,

e.g. cDNA molecules

(hereinafter referred to as template DNA for convenience) is contacted will be of sufficient length to provide for hybridization to complementary template DNA under annealing conditions (described in greater detail below). The length of the primers will depend on the length of the amplification domains, but will generally be at least 10 bp in length, usually at least 15 bp in length and more usually at least 16 bp in length and may be as long as 30 bp in length or longer, where the length of the primers will generally range from 18 to 50 bp in length, usually from about 20 to 35 bp in length. The template DNA may be contacted with a single primer or a set of two primers (forward and reverse primers), depending on whether primer extension, linear or exponential amplification of the template DNA is desired. In addition to the above components, the reaction mixture produced in the subject methods typically includes a polymerase and deoxyribonucleoside triphosphates (dNTPs). The desired polymerase activity may be provided by one or more distinct polymerase enzymes. In many embodiments, the reaction mixture includes at least a Family A polymerase, where representative Family A

polymerases of interest include, but are not limited to: Thermus aquaticus polymerases, including the naturally occurring polymerase (Taq) and derivatives and homologues thereof, such as Klentaq (as described in Barnes et al, Proc. Natl. Acad. Sci USA (1994) 91 :2216-2220); Thermus thermophilus polymerases, including the naturally occurring polymerase (Tth) and derivatives and homologues thereof, and the like. In certain embodiments where the amplification reaction that is carried out is a high fidelity reaction, the reaction mixture may further include a polymerase enzyme having 3'-5' exonuclease activity, e.g., as may be provided by a Family B polymerase, where Family B polymerases of interest include, but are not limited to: Thermococcus litoralis DNA polymerase (Vent) as described in Perler et al., Proc. Natl. Acad. Sci. USA (1992) 89:5577-5581 ; Pyrococcus species GB-D (Deep Vent); Pyrococcus furiosus DNA polymerase (Pfu) as described in Lundberg et al., Gene (1991) 108: 1-6, Pyrococcus woesei (Pwo) and the like. Where the reaction mixture includes both a Family A and Family B polymerase, the Family A polymerase may be present in the reaction mixture in an amount greater than the Family B polymerase, where the difference in activity will usually be at least 10-fold, and more usually at least about 100-fold. Usually the reaction mixture will include four different types of dNTPs corresponding to the four naturally occurring bases present, i.e. dATP, dTTP, dCTP and dGTP. In the subject methods, each dNTP will typically be present in an amount ranging from about 10 to 5000 μM, usually from about 20 to 1000 μM.

The reaction mixtures prepared in the reverse transcriptase and/or amplification steps of the subject methods may further include an aqueous buffer medium that includes a source of monovalent ions, a source of divalent cations and a buffering agent. Any convenient source of monovalent ions, such as KCl, K- acetate, NH4-acetate, K-glutamate, NH4Cl, ammonium sulphate, and the like may be employed. The divalent cation may be magnesium, manganese, zinc and the like, where the cation will typically be magnesium. Any convenient source of magnesium cation may be employed, including MgCl2, Mg-acetate, and the like. The amount of Mg<2+> present in the buffer may range from 0.5 to 10 mM, but will preferably range from about 3 to 6 mM, and will ideally be at about 5 mM.

Representative buffering agents or salts that may be present in the buffer include Tris, Tricine, HEPES, MOPS and the like, where the amount of buffering agent will typically range from about 5 to 150 mM, usually from about 10 to 100 mM, and more usually from about 20 to 50 mM, where in certain preferred embodiments the buffering agent will be present in an amount sufficient to provide a pH ranging from about 6.0 to 9.5, where most preferred is pH 7.3 at 72 °C. Other agents which may be present in the buffer medium include chelating agents, such as EDTA, EGTA and the like.

In preparing the reverse transcriptase, DNA extension or amplification reaction mixture of the steps of the subject methods, the various constituent components may be combined in any convenient order. For example, in the amplification reaction the buffer may be combined with primer, polymerase and then template DNA, or all of the various constituent components may be combined at the same time to produce the reaction mixture.

As discussed above, a preferred embodiment of the invention the DNA, e.g. cDNA molecules may be modified by the addition of amplification domains to the ends of the nucleic acid molecules, which may involve a ligation reaction. A ligation reaction is also required for the in situ synthesis of the capture probe on the array, when the capture probe is immobilized indirectly on the array surface.

As is known in the art, ligases catalyze the formation of a phosphodiester bond between juxtaposed 3'-hydroxyl and 5'-phosphate termini of two immediately adjacent nucleic acids. Any convenient ligase may be employed, where

representative ligases of interest include, but are not limited to: Temperature sensitive and thermostable ligases. Temperature sensitive ligases include, but are

not limited to, bacteriophage T4 DNA ligase, bacteriophage T7 ligase, and E. coli ligase. Thermostable ligases include, but are not limited to, Taq ligase, Tth ligase, and Pfu ligase. Thermostable ligase may be obtained from thermophilic or hyperthermophilic organisms, including but not limited to, prokaryotic, eukaryotic, or archael organisms. Certain RNA ligases may also be employed in the methods of the invention.

In this ligation step, a suitable ligase and any reagents that are necessary and/or desirable are combined with the reaction mixture and maintained under conditions sufficient for ligation of the relevant oligonucleotides to occur. Ligation reaction conditions are well known to those of skill in the art. During ligation, the reaction mixture in certain embodiments may be maintained at a temperature ranging from about 4°C to about 50°C, such as from about 20°C to about 37°C for a period of time ranging from about 5 seconds to about 16 hours, such as from about 1 minute to about 1 hour. In yet other embodiments, the reaction mixture may be maintained at a temperature ranging from about 35°C to about 45°C, such as from about 37°C to about 42°C, e.g., at or about 38°C, 39°C, 40°C or 41 °C, for a period of time ranging from about 5 seconds to about 16 hours, such as from about 1 minute to about 1 hour, including from about 2 minutes to about 8 hours. In a representative embodiment, the ligation reaction mixture includes 50 mM Tris pH7.5, 10 mM MgCl2, 10 mM DTT, 1 mM ATP, 25 mg/ml BSA, 0.25 units/ml RNase inhibitor, and T4 DNA ligase at 0.125 units/ml. In yet another representative embodiment, 2.125 mM magnesium ion, 0.2 units/ml RNase inhibitor; and 0.125 units/ml DNA ligase are employed. The amount of adaptor in the reaction will be dependent on the concentration of the DNA, e.g. cDNA in the sample and will generally be present at between 10-100 times the molar amount of DNA, e.g. cDNA.

By way of a representative example the method of the invention may comprise the following steps:

(a) contacting an array with a tissue sample, wherein the array comprises a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a reverse transcriptase (RT) primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

such that RNA of the tissue sample hybridises to said capture probes;

(b) imaging the tissue sample on the array;

(c) reverse transcribing the captured mRNA molecules to generate cDNA molecules;

(d) washing the array to remove residual tissue;

(e) releasing at least part of the cDNA molecules from the surface of the array; (f) performing second strand cDNA synthesis on the released cDNA molecules;

and

(g) analysing the sequence of (e.g. sequencing) the cDNA molecules. By way of an alternative representative example the method of the invention may comprise the following steps:

(a) contacting an array with a tissue sample, wherein the array comprises a substrate on which at least two species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a reverse transcriptase (RT) primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

such that RNA of the tissue sample hybridises to said capture probes;

(b) optionally rehydrating the tissue sample;

(c) reverse transcribing the captured mRNA molecules to generate first strand cDNA molecules and optionally synthesising second strand cDNA molecules;

(d) imaging the tissue sample on the array;

(e) washing the array to remove residual tissue;

(f) releasing at least part of the cDNA molecules from the surface of the array;

(g) amplifying the released cDNA molecules;

and

(h) analysing the sequence of (e.g. sequencing) the

amplified cDNA molecules.

By way of yet a further representative example the method of the invention may comprise the following steps:

(a) contacting an array with a tissue sample, wherein the array comprises a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a reverse transcriptase (RT) primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

such that RNA of the tissue sample hybridises to said capture probes;

(b) optionally imaging the tissue sample on the array;

(c) reverse transcribing the captured mRNA molecules to generate cDNA molecules;

(d) optionally imaging the tissue sample on the array if not already performed as step (b);

(e) washing the array to remove residual tissue;

(f) releasing at least part of the cDNA molecules from the surface of the array;

(g) performing second strand cDNA synthesis on the released cDNA molecules;

(h) amplifying the double stranded cDNA molecules;

(i) optionally purifying the cDNA molecules to remove components that may interfere with the sequencing reaction;

and

(j) analysing the sequence of (e g. sequencing) the amplified cDNA molecules.

The present invention includes any suitable combination of the steps in the above described methods. It will be understood that the invention also

encompasses variations of these methods, for example where amplification is performed in situ on the array.

Also encompassed are methods which omit the imaging step.

The invention may also be seen to include a method for making or producing an array (i) for use in capturing mRNA from a tissue sample that is contacted with said array; or (ii) for use in determining and/or analysing a (e.g. the partial or global) transcriptome of a tissue sample, said method comprising immobilizing, directly or indirectly, multiple species of capture probe to an array substrate, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3': (i) a positional domain that corresponds to the position of the capture probe on the array; and

(ii) a capture domain.

The method of producing an array of the invention may be further defined such that each species of capture probe is immobilized as a feature on the array.

The method of immobilizing the capture probes on the array may be achieved using any suitable means as described herein. Where the capture probes are immobilized on the array indirectly the capture probe may be synthesized on the array. Said method may comprise any one or more of the following steps:

(a) immobilizing directly or indirectly multiple surface probes to an array substrate, wherein the surface probes comprise:

(i) a domain capable of hybridizing to part of the capture domain oligonucleotide (a part not involved in capturing the nucleic acid, e.g. RNA);

(ii) a complementary positional domain; and

(iii) a complementary universal domain;

(b) hybridizing to the surface probes immobilized on the array capture domain oligonucleotides and universal domain oligonucleotides;

(c) extending the universal domain oligonucleotides, by templated polymerisation, to generate the positional domain of the capture probe; and

(d) ligating the positional domain to the capture domain oligonucleotide to produce the capture oligonucleotide.

Ligation in step (d) may occur simultaneously with extension in step (c).

Thus it need not be carried out in a separate step, although this is course encompassed if desired.

The features of the array produced by the above method of producing the array of the invention, may be further defined in accordance with the above description.

Although the invention is described above with reference to detection or analysis of RNA, and transcriptome analysis or detection, it will be appreciated that the principles described can be applied analogously to the detection or analysis of DNA in cells and to genomic studies. Thus, more broadly viewed, the invention can be seen as being generally applicable to the detection of nucleic acids in general and in a further more particular aspect, as providing methods for the analysis or detection of DNA. Spatial information may be valuable also in a genomics context i.e. detection and/or analysis of a DNA molecule with spatial resolution. This may be achieved by genomic tagging according to the present invention. Such localised or spatial detection methods may be useful for example in the context of studying genomic variations in different cells or regions of a tissue, for example comparing normal and diseased cells or tissues (e.g. normal vs tumour cells or tissues) or in studying genomic changes in disease progression etc. For example, tumour tissues may comprise a heterogeneous population of cells which may differ in the genomic variants they contain (e.g. mutations and/or other genetic aberrations, for example chromosomal rearrangements, chromosomal amplifications/deletions/insertions etc.). The detection of genomic variations, or different genomic loci, in different cells in a localised way may be useful in such a context, e.g. to study the spatial distribution of genomic variations. A principal utility of such a method would be in tumour analysis. In the context of the present invention, an array may be prepared which is designed, for example, to capture the genome of an entire cell on one feature. Different cells in the tissue sample may thus be compared. Of course the invention is not limited to such a design and other variations may be possible, wherein the DNA is detected in a localised way and the position of the DNA captured on the array is correlated to a position or location in the tissue sample.

Accordingly, in a more general aspect, the present invention can be seen to provide a method for localised detection of nucleic acid in a tissue sample comprising:

(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing nucleic acid of the tissue sample to hybridise to the capture domain in said capture probes; (c) generating DNA molecules from the captured nucleic acid molecules using said capture probes as extension or ligation primers, wherein said extended or ligated DNA molecules are tagged by virtue of the positional domain;

(d) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying said tagged DNA;

(e) releasing at least part of the tagged DNA molecules and/or their complements or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof ;

(f) directly or indirectly analysing the sequence of (e.g. sequencing) the released DNA molecules.

As described in more detail above, any method of nucleic acid analysis may be used in the analysis step. Typically this may involve sequencing, but it is not necessary to perform an actual sequence determination. For example sequence- specific methods of analysis may be used. For example a sequence-specific amplification reaction may be performed, for example using primers which are specific for the positional domain and/or for a specific target sequence, e.g. a particular target DNA to be detected (i.e. corresponding to a particular cDNA/RNA or gene or gene variant or genomic locus or genomic variant etc.). An exemplary analysis method is a sequence-specific PCR reaction.

The sequence analysis (e.g. sequencing) information obtained in step (f) may be used to obtain spatial information as to the nucleic acid in the sample. In other words the sequence analysis information may provide information as to the location of the nucleic acid in the sample. This spatial information may be derived from the nature of the sequence analysis information obtained e.g. from a sequence determined or identified, for example it may reveal the presence of a particular nucleic acid molecule which may itself be spatially informative in the context of the tissue sample used, and/or the spatial information (e.g. spatial localisation) may be derived from the position of the tissue sample on the array, coupled with the sequence analysis information. However, as described above, spatial

information may conveniently be obtained by correlating the sequence analysis data to an image of the tissue sample and this represents one preferred embodiment of the invention.

Accordingly, in a preferred embodiment the method also includes a step of:

(g) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (c). The primer extension reaction referred to in step (a) may be defined as a polymerase-catalysed extension reaction and acts to acquire a complementary strand of the captured nucleic acid molecule that is covalently attached to the capture probe, i.e. by synthesising the complementary strand utilising the capture probe as a primer and the captured nucleic acid as a template. In other words it may be any primer extension reaction carried out by any polymerase enzyme. The nucleic acid may be RNA or it may be DNA. Accordingly the polymerase may be any polymerase. It may be a reverse transcriptase or it may be a DNA polymerase. The ligation reaction may be carried out by any ligase and acts to secure the complementary strand of the captured nucleic acid molecule to the capture probe, i.e. wherein the captured nucleic acid molecule (hybridised to the capture probe) is partially double stranded and the complementary strand is ligated to the capture probe.

One preferred embodiment of such a method is the method described above for the determination and/or analysis of a transcriptome, or for the detection of RNA. In alternative preferred embodiment the detected nucleic acid molecule is DNA. In such an embodiment the invention provides a method for localised detection of DNA in a tissue sample comprising:

(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing DNA of the tissue sample to hybridise to the capture domain in said capture probes;

(c) fragmenting DNA in said tissue sample, wherein said fragmentation is carried out before, during or after contacting the array with the tissue sample in step (d) extending said capture probes in a primer extension reaction using the captured DNA fragments as templates to generate extended DNA molecules, or ligating the captured DNA fragments to the capture probes in a ligation reaction to generate ligated DNA molecules, wherein said extended or ligated DNA molecules are tagged by virtue of the positional domain;

(e) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying said tagged DNA;

(f) releasing at least part of the tagged DNA molecules and/or their complements and/or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof ;

(g) directly or indirectly analysing the sequence of the released DNA molecules.

The method may further include a step of:

(h) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (d).

In the context of spatial genomics, where the target nucleic acid is DNA the inclusion of imaging and image correlation steps may in some circumstances be preferred.

In embodiments in which DNA is captured, the DNA may be any DNA molecule which may occur in a cell. Thus it may be genomic, i.e. nuclear, DNA, mitochondrial DNA or plastid DNA, e.g. chloroplast DNA. In a preferred

embodiment, the DNA is genomic DNA.

It will be understood that where fragmentation is carried out after the contacting in step (b), i.e. after the tissue sample is placed on the array,

fragmentation occurs before the DNA is hybridised to the capture domain. In other words the DNA fragments are hybridised (or more particularly, allowed to hybridise) to the capture domain in said capture probes.

Advantageously, but not necessarily, in a particular embodiment of this aspect of the invention, the DNA fragments of the tissue sample may be provided with a binding domain to enable or facilitate their capture by the capture probes on the array. Accordingly, the

binding domain is capable of hybridising to the capture domain of the capture probe. Such a binding domain may thus be regarded as a complement of the capture domain (i.e. it may be viewed as a complementary capture domain), although absolute complementarity between the capture and binding domains is not required, merely that the binding domain is sufficiently complementary to allow a productive hybridisation to take place, i.e. that the DNA fragments in the tissue sample are able to hybridise to the capture domain of the capture probes. Provision of such a binding domain may ensure that DNA in the sample does not bind to the capture probes until after the fragmentation step. The binding domain may be provided to the DNA fragments by procedures well known in the art, for example by ligation of adaptor or linker sequences which may contain the binding domain. For example a linker sequence with a protruding end may be used. The binding domain may be present in the single-stranded portion of such a linker, such that following ligation of the linker to the DNA fragments, the single- stranded portion containing the binding domain is available for hybridisation to the capture domain of the capture probes. Alternatively and in a preferred embodiment, the binding domain may be introduced by using a terminal transferase enzyme to introduce a polynucleotide tail e.g. a homopolymeric tail such as a poly-A domain. This may be carried out using a procedure analogous to that described above for introducing a universal domain in the context of the RNA methods. Thus, in advantageous embodiments a common binding domain may be introduced. In other words, a binding domain which is common to all the DNA fragments and which may be used to achieve the capture of the fragments on the array.

Where a tailing reaction is carried out to introduce a (common) binding domain, the capture probes on the array may be protected from the tailing reaction, i.e. the capture probes may be blocked or masked as described above. This may be achieved for example by hybridising a blocking oligonucleotide to the capture probe e.g. to the protruding end (e.g. single stranded portion) of the capture probe. Where the capture domain comprises a poly-T sequence for example, such a blocking oligonucleotide may be a poly-A oligonucleotide. The blocking

oligonucleotide may have a blocked 3' end (i.e. an end incapable of being extended, or tailed). The capture probes may also be protected, i.e. blocked, by chemical and/or enzymatic modifications, as described in detail above.

Where the binding domain is provided by ligation of a linker as described above, it will be understood that rather than extending the capture probe to generate a

complementary copy of the captured DNA fragment which comprises the positional tag of the capture probe primer, the DNA fragment may be ligated to the 3' end of the capture probe. As noted above ligation requires that the 5' end to be ligated is phosphorylated. Accordingly, in one embodiment, the 5' end of the added linker, namely the end which is to be ligated to the capture probe (i.e. the non-protruding end of the linker added to the DNA fragments) will be

phosphorylated. In such a ligation embodiment, it will accordingly be seen that a linker may be ligated to double stranded DNA fragments, said linker having a single stranded protruding 3' end which contains the binding domain. Upon contact with the array, the protruding end hybridises to the capture domain of the capture probes. This hybridisation brings the 3' end of the capture probe into juxtaposition for ligation to the 5' (non-protruding) end of the added linker. The capture probe, and hence the positional domain, is thus incorporated into the captured DNA fragment by this ligation. Such an embodiment is shown schematically in Figure 21.

Thus, the method of this aspect of the invention may in a more particular embodiment comprise:

(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain;

(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample;

(c) fragmenting DNA in said tissue sample, wherein said fragmentation is carried out before, during or after contacting the array with the tissue sample in step (b);

(d) providing said DNA fragments with a binding domain which is capable of hybridising to said capture domain;

(e) allowing said DNA fragments to hybridise to the capture domain in said capture probes;

(f) extending said capture probes in a primer extension reaction using the captured DNA fragments as

templates to generate extended DNA molecules, or ligating the captured DNA fragments to the capture probes in a ligation reaction to generate ligated DNA molecules, wherein said extended or ligated DNA molecules are tagged by virtue of the positional domain;
(g) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying the tagged DNA;

(h) releasing at least part of the tagged DNA molecules and/or their complements and/or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof;

(i) directly or indirectly analysing the sequence of the released DNA molecules.

The method may optionally include a further step of

(j) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (f).

In the methods of nucleic acid or DNA detection set out above, the optional step of generating a complementary copy of the tagged nucleic acid/DNA or of amplifying the tagged DNA, may involve the use of a strand displacing polymerase enzyme, according to the principles explained above in the context of the

RNA/transcriptome analysis/detection methods. Suitable strand displacing polymerases are discussed above. This is to ensure that the positional domain is copied into the complementary copy or amplicon. This will particularly be the case where the capture probe is immobilized on the array by hybridisation to a surface probe.

However, the use of a strand displacing polymerase in this step is not essential. For example a non-strand displacing polymerase may be used together with ligation of an oligonucleotide which hybridises to the positional domain. Such a procedure is analogous to that described above for the synthesis of capture probes on the array.

In one embodiment, the method of the invention may be used for determining and/or analysing all of the genome of a tissue sample e.g. the global genome of a tissue sample. However, the method is not limited to this and encompasses determining and/or analysing all or part of the genome. Thus, the method may involve determining and/or analysing a part or subset of the genome, e.g. a partial genome corresponding to a subset or group of genes or of

chromosomes, e.g. a set of particular genes or

chromosomes or a particular region or part of the genome, for example related to a particular disease or condition, tissue type etc. Thus, the method may be used to detect or analyse genomic sequences or genomic loci from tumour tissue as compared to normal tissue, or even within different types of cell in a tissue sample. The presence or absence, or the distribution or location of different genomic variants or loci in different cells, groups of cells, tissues or parts or types of tissue may be examined.

Viewed from another aspect, the method steps set out above can be seen as providing a method of obtaining spatial information regarding the nucleic acids, e.g. genomic sequences, variants or loci of a tissue sample. Put another way, the methods of the invention may be used for the labelling (or tagging) of genomes, particularly individual or spatially distributed genomes.

Alternatively viewed, the method of the invention may be seen as a method for spatial detection of DNA in a tissue sample, or a method for detecting DNA with spatial resolution, or for localised or spatial determination and/or analysis of DNA in a tissue sample. In particular, the method may be used for the localised or spatial detection or determination and/or analysis of genes or genomic sequences or genomic variants or loci (e.g. distribution of genomic variants or loci) in a tissue sample. The localised/spatial detection/determination/analysis means that the DNA may be localised to its native position or location within a cell or tissue in the tissue sample. Thus for example, the DNA may be localised to a cell or group of cells, or type of cells in the sample, or to particular regions of areas within a tissue sample. The native location or position of the DNA (or in other words, the location or position of the DNA in the tissue sample), e.g. a genomic variant or locus, may be determined.

It will be seen therefore that the array of the present invention may be used to capture nucleic acid, e.g. DNA of a tissue sample that is contacted with said array. The array may also be used for determining and/or analysing a partial or global genome of a tissue sample or for obtaining a spatially defined partial or global genome of a tissue sample. The methods of the invention may thus be considered as methods of quantifying the spatial distribution of one or more genomic sequences (or variants or loci) in a tissue sample. Expressed another way, the methods of the present invention may be used to detect the spatial distribution of one or more genomic sequences or genomic variants or genomic loci in a tissue sample. In yet another way, the methods of the present invention may be used to determine simultaneously the location or distribution of one or more genomic sequences or genomic variants or genomic loci at one or more positions within a tissue

sample. Still further, the methods may be seen as methods for partial or global analysis of the nucleic acid e.g. DNA of a tissue sample with spatial resolution e.g. two-dimensional spatial resolution. The invention can also be seen to provide an array for use in the methods of the invention comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as an extension or ligation primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array, and

(ii) a capture domain to capture nucleic acid of a tissue sample that is contacted with said array.

In one aspect the nucleic acid molecule to be captured is DNA. The capture domain may be specific to a particular DNA to be detected, or to a particular class or group of DNAs, e.g. by virtue of specific hybridisation to a specific sequence of motif in the target DNA e.g. a conserved sequence, by analogy to the methods described in the context of RNA detection above. Alternatively the DNA to be captured may be provided with a binding domain, e.g. a common binding domain as described above, which binding domain may be recognised by the capture domain of the capture probes. Thus, as noted above, the binding domain may for example be a homopolymeric sequence e.g. poly-A. Again such a binding domain may be provided according to or analogously to the principles and methods described above in relation to the methods for RNA/transcriptome analysis or detection. In such a case, the capture domain may be complementary to the binding domain introduced into the DNA molecules of the tissue sample.

As also described in the RNA context above, the capture domain may be a random or degenerate sequence. Thus, DNA may be captured non-specifically by binding to a random or degenerate capture domain or to a capture domain which comprises at least partially a random or degenerate sequence.

In a related aspect, the present invention also provides use of an array, comprising a substrate on which multiple species of capture probe are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':

(i) a positional domain that corresponds to the position of the capture probe on the array; and (ii) a capture domain;

to capture nucleic acid, e.g. DNA or RNA, of a tissue sample that is contacted with said array.

Preferably, said use is for localised detection of nucleic acid in a tissue sample and further comprises steps of:

(a) generating DNA molecules from the captured nucleic acid molecules using said capture probes as extension or ligation primers, wherein said extended or ligated molecules are tagged by virtue of the positional domain;

(b) optionally generating a complementary strand of said tagged nucleic acid and/or amplifying said tagged nucleic acid;

(c) releasing at least part of the tagged DNA molecules and/or their complements or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof;

(d) directly or indirectly analysing the sequence of the released DNA molecules; and optionally

(e) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (a).

The step of fragmenting DNA in a tissue sample may be carried out using any desired procedure known in the art. Thus physical methods of fragmentation may be used e.g. sonication or ultrasound treatment. Chemical methods are also known. Enzymatic methods of fragmentation may also be used, e.g. with endonucleases, for example restriction enzymes. Again methods and enzymes for this are well known in the art. Fragmentation may be done before during or after preparing the tissue sample for placing on an array, e.g. preparing a tissue section. Conveniently, fragmentation may be achieved in the step of fixing tissue. Thus for example, formalin fixation will result in fragmentation of DNA. Other fixatives may produce similar results.

In terms of the detail of preparing and using the arrays in these aspects of the invention, it will understood that the description and detail given above in the context of RNA methods applies analogously to the more general nucleic acid detection and DNA detection methods set out herein. Thus, all aspects and details discussed above apply analogously. For example, the discussion of reverse transcriptase primers and reactions etc may be applied analogously to any aspect of the extension primers, polymerase reactions etc. referred to above.

Likewise, references and to first and second strand cDNA synthesis may be applied analogously to the tagged DNA molecule and its complement. Methods of sequence analysis as discussed above may be used.

By way of example, the capture domain may be as described for the capture probes above. A poly-T or poly-T-containing capture domain may be used for example where the DNA fragments are provided with a binding domain comprising a poly-A sequence.

The capture probes/tagged DNA molecules (i.e. the tagged extended or ligated molecules) may be provided with universal domains as described above, e.g. for amplification and/or cleavage.

The invention will be further described with reference to the following non- limiting Examples with reference to the following drawings in which:

Figure 1 shows the overall concept using arrayed "barcoded" oligo-dT probes to capture mRNA from tissue sections for transcriptome analysis.

Figure 2 shows the a schematic for the visualization of transcript abundance for corresponding tissue sections.

Figure 3 shows 3' to 5' surface probe composition and synthesis of 5' to 3' oriented capture probes that are indirectly immobilized at the array surface.

Figure 4 shows a bar chart demonstrating the efficiency of enzymatic cleavage (USER or RsaI) from in-house manufactured arrays and by 99°C water from Agilent manufactured arrays, as measured by hybridization of fluorescently labelled probes to the array surface after probe release.

Figure 5 shows a fluorescent image captured after 99°C water mediated release of DNA surface probes from commercial arrays manufactured by Agilent. A fluorescent detection probe was hybridized after hot water treatment. Top array is an untreated control.

Figure 6 shows a fixated mouse brain tissue section on top of the transcriptome capture array post cDNA synthesis and treated with cytoplasmic (top) and nucleic stains (middle), respectively, and merged image showing both stains (bottom).

Figure 7 shows a table that lists the reads sorted for their origin across the low density in-house manufactured DNA-capture array as seen in the schematic representation.

Figure 8 shows a FFPE mouse brain tissue with nucleic and Map2 specific stains using a barcoded microarray.

Figure 9 shows FFPE mouse brain olfactory bulb with nucleic stain (white) and visible morphology.

Figure 10 shows FFPE mouse brain olfactory bulb (approx 2x2mm) with nucleic stain (white), overlaid with theoretical spotting pattern for low resolution array.

Figure 1 1 shows FFPE mouse brain olfactory bulb (approx 2x2mm) with nucleic stain (white), overlaid with theoretical spotting pattern for medium-high resolution array.

Figure 12 shows FFPE mouse brain olfactory bulb zoomed in on glomerular area (top right of Figure 9).

Figure 13 shows the resulting product from a USER release using a random hexamer primer (R6) coupled to the B_handle (B_R6) during amplification; product as depicted on a bioanalyzer.

Figure 14 shows the resulting product from a USER release using a random octamer primer (R8) coupled to the B_handle (B_R8) during amplification; product as depicted on a bioanalyzer.

Figure 15 shows the results of an experiment performed on FFPE brain tissue covering the whole array. ID5 (left) and ID20 (right) amplified with ID specific and gene specific primers (B2M exon 4) after synthesis and release of cDNA from surface; ID5 and ID20 amplified.

Figure 16 shows a schematic illustration of the principle of the method described in Example 4, i.e. use of microarrays with immobilized DNA oligos (capture probes) carrying spatial labeling tag sequences (positional domains). Each feature of oligos of the microarray carries a 1) a unique labeling tag (positional domain) and 2) a capture sequence (capture domain).

Figure 17 shows the results of the spatial genomics protocol described in Example 5 carried out with genomic DNA prefragmented to mean size of 200 bp. Internal products amplified on array labeled and synthesized DNA. The detected peak is of expected size.

Figure 18 shows the results of the spatial genomics protocol described in

Example 5 carried out with genomic DNA prefragmented to mean size of 700 bp. Internal products amplified on array labeled and synthesized DNA. The detected peak is of expected size.

Figure 19 shows the results of the spatial genomics protocol described in Example 5 carried out with genomic DNA prefragmented to mean size of 200 bp.

Products amplified with one internal primer and one universal sequence contained in the surface oligo. Amplification carried out on array labeled and synthesized DNA. The expected product is a smear given that the random fragmentation and terminal transferase labeling of genomic DNA will generate a very diverse sample pool.

Figure 20 shows the results of the spatial genomics protocol described in Example 5 carried out with genomic DNA prefragmented to mean size of 700 bp. Products amplified with one internal primer and one universal sequence contained in the surface oligo. Amplification carried out on array labeled and synthesized DNA. The expected product is a smear given that the random fragmentation and terminal transferase labeling of genomic DNA will generate a very diverse sample pool.

Figure 21 shows a schematic illustration of the ligation of a linker to a DNA fragment to introduce a binding domain for hybridisation to a poly-T capture domain, and subsequent ligation to the capture probe.

Figure 22 shows the composition of 5' to 3' oriented capture probes used on high-density capture arrays.

Figure 23 shows the frame of the high-density arrays, which is used to orientate the tissue sample, visualized by hybridization of fluorescent marker probes.

Figure 24 shows capture probes cleaved and non-cleaved from high-density array, wherein the frame probes are not cleaved since they do not contain uracil bases. Capture probes were labelled with fluorophores coupled to poly-A oligonucleotides.

Figure 25 shows a bioanalyzer image of a prepared sequencing library with transcripts captured from mouse olfactory bulb.

Figure 26 shows a Matlab visualization of captured transcripts from total RNA extracted from mouse olfactory bulb.

Figure 27 shows Olfr (olfactory receptor) transcripts as visualized across the capture array using Matlab visualization after capture from mouse olfactory bulb tissue.

Figure 28 shows a pattern of printing for in-house 41 -ID-tag microarrays. Figure 29 shows a spatial genomics library generated from a A431 specific translocation after capture of poly-A tailed genomic fragments on capture array. Figure 30 shows the detection of A431 specific translocation after capture of spiked 10% and 50% poly-A tailed A431 genomic

fragments into poly-A tailed U20S genomic fragments on capture array.

Figure 31 shows a Matlab visualization of captured ID-tagged transcripts from mouse olfactory bulb tissue on 41-ID-tag in-house arrays overlaid with the tissue image. For clarity, the specific features on which particular genes were identified have been circled.

Example 1

Preparation of the array

The following experiments demonstrate how oligonucleotide probes may be attached to an array substrate by either the 5' or 3' end to yield an array with capture probes capable of hybridizing to mRNA.

Preparation of in-house printed microarray with 5' to 3' oriented probes

20 RNA-capture oligonucleotides with individual tag sequences (Tag 1-20, Table 1 were spotted on glass slides to function as capture probes. The probes were synthesized with a 5'-terminus amino linker with a C6 spacer. All probes where synthesized by Sigma-Aldrich (St. Louis, MO, USA). The RNA-capture probes were suspended at a concentration of 20 μM in 150 mM sodium phosphate, pH 8.5 and were spotted using a Nanoplotter NP2.1/E (Gesim, Grosserkmannsdorf, Germany) onto CodeLink™ Activated microarray slides (7.5cm x 2.5cm; Surmodics, Eden Prairie, MN, USA). After printing, surface blocking was performed according to the manufacturer's instructions. The probes were printed in 16 identical arrays on the slide, and each array contained a pre-defined printing pattern. The 16 sub- arrays were separated during hybridization by a 16-pad mask (ChipClip™

Schleicher & Schuell Bioscience, Keene, NH, USA).
Table 1

Name Sequence Sequences for free 3' capture probes

UUAAGTACAAATCTCGACTGCCACTCTGAACCTTCTCCTTCTCCTTCACC
I I I I I I I I I I I I I I I I I I I I I IVN TAP-ID1 (SEQ ID NO: 1)

Enzymatic recog UUAAGTACAA (SEQ ID NO: 2)

Universal amp handle P ATCTCGACTGCCACTCTGAA
(SEQ ID NO: 3)

ID1 CCTTCTCCTTCTCCTTCACC (SEQ ID NO: 4)

Capture sequence 1111111111111111111 IVN (SEQ ID NO: 5)

ID1 CCTTCTCCTTCTCCTTCACC (SEQ ID NO: 6)

ID2 CCTTGCTGCTTCTCCTCCTC (SEQ ID NO: 7)

ID3 ACCTCCTCCGCCTCCTCCTC (SEQ ID NO: 8)

ID4 GAGACATACCACCAAGAGAC (SEQ ID NO: 9)

ID5 GTCCTCTATTCCGTCACCAT (SEQ ID NO: 10)

ID6 GACTGAGCTCGAACATATGG (SEQ ID NO: 11)

ID7 TGGAGGATTGACACAGAACG (SEQ ID NO: 12)

ID8 CCAGCCTCTCCATTACATCG (SEQ ID NO: 13)

ID9 AAGATCTACCAGCCAGCCAG (SEQ ID NO: 14)

ID10 CGAACTTCCACTGTCTCCTC (SEQ ID NO: 15)

ID11 TTG CG CCTTCTCC A ATAC AC (SEQ ID NO: 16)

ID12 CTCTTCTTAGCATGCCACCT (SEQ ID NO: 17)

ID13 ACCACTTCTGCATTACCTCC (SEQ ID NO: 18)

ID14 ACAGCCTCCTCTTCTTCCTT (SEQ ID NO: 19)

ID15 AATCCTCTCCTTGCCAGTTC (SEQ ID NO: 20)

ID16 GATGCCTCCACCTGTAGAAC (SEQ ID NO: 21)

ID17 GAAGGAATGGAGGATATCGC (SEQ ID NO: 22)

ID18 GATCCAAGGACCATCGACTG (SEQ ID NO: 23)

ID19 CCACTGGAACCTGACAACCG (SEQ ID NO: 24)

ID20 CTGCTTCTTCCTGGAACTCA (SEQ ID NO: 25)

Sequences for free 5' surface probes and on-chip free 3' capture probe synthesis

Free 5' surface probe - A GCGTTCAGAGTGGCAGTCGAGATCACGCGGCAATCATATCGGACAGATCGGAAGAGCGTAGTGTAG (SEQ ID NO: 26) Amino C7 Free 5' surface probe - U GCGTTCAGAGTGGCAGTCGAGATCACGCGGCAATCATATCGGACGGCTGCTGGTAAATAGAGATCA (SEQ ID NO: 27) Amino C7

Nick GCG

LP' TTCAGAGTGGCAGTCGAGATCAC (SEQ ID NO: 28)

ID' GCGGCAATCATATCGGAC (SEQ ID NO: 29)

A' 22bp MutY mismatch
AGATCGGAAGAGCGTAGTGTAG (SEQ ID NO: 30)

U' 22bp MutY mismatch G G CTG CTG GT AA AT AG
AG ATC A (SEQ ID NO: 31)

Hybridized sequences for capture probe synthesis

Illumina amp handle A
ACACTCTTTCCCTACACGACGCTCTTCCGATCT
(SEQ ID NO: 32)

Universa ampl handle U
AAGTGTGGAAAGTTGATCGCTATTTACCAGCAGCC
(SEQ ID NO: 33)

Capture_LP_Poly-dTVN
GTGATCTCGACTGCCACTCTGAA I I I I I I I I I I I I I I I I
I I I I VN (SEQ ID NO: 34) Phosphorylated
Capture_LP_Poly-d24T
GTGATCTCGACTGCCACTCTGAA I I I I I I I I I I I I I I I I
I I I I I I I T (SEQ ID NO: 35) Phosphorylated

Additional secondary universal amplification handl
Illumina amp handle B AGACGTGTGCTCTTCCGATCT
(SEQ ID NO: 36) 21 Universal amp handle X
ACGTCTGTGAATAGCCGCAT (SEQ ID NO: 37) 20

B_ 6 handle (or X) AG ACGTGTGCTCTTCCG ATCTN
N N N N N N N (SEQ I D NO: 38) 27 (26)

B_R8 handle (or X) AG ACGTGTGCTCTTCCG ATCTN
N NN N NN NN N (SEQ I D NO: 39) 29 (28)

B_polyTVN (or X) AGACGTGTGCTCTTCCGATC I I I I I
I I I I I I I I I I I I I I I I VN (SEQ I D NO: 40) 43 (42)

B_poly24T (or X) AGACGTGTGCTCTTCCGATCT I I I I
I I I I I I I I I I I I I I I I I I I I T (SEQ I D NO: 41) 45 (44)

Amplification handle to incorporate A handle into P
handle products

A_P handle
ACACTCTTTCCCTACACGACGCTCTTCCGATCTATCTCGACTGCCACTCTGAA
(SEQ ID NO: 42)

Preparation of in-house printed microarray with 3' to 5'
oriented probes and synthesis of 5' to 3' oriented
capture probes

Printing of surface probe oligonucleotides was
performed as in the case with 5' to 3' oriented probes
above, with an amino-C7 linker at the 3' end, as shown
in Table I

To hybridize primers for capture probe synthesis,

hybridization solution containing 4xSSC and 0.1 % SDS, 2µM extension primer (the universal domain oligonucleotide) and 2µM thread joining primer (the capture domain oligonucleotide) was incubated for 4 min at 50°C. Meanwhile the in-house array was attached to a ChipClip (Whatman). The array was subsequently incubated at 50°C for 30 min at 300 rpm shake with 50µl_ of hybridization solution per well.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake; 2) 0.2xSSC for 1 min at 300 rpm shake; and 3) O.lxSSC for 1 min at 300 rpm shake. The array was then spun dry and placed back in the ChipClip.

For extension and ligation reaction (to generate the positional domain of the capture probe) 50µl_ of enzyme mix containing l OxAmpligase buffer, 2.5 U

AmpliTaq DNA Polymerase Stoffel Fragment (Applied Biosystems), 10 U

Ampligase (Epicentre Biotechnologies), dNTPs 2 mM each (Fermentas) and water, was pipetted to each well. The array was subsequently incubated at 55°C for 30 min. After incubation the array was washed according to the previously described array washing method but the first step has the duration of 10 min instead of 6 min.

The method is depicted in Figure 3.

Tissue Preparation

The following experiments demonstrate how tissue sample sections may be prepared for use in the methods of the invention.

Preparation of fresh frozen tissue and sectioning onto capture probe arrays Fresh non-fixed mouse brain tissue was trimmed if necessary and frozen down in -40°C cold isopentane and subsequently mounted for sectioning with a cryostat at 10µηι. A slice of tissue was applied onto each capture probe array to be used.

Preparation of formalin-fixed paraffin-embedded (FFPE) tissue Mouse brain tissue was fixed in 4% formalin at 4° C for 24h. After that it was incubated as follows: 3x incubation in 70% ethanol for 1 hour; 1x incubation in 80% ethanol for 1 hour; 1x incubation in 96% ethanol for 1 hour; 3x incubation in 100% ethanol for 1 hour; and 2x incubation in xylene at room temperature for 1 h.

The dehydrated samples were then incubated in liquid low melting paraffin

52-54°C for up to 3 hours, during which the paraffin was

changed once to wash out residual xylene. Finished tissue blocks were then stored at RT. Sections were then cut at 4μηι in paraffin with a microtome onto each capture probe array to be used.

The sections were dried at 37°C on the array slides for 24 hours and stored at RT.

Deparaffinization of FFPE tissue

Formalin fixed paraffinized mouse brain 10μηι sections attached to

CodeLink slides were deparaffinised in xylene twice for: 10 min, 99.5% ethanol for 2 min; 96% ethanol for 2 min; 70% ethanol for 2 min; and were then air dried. cDNA synthesis

The following experiments demonstrate that mRNA captured on the array from the tissue sample sections may be used as template for cDNA synthesis. cDNA synthesis on chip

A 16 well mask and Chip Clip slide holder from Whatman was attached to a CodeLink slide. The Superscript™ I II One-step RT-PCR System with PlatinumOTaq DNA Polymerase from Invitrogen was used when performing the cDNA synthesis. For each reaction 25 μl 2x reaction mix (Superscript™ 111 One-step RT-PCR System with PlatinumOTaq DNA Polymerase, Invitrogen), 22.5 μl H20 and 0.5 μl 100xBSA were mixed and heated to 50°C. Superscript lll/Platinum Taq enzyme mix was added to the reaction mix, 2 μl per reaction, and 50 μl of the reaction mix was added to each well on the chip. The chip was incubated at 50°C for 30 min

(Thermomixer Comfort, Eppendorf).

The reaction mix was removed from the wells and the slide was washed with: 2xSSC, 0.1 % SDS at 50°C for 10 min; 0.2xSSC at room temperature for 1 min; and O. lxSSC at room temperature for 1 min. The chip was then spin dried.

In the case of FFPE tissue sections, the sections could now be stained and visualized before removal of the tissue, see below section on visualization. Visualization

Hybridization of fluorescent marker probes prior to staining

Prior to tissue application fluorescent marker probes were hybridized to features comprising marker oligonucleotides printed on the capture probe array. The fluorescent marker probes aid in the orientation of the resulting image after tissue visualization, making it

possible to combine the image with the resulting expression profiles for individual capture probe "tag" (positional domain) sequences obtained after sequencing. To hybridize fluorescent probes a hybridization solution containing 4xSSC and 0.1 % SDS, 2μM detection probe (P) was incubated for 4 min at 50° C. Meanwhile the in-house array was attached to a ChipClip (Whatman). The array was subsequently incubated at 50°C for 30 min at 300 rpm shake with 50μl_ of hybridization solution per well.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O.lxSSC for 1 min at 300 rpm shake. The array was then spun dry.

General histological staining of FFPE tissue sections prior to or post cDNA synthesis

FFPE tissue sections immobilized on capture probe arrays were washed and rehydrated after deparaffinization prior to cDNA synthesis as described previously, or washed after cDNA synthesis as described previously. They are then treated as follows: incubate for 3 minutes in Hematoxylin; rinse with deionized water; incubate 5 minutes in tap water; rapidly dip 8 to 12 times in acid ethanol; rinse 2x1 minute in tap water; rinse 2 minutes in deionized water; incubate 30 seconds in Eosin; wash 3x5 minutes in 95% ethanol; wash 3x5 minutes in 100% ethanol; wash 3x10 minutes in xylene (can be done overnight); place coverslip on slides using DPX; dry slides in the hood overnight.

General immunohistochemistry staining of a target protein in FFPE tissue sections prior to or post cDNA synthesis

FFPE tissue sections immobilized on capture probe arrays were washed and rehydrated after deparaffinization prior to cDNA synthesis as described previously, or washed after cDNA synthesis as described previously. They were then treated as follows without being allowed to dry during the whole staining process: sections were incubated with primary antibody (dilute primary antibody in blocking solution comprising lxTris Buffered Saline (50mM Tris, 150mM NaCl, pH 7.6), 4% donkey serum and 0.1 % triton-x) in a wet chamber overnight at RT; rinse three times with 1xTBS; incubate section with matching secondary antibody conjugated to a fluorochrome (FITC, Cy3 or Cy5) in a wet chamber at RT for 1 hour. Rinse 3x with 1xTBS, remove as much as possible of TBS and mount section with ProLong Gold +DAPI (Invitrogen) and analyze with fluorescence microscope and matching filter sets.

Removal of residual tissue

Frozen tissue

For fresh frozen mouse brain tissue the washing step directly following cDNA synthesis was enough to remove the tissue completely.

FFPE tissue

The slides with attached formalin fixed paraffinized mouse brain tissue sections were attached to ChipClip slide holders and 16 well masks (Whatman). For each 150 µl Proteinase K Digest Buffer from the RNeasy FFPE kit (Qiagen), 10 µl Proteinase K Solution (Qiagen) was added. 50 µl of the final mixture was added to each well and the slide was incubated at 56°C for 30 min.

Capture probe (cDNA) release

Capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently attached probes)

A 16 well mask and CodeLink slide was attached to the ChipClip holder (Whatman). 50µl of a mixture containing 1x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 (Roche), 200 µM dNTPs (New England Biolabs) and 0.1 U/1 µl USER Enzyme (New England Biolabs) was heated to 37°C and was added to each well and incubated at 37°C for 30 min with mixing (3 seconds at 300 rpm, 6 seconds at rest) (Thermomixer comfort; Eppendorf). The reaction mixture containing the released cDNA and probes was then recovered from the wells with a pipette. Capture probe release with uracil cleaving USER enzyme mixture in TdT (terminal transferase) buffer (covalentlv attached probes)

50µl of a mixture containing: 1x TdT buffer (20mM Tris-acetate (pH 7.9), 50mM Potassium Acetate and 10mM Magnesium Acetate) (New England Biolabs, www.neb.com); O.^g^l BSA (New England Biolabs); and 0.1 U/µl USER Enzyme (New England Biolabs) was heated to 37°C and was added to each well and incubated at 37°C for 30 min with mixing (3 seconds at 300 rpm, 6 seconds at rest) (Thermomixer comfort; Eppendorf). The reaction mixture containing the released cDNA and probes was then recovered from the wells with a pipette.

Capture probe release with boiling hot water (covalentlv attached probes) A 16 well mask and CodeLink slide was attached to the ChipClip holder (Whatman). 50µl of 99°C water was pipetted into each well. The 99°C water was allowed to react for 30 minutes. The reaction mixture containing the released cDNA and probes was then recovered from the wells with a pipette.

Capture probe release with heated PCR buffer (hybridized in situ synthesized capture probes, i.e. capture probes hybridized to surface probes)

50µl of a mixture containing: 1x TdT buffer (20mM Tris-acetate (pH 7.9), 50mM Potassium Acetate and 10mM Magnesium Acetate) (New England Biolabs, www.neb.com); 0.1 Mg/µl BSA (New England Biolabs); and 0.1 U/µl USER Enzyme (New England Biolabs) was preheated to 95°C. The mixture was then added to each well and incubated for 5 minutes at 95°C with mixing (3 seconds at 300 rpm, 6 seconds at rest) (Thermomixer comfort; Eppendorf). The reaction mixture containing the released probes was then recovered from the wells.

Capture probe release with heated TdT (terminal transferase) buffer (hybridized in situ synthesized capture probes, i.e. capture probes hybridized to surface probes)

50µl of a mixture containing: 1x TdT buffer (20mM Tris-acetate (pH 7.9),

50mM Potassium Acetate and 10mM Magnesium Acetate) (New England Biolabs, www.neb.com); O.^g^l BSA (New England Biolabs); and 0.1 U/µl USER Enzyme (New England Biolabs) was preheated to 95°C. The mixture was then added to each well and incubated for 5 minutes at 95°C with mixing (3 seconds at 300 rpm, 6 seconds at rest) (Thermomixer comfort; Eppendorf). The reaction mixture containing the released probes was then recovered from the wells.

The efficacy of treating the array with the USER enzyme and water heated to 99°C can be seen in Figure 3. Enzymatic cleavage using the USER enzyme and the RsaI enzyme was performed using the "in-house" arrays described above

(Figure 4). Hot water mediated release of DNA surface probes was performed using commercial arrays manufactured by Agilent (see Figure 5).

Probe collection and linker introduction

The experiments demonstrate that first strand cDNA released from the array surface may be modified to produce double stranded DNA and subsequently amplified.

Whole Transcriptome Amplification by the Picoplex whole genome amplification kit (capture probe sequences including positional domain (tag) sequences not retained at the edge of the resulting dsDNA)

Capture probes were released with uracil cleaving

USER enzyme mixture in PCR buffer (covalently attached capture probes) or with heated PCR buffer (hybridized in situ synthesized capture probes, i.e. capture probes hybridized to surface probes).

The released cDNA was amplified using the Picoplex (Rubicon Genomics) random primer whole genome amplification method, which was carried out according to manufacturers instructions.

Whole Transcriptome Amplification by dA tailing with Terminal Transferase (TdT) (capture probe sequences including positional domain (tag) sequences retained at the end of the resulting dsDNA)

Capture probes were released with uracil cleaving USER enzyme mixture in TdT (terminal transferase) buffer (covalently attached capture probes) or with heated TdT (terminal transferase) buffer (hybridized in situ synthesized capture probes, i.e. capture probes hybridized to surface probes).

38µl of cleavage mixture was placed in a clean 0.2ml PCR tube. The mixture contained: 1x TdT buffer (20mM Tris-acetate (pH 7.9), 50mM Potassium Acetate and 10mM Magnesium Acetate) (New England Biolabs, www.neb.com), 0.1 Mg/µl BSA (New England Biolabs); O. I U/µl USER Enzyme (New England Biolabs) (not for heated release); released cDNA (extended from surface probes); and released surface probes. To the PCR tube, 0.5µl RNase H (511/µl, final concentration of 0.0611/µl), 1 µl TdT (201/µl, final concentration of 0.511/µl), and 0.5µl dATPs (100mM, final concentration of 1.25mM), were added. For dA tailing, the tube was incubated in a thermocycler (Applied Biosystems) at 37°C for 15 min followed by an inactivation of TdT at 70°C for 10 min. After dA tailing, a PCR master mix was prepared. The mix contained: 1x Faststart HiFi PCR Buffer (pH 8.3) with 1.8mM MgCl2 (Roche); 0.2mM of each dNTP (Fermentas); 0.2µM of each primer, A (complementary to the amplification domain of the capture probe) and B_ (dT)24 (Eurofins MWG Operon) (complementary to the poly-A tail to be added to the 3' end of the first cDNA strand); and 0.1 U/µl Faststart HiFi DNA polymerase (Roche). 23µl of PCR Master mix was placed into nine clean 0.2ml PCR tubes. 2µl of dA tailing mixture were added to eight of the tubes, while 2µl water (RNase/DNase free) was added to the last tube (negative control). PCR amplification was carried out with the following program: Hot start at 95°C for 2 minutes, second strand synthesis at 50°C for 2 minutes and 72°C for 3 minutes, amplification with 30 PCR cycles at 95°C for 30 seconds, 65°C for 1 minutes, 72°C for 3 minutes, and a final extension at 72°C for 10 minutes.

Post-reaction cleanup and analysis

Four amplification products were pooled together and were processed through a Qiaquick PCR purification column (Qiagen) and eluted into 30µl EB (10mM Tris-Cl, pH 8.5). The product was analyzed on a Bioanalyzer (Agilent). A DNA 1000 kit was used according to manufacturers instructions.

Sequencing

Illumina sequencing

dsDNA library for illumina sequencing using sample indexing was carried out according to manufacturers instructions. Sequencing was carried out on an HiSeq2000 platform (Illumina).

Bioinformatics

Obtaining digital transcriptomic information from sequencing data from whole transcriptome libraries amplified using the dA tailing terminal transferase approach

The sequencing data was sorted through the FastX toolkit FASTQ Barcode splitter tool into individual files for the respective capture probe positional domain (tag) sequences. Individually tagged sequencing data was then analyzed through mapping to the mouse genome with the Tophat mapping tool. The resulting SAM file was processed for transcript counts through the HTseq-count software. Obtaining digital transcriptomic information from sequencing data from whole transcriptome libraries amplified using the Picoplex whole genome amplification kit approach

The sequencing data was converted from FASTQ format to FASTA format using the FastX toolkit FASTQ-to- FASTA converter. The sequencing reads was aligned to the capture probe positional domain (tag) sequences using Blastn and the reads with hits better than 1e<"6> to one of tag sequences were sorted out to individual files for each tag sequence respectively. The file of tag sequence reads was then aligned using Blastn to the mouse transcriptome, and hits were collected. Combining visualization data and expression profiles

The expression profiles for individual capture probe positional domain (tag) sequences are combined with the spatial information obtained from the tissue sections through staining. Thereby the transcriptomic data from the cellular compartments of the tissue section can be analyzed in a directly comparative fashion, with the availability to distinguish distinct expression features for different cellular subtypes in a given structural context

Example 2

Figures 8 to 12 show successful visualisation of stained FFPE mouse brain tissue (olfactory bulb) sections on top of a bar-coded transcriptome capture array, according to the general procedure described in Example 1. As compared with the experiment with fresh frozen tissue in Example 1 , Figure 8 shows better morphology with the FFPE tissue. Figures 9 and 10 show how tissue may be positioned on different types of probe density arrays.

Example 3

Whole Transcriptome Amplification by Random primer second strand synthesis followed by universal handle amplification (capture probe sequences including tag sequences retained at the end of the resulting dsDNA) Following capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently attached probes)

OR

Following capture probe release with heated PCR buffer (hybridized in situ synthesized capture probes)

1 µl RNase H (51l/µl) was added to each of two tubes, final concentration of

0.1211/µl, containing 40µl 1x Faststart HiFi PCR Buffer (pH 8.3) with 1.8mM MgCl2 (Roche, www.roche-applied-science.com), 0.2mM of each dNTP (Fermentas, www.fermentas.com), O. ^/µl BSA (New England Biolabs, www.neb.com), 0.1 U/µl USER Enzyme (New England Biolabs), released cDNA (extended from surface probes) and released surface probes. The tubes were incubated at 37°C for 30 min followed by 70°C for 20 min in a thermo cycler (Applied Biosystems,

www.appliedbiosystems.com). 1 µl Klenow Fragment (3' to 5' exo minus) (Illumina, www.illumina.com) and 1 µl handle coupled random primer (10µM) (Eurofins MWG Operon, www.eurofinsdna.com) was added to the two tubes (B_R8 (octamer) to one of the tubes and B_R6 (hexamer) to the other tube), final concentration of 0.23µM. The two tubes were incubated at 15°C for 15 min, 25°C for 15 min, 37°C for 15 min and finally 75°C for 20 min in a thermo cycler (Applied Biosystems). After the incubation, 1 µl of each primer, A_P and B (10µM) (Eurofins MWG

Operon), was added to both tubes, final concentration of 0.22µM each. 1 µl Faststart HiFi DNA polymerase (5u/µl) (Roche) was also added to both tubes, final concentration of 0.11 U/µl. PCR amplification were carried out in a thermo cycler (Applied Biosystems) with

the following program: Hot start at 94°C for 2 min, followed by 50 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 1 minute, and a final extension at 68°C for 5 minutes. After the amplification, 40µl from each of the two tubes were purified with Qiaquick PCR purification columns (Qiagen, www.qiagen.com) and eluted into 30µl EB (10mM Tris-Cl, pH 8.5). The Purified products were analyzed with a Bioanalyzer (Agilent,

www.home.agilent.com), DNA 7500 kit were used. The results are shown in Figures 13 and 14.

This Example demonstrates the use of random hexamer and random octamer second strand synthesis, followed by amplification to generate the population from the released cDNA molecules. Example 4

Amplification of ID-specific and gene specific products after cDNA synthesis and probe collection

Following capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently attached probes).

The cleaved cDNA was amplified in final reaction volumes of 10 µl. 7 µl cleaved template, 1 µl ID-specific forward primer (2 µM), 1 µl gene-specific reverse primer (2 µM) and 1 µl FastStart High Fidelity Enzyme Blend in 1.4x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 to give a final reaction of 10 µl with 1x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 and 1 U FastStart High Fidelity Enzyme Blend. PCR amplification were carried out in a thermo cycler (Applied Biosystems) with the following program: Hot start at 94°C for 2 min, followed by 50 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 1 minute, and a final extension at 68°C for 5 minutes.

Primer sequences, resulting in a product of approximately 250 bp,

Beta-2 microglobulin (B2M) primer

5'-TGGGGGTGAGAATTGCTAAG-3' (SEQ ID NO: 43)

ID-1 primer

5'-CCTTCTCCTTCTCCTTCACC-3' (SEQ ID NO: 44)

ID-5 primer

5'-GTCCTCTATTCCGTCACCAT-3' (SEQ ID NO: 45)

ID-20 primer

5'-CTGCTTCTTCCTGGAACTCA-3' (SEQ ID NO: 46)

The results are shown in Figure 15. This shows successful amplification of ID-specific and gene-specific products using two different ID primers (i.e.

specific for ID tags positioned at different locations on the microarray and the same gene specific primer from a brain tissue covering all the probes. Accordingly this experiment establishes that products may be identified by an ID tag -specific or target nucleic acid specific amplification reaction. It is further established that different ID tags may be distinguished. A second experiment, with tissue covering only half of the ID probes (i.e. capture probes) on the array resulted in a positive result (PCR product) for spots that were covered with tissue. Example 5

Spatial Genomics

Background. The method has as its purpose to capture DNA molecules from a tissue sample with retained spatial resolution, making it possible to determine from what part of the tissue a particular DNA fragment stems.

Method. The principle of the method is to use microarrays with immobilized DNA oligos (capture probes) carrying spatial labeling tag sequences (positional domains). Each feature of oligos of the microarray carries a 1) a unique labeling tag (positional domain) and 2) a capture sequence (capture domain). Keeping track of where which labeling tag is geographically placed on the array surface makes it possible to extract positional information in two dimensions from each labeling tag. Fragmented genomic DNA is added to the microarray, for instance through the addition of a thin section of FFPE treated tissue. The genomic DNA in this tissue section is pre-fragmented due to the fixation treatment.

Once the tissue slice has been placed on the array, a universal tailing reaction is carried out through the use of a terminal transferase enzyme. The tailing reaction adds polydA tails to the protruding 3' ends of the genomic DNA fragments in the tissue. The oligos on the surface are blocked from tailing by terminal transferase through a hybridized and 3' blocked polydA probe.

Following the terminal transferase tailing, the genomic DNA fragments are able to hybridize to the spatially tagged oligos in their vicinity through the polydA tail meeting the polydT capture sequence on the surface oligos. After hybridization is completed a strand displacing polymerase such as Klenow exo- can use the oligo on the surface as a primer for creation of a new DNA strand complementary to the hybridized genomic DNA fragment. The new DNA strand will now also contain the positional information of the surface oligo's labeling tag.

As a last step the newly generated labeled DNA strands are cleaved from the surface through either enzymatic means, denaturation or physical means. The strands are then collected and can be subjected to downstream amplification of the entire set of strands through introduction of universal handles, amplification of specific amplicons, and/or sequencing.

Figure 16 is a schematic illustration of this process.

Materials and methods

Preparation of in-house printed microarray with 5' to 3' oriented probes 20 DNA-capture oligos with individual tag sequences (Table 1) were spotted on glass slides to function as capture probes. The probes were synthesized with a 5'-terminus amino linker with a C6 spacer. All probes where synthesized by Sigma- Aldrich (St. Louis, MO, USA). The DNA-capture probes were suspended at a concentration of 20 µM in 150 mM sodium phosphate, pH 8.5 and were spotted using a Nanoplotter NP2.1/E (Gesim, Grosserkmannsdorf, Germany) onto

CodeLink™ Activated microarray slides (7.5cm x 2.5cm; Surmodics, Eden Prairie, MN, USA). After printing, surface blocking was performed according to the manufacturer's instructions. The probes were printed in 16 identical arrays on the slide, and each array contained a pre-defined printing pattern. The 16 sub-arrays were separated during hybridization by a 16-pad mask (ChipClip™ Schleicher & Schuell Bioscience, Keene, NH, USA).

Preparation of in-house printed microarray with 3' to 5' oriented probes and synthesis of 5' to 3' oriented capture probes

Printing of oligos was performed as in the case with 5' to 3' oriented probes above.

To hybridize primers for capture probe synthesis hybridization solution containing 4xSSC and 0.1 % SDS, 2 µM extension primer (A_primer) and 2 µM thread joining primer (p_poly_dT) was incubated for 4 min at 50°C. Meanwhile the in-house array was attached to a ChipClip (Whatman). The array was subsequently incubated at 50°C for 30 min at 300 rpm shake with 50 µl_ of hybridization solution per well.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O. lxSSC for 1 min at 300 rpm shake. The array was then spun dry and placed back in the ChipClip.

For extension and ligation 50 µl_ of enzyme mix containing lOxAmpligase buffer, 2.5 U AmpliTaq DNA Polymerase Stoffel Fragment (Applied Biosystems), 10 U Ampligase (Epicentre Biotechnologies), dNTPs 2 mM each (Fermentas) and water, is pipetted to each well. The array is subsequently incubated at 55°C for 30 min. After incubation the array is washed according to previously described array washing method but the first step has the duration of 10 min instead of 6 min. Hybridization of polydA probe for protection of surface oligo capture sequences from dA tailing

To hybridize a 3'-biotin blocked polydA probe for protection of the surface oligo capture sequences a hybridization solution containing 4xSSC and 0.1 % SDS, 2 µM 3'bio-polydA was incubated for 4 min at 50°C. Meanwhile the in-house array was attached to a ChipClip (Whatman). The array was subsequently incubated at 50°C for 30 min at 300 rpm shake with 50 µl_ of hybridization solution per well.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O. lxSSC for 1 min at 300 rpm shake. The array was then spun dry and placed back in the ChipClip.

Preparation of formalin-fixed paraffin-embedded (FFPE) tissue

Mouse brain tissue was fixed in 4% formalin at 4°C for 24h. After that it was incubated as follows: 3x incubation in 70% ethanol for 1 hour, 1x incubation in 80% ethanol for 1 hour, 1x incubation in 96% ethanol for 1 hour, 3x incubation in 100% ethanol for 1 hour, 2x incubation in xylene at room temperature for 1 h.

The dehydrated samples were then incubated in liquid low melting paraffin 52-54 °C for up to 3 hours, during which the paraffin in changed once to wash out residual xylene. Finished tissue blocks were then stored at RT. Sections were then cut at 4 µη in paraffin with a microtome onto each capture probe array to be used.

The sections are dried at 37°C on the array slides for 24 hours and store at

Deparaffinization of FFPE tissue

Formalin fixed paraffinized mouse brain 10 µη sections attached to

CodeLink slides were deparaffinised in xylene twice for 10 min, 99.5% ethanol for 2 min, 96% ethanol for 2 min, 70% ethanol for 2 min and were then air dried.

Universal tailing of genomic DNA

For dA tailing a 50 µl reaction mixture containing 1x TdT buffer (20mM Tris- acetate (pH 7.9), 50mM Potassium Acetate and 10mM Magnesium Acetate) (New England Biolabs, www.neb.com), 0.1 µ9/µl BSA (New England Biolabs) , 1 µl TdT (20u/µl) and 0.5µl dATPs (100mM) was prepared. The mixture was added to the array surface and the array was incubated in a thermo cycler (Applied Biosystems) at 37°C for 15 min followed by an inactivation of TdT at 70°C for 10 min. After this the temperature was lowered to 50°C again to allow for hybridization of dA tailed genomic fragments to the surface oligo capture sequences.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O.lxSSC for 1 min at 300 rpm shake. The array was then spun dry. Extension of labeled DNA

A 50 µl reaction mixture containing 50 µl of a mixture containing 1x Klenow buffer, 200 µM dNTPs (New England Biolabs) and 1 µl Klenow Fragment (3' to 5' exo minus) and was heated to 37°C and was added to each well and incubated at 37°C for 30 min with mixing (3 s. 300 rpm, 6 s. rest) (Thermomixer comfort;

Eppendorf).

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O. lxSSC for 1 min at 300 rpm shake. The array was then spun dry.

Removal of residual tissue

The slides with attached formalin fixed paraffinized mouse brain tissue sections were attached to ChipClip slide holders and 16 well masks (Whatman). For each 150 µl Proteinase K Digest Buffer from the RNeasy FFPE kit (Qiagen) 10 µl Proteinase K Solution (Qiagen) was added. 50 µl of the final mixture was added to each well and the slide was incubated at 56°C for 30 min.

Capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently attached probes)

A 16 well mask and CodeLink slide was attached to the ChipClip holder

(Whatman). 50 µl of a mixture containing 1x FastStart

High Fidelity Reaction Buffer with 1.8 mM MgCl2 (Roche), 200 µM dNTPs (New England Biolabs) and 0.1 U/1 µl USER Enzyme (New England Biolabs) was heated to 37°C and was added to each well and incubated at 37°C for 30 min with mixing (3 s. 300 rpm, 6 s. rest) (Thermomixer comfort; Eppendorf). The reaction mixture containing the released cDNA and probes was then recovered from the wells with a pipette.

Amplification of ID-specific and gene specific products after synthesis of labelled DNA and probe collection

Following capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently attached probes).

The cleaved DNA was amplified in final reaction volumes of 10 µl. 7 µl cleaved template, 1 µl ID-specific forward primer (2 µM), 1 µl gene-specific reverse primer (2 µM) and 1 µl FastStart High Fidelity Enzyme Blend in 1.4x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 to give a final reaction of 10 µl with 1x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 and 1 U FastStart High Fidelity Enzyme Blend. PCR amplification were carried out in a thermo cycler (Applied Biosystems) with the following program: Hot start at 94°C for 2 min, followed by 50 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 1 minute, and a final extension at 68°C for 5 minutes.

Whole Genome Amplification by Random primer second strand synthesis followed by universal handle amplification (capture probe sequences including tag sequences retained at the end of the resulting dsDNA)

Following capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently attached probes).

A reaction mixture containing 40µl 1x Faststart HiFi PCR Buffer (pH 8.3) with 1.8mM MgCl2 (Roche, www.roche-applied-science.com), 0.2mM of each dNTP (Fermentas, www.fermentas.com), O. ^g^l BSA (New England Biolabs, www.neb.com), 0.1 U/µl USER Enzyme (New England Biolabs), released DNA (extended from surface probes) and released surface probes. The tubes were incubated at 37°C for 30 min followed by 70°C for 20 min in a thermo cycler (Applied Biosystems, www.appliedbiosystems.com). 1 µl Klenow Fragment (3' to 5' exo minus) (Illumina, www.illumina.com) and 1 µl handle coupled random primer (10µM) (Eurofins MWG Operon, www.eurofinsdna.com) was added to the tube. The tube was incubated at 15°C for 15 min, 25°C for 15 min, 37° C for 15 min and finally 75°C for 20 min in a thermo cycler (Applied Biosystems). After the incubation, 1 µl of each primer, A_P and B (10µM) (Eurofins MWG

Operon), was added to the tube. 1 µl Faststart HiFi DNA polymerase (5u/µl) (Roche) was also added to the tube. PCR amplification were carried out in a thermo cycler (Applied Biosystems) with the following program: Hot start at 94°C for 2 min, followed by 50 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 1 minute, and a final extension at 68°C for 5 minutes. After the amplification, 40µl from the tube was purified with Qiaquick PCR purification columns (Qiagen, www.qiagen.com) and eluted into 30µl EB (10mM Tris-Cl, pH 8.5). The Purified product was analyzed with a Bioanalyzer (Agilent, www.home.agilent.com), DNA 7500 kit were used.

Visualization

Hybridization of fluorescent marker probes prior to staining

Prior to tissue application fluorescent marker probes are hybridized to designated marker sequences printed on the capture probe array. The fluorescent marker probes aid in the orientation of the resulting image after tissue visualization, making it possible to combine the image with the resulting expression profiles for individual capture probe tag sequences obtained after sequencing. To hybridize fluorescent probes a hybridization solution containing 4xSSC and 0.1 % SDS, 2 µM detection probe (P) was incubated for 4 min at 50° C. Meanwhile the in-house array was attached to a ChipClip (Whatman). The array was subsequently incubated at 50°C for 30 min at 300 rpm shake with 50 µl_ of hybridization solution per well.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O. lxSSC for 1 min at 300 rpm shake. The array was then spun dry. General histological staining of FFPE tissue sections prior to or post synthesis of labeled DNA

FFPE tissue sections immobilized on capture probe arrays are washed and rehydrated after deparaffinization prior to synthesis of labeled as described previously, or washed after synthesis of labeled DNA as described previously. They are then treated as follows: incubate for 3 minutes in Hematoxylin, rinse with deionized water, incubate 5 minutes in tap water, rapidly dip 8 to 12 times in acid ethanol, rinse 2x1 minute in tap water, rinse 2 minutes in deionized water, incubate 30 seconds in Eosin, wash 3x5 minutes in 95% ethanol, wash 3x5 minutes in 100% ethanol, wash 3x10 minutes in xylene (can be done overnight), place coverslip on slides using DPX, dry slides in the hood overnight.

General immunohistochemistry staining of a target protein in FFPE tissue sections prior to or post synthesis of labeled DNA

FFPE tissue sections immobilized on capture probe arrays are washed and rehydrated after deparaffinization prior to synthesis of labeled DNA as described previously, or washed after synthesis of labeled DNA as described previously. They are then treated as follows without being let to dry during the whole staining process: Dilute primary antibody in blocking solution (1xTBS (Tris Buffered Saline (50mM Tris, 150mM NaCl, pH 7.6), 4% donkey serum, 0.1 % triton-x), incubate sections with primary antibody in a wet chamber overnight at RT, rinse 3x with 1xTBS, incubate section with matching secondary antibody conjugated to a fluorochrome (FITC, Cy3 or Cy5) in a wet chamber at RT for 1 h, Rinse 3x with 1xTBS, remove as much as possible of TBS and mount section with ProLong Gold +DAPI (Invitrogen) and analyze with fluorescence microscope and matching filter sets.

Example 6

This experiment was conducted following the principles of Example 5, but using fragmented genomic DNA on the array rather than tissue. The genomic DNA was pre-fragmented to a mean size of 200bp and 700bp respectively. This experiment shows that the principle works. Fragmented genomic DNA is very similar to FFPE tissue.

Amplification of internal gene specific products after synthesis of labelled DNA and probe collection

Following capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently attached probes) containing 1x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 (Roche), 200 µM dNTPs (New England Biolabs) and 0.1 U/1 µl USER Enzyme (New England Biolabs).

The cleaved DNA was amplified in a final reaction volume of 50 µl. To 47 µl cleaved template was added 1 µl ID-specific forward primer (10 µM), 1 µl gene- specific reverse primer (10 µM) and 1 µl FastStart High Fidelity Enzyme Blend. PCR amplification were carried out in a thermo cycler (Applied Biosystems) with the following program: Hot start at 94°C for 2 min, followed by 50 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68° C for 1 minute, and a final extension at 68°C for 5 minutes. Amplification of label-specific and gene specific products after synthesis of labelled DNA and probe collection

Following capture probe release with uracil cleaving USER enzyme mixture in PCR buffer (covalently

attached probes) containing 1x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 (Roche), 200 µM dNTPs (New England Biolabs) and 0.1 U/1 µl USER Enzyme (New England Biolabs).

The cleaved DNA was amplified in a final reaction volume of 50 µl. To 47 µl cleaved template was added 1 µl label-specific forward primer (10 µM), 1 µl gene-specific reverse primer (10 µM) and 1 µl FastStart High Fidelity Enzyme Blend. PCR amplification were carried out in a thermo cycler (Applied Biosystems) with the following program: Hot start at 94°C for 2 min, followed by 50 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 68°C for 1 minute, and a final extension at 68° C for 5 minutes.

Forward - Genomic DNA Human Primer

5'- GACTGCTCTTTTCACCCATC-3' (SEQ ID NO: 47)

Reverse - Genomic DNA Human Primer

5'-GGAGCTGCTGGTGCAGGG-3' (SEQ ID NO: 48)

P - label specific primer

5'- ATCTCGACTGCCACTCTGAA-3' (SEQ ID NO: 49)

The results are shown in Figures 17 to 20. The Figures show internal products amplified on the array - the detected peaks in Figures 17 and 18 are of the expected size. This thus demonstrates that genomic DNA may be captured and amplified. In Figures 19 and 20, the expected product is a smear given that the random fragmentation and terminal transferase labeling of genomic DNA will generate a very diverse sample pool.

Example 7

Alternative synthesis of 5' to 3' oriented capture probes using polymerase extension and terminal transferase tailing To hybridize primers for capture probe synthesis hybridization solution containing 4xSSC and 0.1 % SDS and 2 µM extension primer (A_primer) was incubated for 4 min at 50°C. Meanwhile the in-house array (see Example 1) was attached to a ChipClip (Whatman). The array was subsequently incubated at 50°C for 30 min at 300 rpm shake with 50 µl_ of hybridization solution per well.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O. lxSSC for 1 min at 300 rpm shake. The array was then spun dry and placed back in the ChipClip.

1 µl Klenow Fragment (3' to 5' exo minus) (Illumina, www.illumina.com) together with 10x Klenow buffer, dNTPs 2 mM each (Fermentas) and water, was mixed into a 50 µl reaction and was pipetted into each well.

The array was incubated at 15°C for 15 min, 25°C for 15 min, 37°C for 15 min and finally 75°C for 20 min in an Eppendorf Thermomixer.

After incubation, the array was removed from the ChipClip and washed with the 3 following steps: 1) 50°C 2xSSC solution with 0.1 % SDS for 6 min at 300 rpm shake, 2) 0.2xSSC for 1 min at 300 rpm shake and 3) O. lxSSC for 1 min at 300 rpm shake. The array was then spun dry and placed back in the ChipClip.

For dT tailing a 50 µl reaction mixture containing 1x TdT buffer (20mM Tris- acetate (pH 7.9), 50mM Potassium Acetate and 10mM Magnesium Acetate) (New England Biolabs, www.neb.com), 0.1 µ9/µl BSA (New England Biolabs), 0.5µl RNase H (51l/µl) , 1 µl TdT (201l/µl) and 0.5µl dTTPs (100mM) was prepared. The mixture was added to the array surface and the array was incubated in a thermo cycler (Applied Biosystems) at 37°C for 15 min followed by an inactivation of TdT at 70°C for 10 min.

Example 8

Spatial transcriptomics using 5' to 3' high probe density arrays and formalin- fixed frozen (FF-frozen) tissue with USER system cleavage and amplification via terminal transferase

Array preparation

Pre-fabricated high-density microarrays chips were ordered from Roche- Nimblegen (Madison, Wi, USA). Each capture probe array contained 135,000 features of which 132,640 features carried a capture probe comprising a unique ID- tag sequence (positional domain) and a capture region (capture domain). Each feature was 13x13 µn; in size. The capture probes were composed 5' to 3' of a universal domain containing five dUTP bases (a cleavage domain) and a general amplification domain, an ID tag (positional domain) and a capture region (capture domain) (Figure 22 and Table 2). Each array was also fitted with a frame of marker probes (Figure 23) carrying a generic 30 bp sequence (Table 2) to enable hybridization of fluorescent probes to help with orientation during array

visualization.

Tissue preparation - preparation of formalin-fixed frozen tissue

The animal (mouse) was perfused with 50ml PBS and 100ml 4% formalin solution. After excision of the olfactory bulb, the tissue was put into a 4% formalin bath for post-fixation for 24 hrs. The tissue was then sucrose treated in 30% sucrose dissolved in PBS for 24 hrs to stabilize morphology and to remove excess formalin. The tissue was frozen at a controlled rate down to -40°C and kept at -20°C between experiments. Similar preparation of tissue postfixed for 3 hrs or without post-fixation was carried out for a parallel specimen. Perfusion with 2% formalin without post-fixation was also used successfully. Similarly the sucrose treatment step could be omitted. The tissue was mounted into a cryostat for sectioning at 10μηι. A slice of tissue was applied onto each capture probe array to be used. Optionally for better tissue adherence, the array chip was placed at 50°C for 15 minutes. Optional control - Total RNA preparation from sectioned tissue

Total RNA was extracted from a single tissue section (10μηι) using the RNeasy FFPE kit (Qiagen) according to manufacturers instructions. The total RNA obtained from the tissue section was used in control experiments for a comparison with experiments in which the RNA was captured on the array directly from the tissue section. Accordingly, in the case where totalRNA was applied to the array the staining, visualization and degradation of tissue steps were omitted.

On-chip reactions

The hybridization of marker probe to the frame probes, reverse transcription, nuclear staining, tissue digestion and probe cleavage reactions were all performed in a 16 well silicone gasket (Arrayit, Sunnyvale, CA, USA) with a reaction volume of 50 µl per well. To prevent evaporation, the cassettes were covered with plate sealers (In Vitro AB, Stockholm, Sweden). Optional - tissue permeabilization prior to cDNA synthesis

For permeabilization using Proteinase K, proteinase K (Qiagen, Hilden, Germany) was diluted to 1 µg/ml in PBS. The solution was added to the wells and the slide incubated at room temperature for 5 minutes, followed by a gradual increase to 80°C over 10 minutes. The slide was washed briefly in PBS before the reverse transcription reaction.

Alternatively for permeabilization using microwaves, after tissue attachment, the slide was placed at the bottom of a glass jar containing 50ml 0.2xSSC (Sigma-Aldrich) and was heated in a microwave oven for 1 minute at 800W. Directly after microwave treatment the slide was placed onto a paper tissue and was dried for 30 minutes in a chamber protected from unnecessary

air exposure. After drying, the slide was briefly dipped in water (RNase/DNase free) and finally spin-dried by a centrifuge before cDNA synthesis was initiated. cDNA synthesis

For the reverse transcription reaction the Superscript III One-Step RT-PCR

System with Platinum Taq (Life Technologies/Invitrogen, Carlsbad, CA, USA) was used. Reverse transcription reactions contained 1x reaction mix, 1x BSA (New England Biolabs, Ipswich, MA, USA) and 2 µl Superscript III RT/Platinum Taq mix in a final volume of 50 µl. This solution was heated to 50°C before application to the tissue sections and the reaction was performed at 50°C for 30 minutes. The reverse transcription solution was subsequently removed from the wells and the slide was allowed to air dry for 2 hours.

Tissue visualization

After cDNA synthesis, nuclear staining and hybridization of the marker probe to the frame probes (probes attached to the array substrate to enable orientation of the tissue sample on the array) was done simultaneously. A solution with DAPI at a concentration of 300nM and marker probe at a concentration of 170 nM in PBS was prepared. This solution was added to the wells and the slide was incubated at room temperature for 5 minutes, followed by brief washing in PBS and spin drying.

Alternatively the marker probe was hybridized to the frame probes prior to placing the tissue on the array. The marker probe was then diluted to 170 nM in hybridization buffer (4xSSC, 0.1 % SDS). This solution was heated to 50°C before application to the chip and the hybridization was performed at 50°C for 30 minutes at 300 rpm. After hybridization, the slide was washed in 2xSSC, 0.1 % SDS at 50°C and 300 rpm for 10 minutes, 0.2xSSC at 300 rpm for 1 minute and O. lxSSC at 300 rpm for 1 minute. In that case the staining solution after cDNA synthesis only contained the nuclear DAPI stain diluted to 300nM in PBS. The solution was applied to the wells and the slide was incubated at room temperature for 5 minutes, followed by brief washing in PBS and spin drying.

The sections were microscopically examined with a Zeiss Axio Imager Z2 and processed with MetaSystems software.

Tissue removal

The tissue sections were digested using Proteinase K diluted to 1.25 g/µl in PKD buffer from the RNeasy

FFPE Kit (both from Qiagen) at 56°C for 30 minutes with an interval mix at 300 rpm for 3 seconds, then 6 seconds rest. The slide was subsequently washed in 2xSSC, 0.1 % SDS at 50°C and 300 rpm for 10 minutes, 0.2xSSC at 300 rpm for 1 minute and O. lxSSC at 300 rpm for 1 minute.

Probe release

The 16-well Hybridization Cassette with silicone gasket (Arrayit) was preheated to 37°C and attached to the Nimblegen slide. A volume of 50µl of cleavage mixture preheated to 37°C, consisting of Lysis buffer at an unknown concentration (Takara), 0.1 U/µl USER Enzyme (NEB) and O. ^g^l BSA was added to each of wells containing surface immobilized cDNA. After removal of bubbles the slide was sealed and incubated at 37°C for 30 minutes in a Thermomixer comfort with cycled shaking at 300 rpm for 3 seconds with 6 seconds rest in between. After the incubation 45µl cleavage mixture was collected from each of the used wells and placed into 0.2ml PCR tubes (Figure 24). Library preparation

Exonuclease treatment

After cooling the solutions on ice for 2 minutes, Exonuclease I (NEB) was added, to remove unextended cDNA probes, to a final volume of 46.2µl and a final concentration of 0.5211/µl. The tubes were incubated in a thermo cycler (Applied Biosystems) at 37°C for 30 minutes followed by inactivation of the exonuclease at 80°C for 25 minutes. dA-tailing by terminal transferase

After the exonuclease step, 45µl polyA-tailing mixture, according to manufacturers instructions consisting of TdT Buffer (Takara), 3mM dATP (Takara) and manufacturers TdT Enzyme mix (TdT and RNase H) (Takara), was added to each of the samples. The mixtures were incubated in a thermocycler at 37°C for 15 minutes followed by inactivation of TdT at 70°C for 10 minutes.

Second-strand synthesis and PCR-amplification

After dA-tailing, 23µl PCR master mix was placed into four new 0.2ml PCR tubes per sample, to each tube 2µl sample was added as a template. The final PCRs consisted of 1x Ex Taq buffer (Takara), 200µM of each dNTP (Takara), 600nM A_primer (MWG), 600nM B_dT20VN_primer (MWG) and 0.0251I/µl Ex Taq polymerase (Takara)(Table 2). A second cDNA strand was created by running one cycle in a thermocycler at 95°C for 3 minutes, 50°C for 2 minutes and 72°C for 3 minutes. Then the samples were amplified by running 20 cycles (for library preparation) or 30 cycles (to confirm the presence of cDNA) at 95°C for 30 seconds,

67°C for 1 minute and 72°C for 3 minutes, followed by a final extension at 72°C for 10 minutes.

Library cleanup

After amplification, the four PCRs (100µl) were mixed with 500µl binding buffer (Qiagen) and placed in a Qiaquick PCR purification column (Qiagen) and spun for 1 minute at 17,900 x g in order to bind the amplified cDNA to the membrane. The membrane was then washed with wash buffer (Qiagen) containing ethanol and finally eluted into 50µl of 10mM Tris-Cl, pH 8.5. The purified and concentrated sample was further purified and concentrated by CA-purification (purification by superparamagnetic beads conjugated to carboxylic acid) with an MBS robot (Magnetic Biosolutions). A final PEG

concentration of 10% was used in order to remove fragments below 150-200bp. The amplified cDNA was allowed to bind to the CA-beads (invitrogen) for 10 min and were then eluted into 15µl of 10mM Tris-Cl, pH 8.5.

Library Quality analysis

Samples amplified for 30 cycles were analyzed with an Agilent Bioanalyzer (Agilent) in order to confirm the presence of an amplified cDNA library, the DNA High Sensitivity kit or DNA 1000 kit were used depending on the amount of material.

Sequencing library preparation

Library indexing

Samples amplified for 20 cycles were used further to prepare sequencing libraries. An index PCR master mix was prepared for each sample and 23µl was placed into six 0.2ml tubes. 2µl of the amplified and purified cDNA was added to each of the six PCRs as template making the PCRs containing 1x Phusion master mix (Fermentas), 500nM InPELO (Illumina), 500nM Index 1-12 (illumina), and 0.4nM inPE2.0 (illumina). The samples were amplified in a thermocycler for 18 cycles at 98°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. Sequencing library cleanup

After amplification, the six PCRs (150µl) were mixed with 750µl binding buffer and placed in a Qiaquick PCR purification column and spun for 1 minute at 17,900 x g in order to bind the amplified cDNA to the membrane (because of the large sample volume (900µl), the sample was split in two (each 450µl) and was bound in two separate steps). The membrane was then washed with wash buffer containing ethanol and finally eluted into 50µl of 10mM Tris-Cl, pH 8.5.

The purified and concentrated sample was further purified and concentrated by CA-purification with an MBS robot. A final PEG concentration of 7.8% was used in order to remove fragments below 300-350bp. The amplified cDNA was allowed to bind to the CA-beads for 10 min and were then eluted into 15µl of 10mM Tris-Cl, pH 8.5. Samples were analyzed with an Agilent Bioanalyzer in order to confirm the presence and size of the finished libraries, the DNA High Sensitivity kit or DNA 1000 kit were used according to manufacturers instructions depending on the amount of material (Figure 25).

Sequencing

The libraries were sequenced on the Illumina Hiseq2000 or Miseq depending on desired data throughput according to manufacturers instructions. Optionally for read 2, a custom sequencing primer B_r2 was used to avoid sequencing through the homopolymeric stretch of 20T.

Data analysis

Read 1 was trimmed 42 bases at 5' end. Read 2 was trimmed 25 bases at 5' end (optionally no bases were trimmed from read 2 if the custom primer was used). The reads were then mapped with bowtie to the repeat masked Mus musculus 9 genome assembly and the output was formatted in the SAM file format. Mapped reads were extracted and annotated with UCSC refGene gene annotations. Indexes were retrieved with 'indexFinder' (an inhouse software for index retrieval). A mongo DB database was then created containing information about all caught transcripts and their respective index position on the chip.

A matlab implementation was connected to the database and allowed for spatial visualization and analysis of the data (Figure 26).

Optionally the data visualization was overlaid with the microscopic image using the fluorescently labelled frame probes for exact alignment and enabling spatial transcriptomic data extraction.

Example 9

Spatial transcriptomics using 3' to 5' high probe density arrays and FFPE tissue with MutY system cleavage and amplification via TdT

Array preparation

Pre-fabricated high-density microarrays chips were ordered from Roche- Nimblegen (Madison, WI, USA).

Each used capture probe array contained 72k features out of which 66,022 contained a unique ID-tag complementary sequence. Each feature was 16x16 µm in size. The capture probes were composed 3' to 5' in the same way as the probes used for the in-house printed 3' to 5' arrays with the exeception to 3 additional bases being added to the upper (P') general handle of the probe to make it a long version of P', LP' (Table 2). Each array was also fitted with a frame of probes carrying a generic 30 bp sequence to enable hybridization of fluorescent probes to help with orientation during array visualization.

Synthesis of 5' to 3' oriented capture probes

The synthesis of 5' to 3' oriented capture probes on the high-density arrays was carried out as in the case with in-house printed arrays, with the exception that the extension and ligation steps were carried out at 55°C for 15 mins followed by 72°C for 15 mins. The A-handle probe (Table 2) included an A/G mismatch to allow for subsequent release of probes through the MutY enzymatic system described below. The P-probe was replaced by a longer LP version to match the longer probes on the surface.

Preparation of formalin-fixed paraffin-embedded tissue and deparaffinization This was carried out as described above in the in-house protocol. cDNA synthesis and staining

cDNA synthesis and staining was carried out as in the protocol for 5' to 3' oriented high-density Nimblegen arrays with the exception that biotin labeled dCTPs and dATPs were added to the cDNA synthesis together with the four regular dNTPs (each was present at 25x times more than the biotin labeled ones).

Tissue removal

Tissue removal was carried out in the same way as in the protocol for 5' to 3' oriented high-density Nimblegen arrays described in Example 8. Probe cleavage by MutY

A 16-well Incubation chamber with silicone gasket (ArrayIT) was preheated to 37°C and attached to the Codelink slide. A volume of 50µl of cleavage mixture preheated to 37°C, consisting of 1x Endonucelase VIII Buffer (NEB), IOU/µl MutY (Trevigen), IOU/µl Endonucelase VIII (NEB), O. ^/µl BSA was added to each of wells containing surface immobilized cDNA. After removal of bubbles the slide was sealed and incubated at 37°C for 30 minutes in a Thermomixer comfort with cycled shaking at 300rpm for 3 seconds with 6 seconds rest in between. After the incubation, the plate sealer was removed and 40µl cleavage mixture was collected from each of the used wells and placed

into a PCR plate.

Library preparation

Biotin-streptavidin mediated library cleanup

To remove unextended cDNA probes and to change buffer, the samples were purified by binding the biotin labeled cDNA to streptavidin coated C1 -beads (Invitrogen) and washing the beads with 0.1 M NaOH (made fresh). The purification was carried out with an MBS robot (Magnetic Biosolutions), the biotin labelled cDNA was allowed to bind to the C1 -beads for 10 min and was then eluted into 20µl of water by heating the bead-water solution to 80°C to break the biotin-streptavidin binding. dA-tailing by terminal transferase

After the purification step, 18µl of each sample was placed into new 0.2ml PCR tubes and mixed with 22µl of a polyA-tailing master mix leading to a 40µl reaction mixture according to manufacturers instructions consisting of lysis buffer (Takara, Cellamp Whole Transcriptome Amplification kit), TdT Buffer (Takara), 1.5mM dATP (Takara) and TdT Enzyme mix (TdT and RNase H) (Takara). The mixtures were incubated in a thermocycler at 37°C for 15 minutes followed by inactivation of TdT at 70°C for 10 minutes. Second-strand synthesis and PCR-amplification

After dA-tailing, 23µl PCR master mix was placed into four new 0.2ml PCR tubes per sample, to each tube 2µl sample was added as a template. The final PCRs consisted of 1x Ex Taq buffer (Takara), 200µM of each dNTP (Takara), 600nM A_primer (MWG), 600nM B_dT20VN_primer (MWG) and 0.025Il/µl Ex Taq polymerase (Takara). A second cDNA strand was created by running one cycle in a thermo cycler at 95°C for 3 minutes, 50°C for 2 minutes and 72°C for 3 minutes. Then the samples were amplified by running 20 cycles (for library preparation) or 30 cycles (to confirm the presence of cDNA) at 95°C for 30 seconds, 67°C for 1 minute and 72°C for 3 minutes, followed by a final extension at 72°C for 10 minutes. Library cleanup

After amplification, the four PCRs (1 OOµl) were mixed with 500µl binding buffer (Qiagen) and placed in a Qiaquick PCR purification column (Qiagen) and spun for 1 minute at 17,900 x g in order to bind the amplified cDNA to the membrane. The membrane was then washed with wash buffer (Qiagen) containing ethanol and finally eluted into 50µl of 10mM Tris-HCl, pH 8.5.

The purified and concentrated sample was further purified and concentrated by CA-purification (purification by superparamagnetic beads conjugated to carboxylic acid) with an MBS robot (Magnetic Biosolutions). A final PEG

concentration of 10% was used in order to remove fragments below 150-200bp. The amplified cDNA was allowed to bind to the CA-beads (invitrogen) for 10 min and were then eluted into 15µl of 10mM Tris-HCl, pH 8.5.

Second PCR-amplification

The final PCRs consisted of 1x Ex Taq buffer (Takara), 200µM of each dNTP (Takara), 600nM A_primer (MWG), 600nM B_ primer (MWG) and 0.025U/µl Ex Taq polymerase (Takara). The samples were heated to 95°C for 3 minutes, and then amplified by running 10 cycles at 95°C for 30 seconds, 65°C for 1 minute and 72°C for 3 minutes, followed by a final extension at 72° C for 10 minutes.

Second library cleanup

After amplification, the four PCRs (100µl) were mixed with 500µl binding buffer (Qiagen) and placed in a Qiaquick PCR purification column (Qiagen) and spun for 1 minute at 17,900 x g in order to bind the amplified cDNA to the membrane. The membrane was then washed with wash buffer (Qiagen) containing ethanol and finally eluted into 50µl of 10mM Tris-Cl, pH 8.5.

The purified and concentrated sample was further purified and concentrated by CA-purification (purification by super-paramagnetic beads conjugated to carboxylic acid) with an MBS robot (Magnetic Biosolutions). A final PEG

concentration of 10% was used in order to remove fragments below 150-200bp.

The amplified cDNA was allowed to bind to the CA-beads (invitrogen) for 10 min and were then eluted into 15µl of 10mM Tris-HCl, pH 8.5. Sequencing library preparation

Library indexing

Samples amplified for 20 cycles were used further to prepare sequencing libraries. An index PCR master mix was prepared for each sample and 23µl was placed into six 0.2ml tubes. 2µl of the amplified and purified cDNA was added to each of the six PCRs as template making the PCRs containing 1x Phusion master mix (Fermentas), 500nM InPELO (Illumina), 500nM Index 1-12 (Illumina), and 0.4nM InPE2.0 (Illumina). The samples were amplified in a thermo cycler for 18 cycles at 98°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes.

Sequencing library cleanup

After amplification, the samples was purified and concentrated by CA- purification with an MBS robot. A final PEG concentration of 7.8% was used in order to remove fragments below 300-350bp. The amplified cDNA was allowed to bind to the CA-beads for 10 min and were then eluted into 15µl of 10mM Tris-HCl, pH 8.5.

10µl of the amplified and purified samples were placed on a Caliper XT chip and fragments between 480bp and 720bp were cut out with the Caliper XT

(Caliper). Samples were analyzed with an Agilent Bioanalyzer in order to confirm the presence and size of the finished libraries, the DNA High Sensitivity kit was used.

Sequencing and Data analysis

Sequencing and Bioinformatic was carried out in the same way as in the protocol for 5' to 3' oriented high-density Nimblegen arrays described in Example 8. However, in the data analysis, read 1 was not used in the mapping of transcripts. Specific Olfr transcripts could be sorted out using the Matlab visualization tool (Figure 27).

Example 10

Spatial transcriptomics using in house printed 41-taq microarray with 5' to 3' oriented probes and formalin-fixed frozen (FF-frozen) tissue with permeabilization through ProteinaseK or microwaving with USER system cleavage and amplification via TdT

Array preparation

In-house arrays were printed as previously described but with a pattern of

41 unigue ID-tag probes with the same composition as the probes in the 5' to 3' oriented high-density array in Example 8 (Figure 28).

All other steps were carried out in the same way as in the protocol described in Example 8.

Example 11

Alternative method for performing the cDNA synthesis step

cDNA synthesis on chip as described above can also be combined with template switching to create a second strand by adding a template switching primer to the

cDNA synthesis reaction (Table 2). The second amplification domain is introduced by coupling it to terminal bases added by the reverse transcriptase at the 3' end of the first cDNA strand, and primes the synthesis of the second strand. The library can be readily amplified directly after release of the double-stranded complex from the array surface.

Example 12

Spatial genomics using in house printed 41 -tag microarray with 5' to 3' oriented probes and fragmented poly-A tailed gDNA with USER system cleavage and amplification via TdT -tailing or translocation specific primers

Array preparation

In-house arrays were printed using Codelink slides (Surmodics) as previously described but with a pattern of 41 unigue ID-tag probes with the same composition as the probes in the 5' to 3' oriented high-density in Example 8.

Total DNA preparation from cells

DNA fragmentation

Genomic DNA (gDNA) was extracted by DNeasy kit (Qiagen) according to the manufacturer's instructions from A431 and U20S cell lines. The DNA was fragmented to 500 bp on a Covaris sonicator (Covaris) according to manufacturer's instructions.

The sample was purified and concentrated by CA-purification (purification by super-paramagnetic beads conjugated to carboxylic acid) with an MBS robot (Magnetic Biosolutions). A final PEG concentration of 10% was used in order to remove fragments below 150-200bp. The fragmented DNA was allowed to bind to the CA-beads (Invitrogen) for 10 min and were then eluted into 15µl of 10mM Tris- HCl, pH 8.5. Optional control - spiking of different cell lines

Through spiking of A431 DNA into U20S DNA different levels of capture sensitivity can be measured, such as from spiking of 1 %, 10% or 50% of A431 DNA. dA-tailing by terminal transferase

A 45µl polyA-tailing mixture, according to manufacturer's instructions consisting of TdT Buffer (Takara), 3mM dATP (Takara) and TdT Enzyme mix (TdT and RNase H) (Takara), was added to 0^g of fragmented DNA. The mixtures were incubated in a thermocycler at 37°C for 30 minutes followed by inactivation of TdT at 80°C for 20 minutes. The dA-tailed fragments were then cleaned through a Qiaquick

(Qiagen) column according to manufacturer's instructions and the concentration was measured using the Qubit system (Invitrogen) according to manufacturer's instructions. On-chip experiments

The hybridization, second strand synthesis and cleavage reactions were performed on chip in a 16 well silicone gasket (Arrayit, Sunnyvale, CA, USA). To prevent evaporation, the cassettes were covered with plate sealers (in Vitro AB, Stockholm, Sweden).

Hybridization

1 17 ng of DNA was deposited onto a well on a prewarmed array (50°C) in a total volume of 45 µl consisting of 1x NEB buffer (New England Biolabs) and 1x BSA. The mixture was incubated for 30mins at 50°C in a Thermomixer Comfort (Eppendorf) fitted with an MTP block at 300 rpm shake.

Second strand synthesis

Without removing the hybridization mixture, 15µl of a Klenow extension reaction mixture consisting of 1x NEB buffer 1.5µl Klenow polymerase, and 3.75µl dNTPs (2mM each) was added to the well. The reaction mixture was incubated in a Thermomixer Comport (Eppendorf) 37°C for 30 mins without shaking.

The slide was subsequently washed in 2xSSC, 0.1 % SDS at 50°C and 300 rpm for 10 minutes, 0.2xSSC at 300 rpm for 1 minute and O. lxSSC at 300 rpm for 1 minute.

Probe release

A volume of 50µl of a mixture containing 1x FastStart High Fidelity Reaction Buffer with 1.8 mM MgCl2 (Roche), 200 µM dNTPs (New England Biolabs), 1x BSA and 0.1 U/1 µl USER Enzyme (New England Biolabs) was heated to 37°C and was added to each well and incubated at 37°C for 30 min with mixing (3 seconds at 300 rpm, 6 seconds at rest) (Thermomixer comfort; Eppendorf). The reaction mixture containing the released DNA which was then recovered from the wells with a pipette.

Library preparation

Amplification reaction

Amplification was carried out in 10µl reactions consisting of 7.5 µl released sample, 1 µl of each primer and 0.5µl enzyme (Roche, FastStart HiFi PCR system). The reaction was cycled as 94°C for 2 mins, one cycle of 94°C 15 sec, 55°C for 2mins, 72°C for 2mins, 30 cycles of 94°C for 15 sees, 65°C for 30 sees, 72°C for

90 sees, and a final elongation at 72°C for 5 mins.

In the preparation of a library for sequencing the two primers consisted of the surface probe A-handle and either of a specific translocation primer (for A431) or a specific SNP primer coupled to the B-handle (Table 2).

Library cleanup

The purified and concentrated sample was further purified and concentrated by CA-purification (purification by superparamagnetic beads conjugated to carboxylic acid) with an MBS robot (Magnetic Biosolutions). A final PEG

concentration of 10% was used in order to remove fragments below 150-200bp. The amplified DNA was allowed to bind to the CA-beads (Invitrogen) for 10 min and was then eluted into 15µl of 10mM Tris-HCl, pH 8.5.

Library Quality analysis

Samples were analyzed with an Agilent Bioanalyzer (Agilent) in order to confirm the presence of an amplified DNA library, the DNA High Sensitivity kit or DNA 1000 kit were used depending on the amount of material.

Library indexing

Samples amplified for 20 cycles were used further to prepare sequencing libraries. An index PCR master mix was prepared for each sample and 23µl was placed into six 0.2ml tubes. 2µl of the amplified and purified cDNA was added to each of the six PCRs as template making the PCRs containing 1x Phusion master mix (Fermentas), 500nM InPELO (Illumina), 500nM Index 1-12 (Illumina), and 0.4nM InPE2.0 (Illumina). The samples were amplified in a thermo cycler for 18 cycles at 98°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes.

Sequencing library cleanup

The purified and concentrated sample was further purified and concentrated by CA-purification with an MBS robot. A final PEG concentration of 7.8% was used in order to remove fragments below 300-350bp. The amplified DNA was allowed to bind to the CA-beads for 10 min and were then eluted into 15µl of 10mM Tris-Cl, pH 8.5. Samples were analyzed with an Agilent Bioanalyzer in order to confirm the presence and size of the finished libraries, the DNA High Sensitivity kit or DNA 1000 kit were used according to manufacturers instructions depending on the amount of material (Figure 29).

Sequencing

Sequencing was carried out in the same way as in the protocol for 5' to 3' oriented high-density Nimblegen arrays described in Example 8. Data analysis

Data analysis was carried out to determine the sensitivity of capture of the arrayed ID-capture probes. Read 2 was sorted based on its content of either of the translocation or SNP primers. These reads were then sorted per their ID contained in Read 1.

Optional Control - Direct amplification of cell-line specific translocations This was used to measure the capture sensitivity of spiked cell lines directly by PCR. The forward and reverse primers (Table 2) for the A431 translocations were used to try and detect the presence of the translocation in the second strand copied and released material (Figure 30).

Table 2. Oligos used for spatial transcriptomics and spatial genomics

Example 8

Nimblegen 5' to 3' arrays with free 3' end Array probes

5' to 3'

Probel (SEQ ID NO: 50)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCCGATATGATTGCCGC
I I I I I I I I I I I I I I I I I I I I I VN Probe2 (SEQ ID NO: 51)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATGAGCCGGGTTCATC
I I I I I I I I I I I I I I I I I I I I I I I VN Probe3 (SEQ ID NO: 52)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTGAGGCACTCTGTTGGGA
I I I I I I I I I I I I I I I I I I I I I I VN Probe4 (SEQ ID NO: 53)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATGATTAGTCGCCATTCG
I I I I I I I I I I I I I I I I I I I I I I VN Probe5 (SEQ ID NO: 54)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTACTTGAGGGTAGATG
I I I I I I I I I I I I I I I I I I I I I I I I VN Probe6 (SEQ ID NO:
55)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATGGCCAATACTGTTATC
I I I I I I I I I I I I I I I I I I I I I I VN Probe7 (SEQ ID NO: 56)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCGCTACCCTGATTCGACC
I I I I I I I I I I I I I I I I I I I I I I VN Probe8 (SEQ ID NO: 57) U
U U
UUACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCCACTTTCGCCGTAG
I I I I I I I I I I I I I I I I I I I I I V N Probe9 (SEQ ID NO: 58)
U U U U U AC ACTCTTTCCCTAC ACG
ACGCTCTTCCG ATCTAGC AACTTTG AGC A AG A I I
I I I I I I I I I I I I I I I I I I VN

ProbelO (SEQ ID NO: 59)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCAATTCGGAATTCCGG
I I I I I I I I I I I I I I I I I I I I I VN

Probe1 (SEQ ID NO: 60)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGCCCAAGGTAATACA
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe2 (SEQ ID NO: 61) U U U U U U AC
ACTCTTTCCCTAC ACG ACGCTCTTCCG
ATCTTCGCATTTCCTATTCG AG I I I I I I I I I I I I I I I I I I I
I I I VN

Probe3 (SEQ ID NO: 62)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGCTAAATCTAACCGCC
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe4 (SEQ ID NO: 63)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAATTAAATTCTGATGG
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe5 (SEQ ID NO: 64)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCATTACATAGGTGCTAAG
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe6 (SEQ ID NO: 65)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATTGACTTGCGCTCGCAC
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe7 (SEQ ID NO: 66)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTATAGTATCTCCCAAGTTC
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe8 (SEQ ID NO: 67) U U U U U U AC
ACTCTTTCCCTAC ACG ACGCTCTTCCG
ATCTGTGCGCCTGTAATCCGC A I I I I I I I I I I I I I I I I I I I
I I I I VN

Probe9 (SEQ ID NO: 68)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGCCACTCTTTAGGTAG
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe20 (SEQ ID NO: 69)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTATGCAAGTGATTGGC
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe21 (SEQ ID NO: 70)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCCAAGCCACGTTTATACG
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe22 (SEQ ID NO: 71) U U U
UUACACTCTTTCCCTACACGACGCTCTTCCGATCTACCTGATTGCTGTATAAC
I I I I I I I I I I I I I I I I I I I I I I I I I I I I V N

Probe23 (SEQ ID NO: 72)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGCGCATCTATCCTCTA
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe24 (SEQ ID NO: 73)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCACGCGTAGGACTAG
I I I I I I I I I I I I I I I I I I I I I I I I I I I I I VN

Probe25 (SEQ ID NO: 74)
UUUUUACACTCTTTCCCTACACGACGCTCTTCCGATCTCGACTAAGTATGTAGCGC
I I I I I I I I I I I I I I I I I I I I I VN

Frame probe

Layoutl (SEQ ID NO: 75)
AAATTTCGTCTGCTATCGCGCTTCTGTACC

Fluorescent marker probe

PS_1 (SEQ ID NO: 76)
GGTACAGAAGCGCGATAGCAG

Second strand synthesis and first PCR Amplification handles

A_primer (SEQ ID NO: 77)
ACACTCTTTCCCTACACGACGCTCTTCCGATCT

B_dt20VN_primer

(SEQ ID NO: 78) AGACGTGTGCTCTTCCGATC I I I I I
I I I I I I I I I I I I I I I VN

Custom sequencing primer

B_r2 (SEQ ID NO: 79) TCA GAC GTG TGC TCT TCC
GAT CTT TIT TIT TTT TTT TTT TTT T

Example 9

Nimblegen 3' to 5' arrays with free 5' end Array probes

5' to 3'

Probel (SEQ ID NO 80)
GCGTTCAGAGTGGCAGTCGAGATCACGCGGCAATCATATCGGACAGATCGGAAGAGCGTAGTGTAG

Probe2 (SEQ ID NO 81)
GCGTTCAGAGTGGCAGTCGAGATCACAAGATGAACCCGGCTCATAGATCGGAAGAGCGTAGTGTAG

Probe3 (SEQ ID NO 82)
GCGTTCAGAGTGGCAGTCGAGATCACTCCCAACAGAGTGCCTCAAGATCGGAAGAGCGTAGTGTAG

Probe4 (SEQ ID NO 83)
GCGTTCAGAGTGGCAGTCGAGATCACCGAATGGCGACTAATCATAGATCGGAAGAGCGTAGTGTAG

Probe5 (SEQ ID NO 84)
GCGTTCAGAGTGGCAGTCGAGATCACAAACATCTACCCTCAAGTAGATCGGAAGAGCGTAGTGTAG

Probe6 (SEQ ID NO 85)
GCGTTCAGAGTGGCAGTCGAGATCACGATAACAGTATTGGCCATAGATCGGAAGAGCGTAGTGTAG

Probe7 (SEQ ID NO 86)
GCGTTCAGAGTGGCAGTCGAGATCACGGTCGAATCAGGGTAGCGAGATCGGAAGAGCGTAGTGTAG

Probe8 (SEQ ID NO 87)
GCGTTCAGAGTGGCAGTCGAGATCACACTACGGCGAAAGTGGGCAGATCGGAAGAGCGTAGTGTAG

Probe9 (SEQ ID NO 88)
GCGTTCAGAGTGGCAGTCGAGATCACATCTTGCTCAAAGTTGCTAGATCGGAAGAGCGTAGTGTAG

ProbeIO (SEQ ID NO: 89)
GCGTTCAGAGTGGCAGTCGAGATCACCCGGAATTCCGAATTGGCAGATCGGAAGAGCGTAGTGTAG
Probel 1 (SEQ ID NO: 90)
GCGTTCAGAGTGGCAGTCGAGATCACATGTATTACCTTGGGCGAAGATCGGAAGAGCGTAGTGTAG
Probel2 (SEQ ID NO: 91)
GCGTTCAGAGTGGCAGTCGAGATCACCTCGAATAGGAAATGCGAAGATCGGAAGAGCGTAGTGTAG
Probel3 (SEQ ID NO: 92)
GCGTTCAGAGTGGCAGTCGAGATCACGGCGGTTAGATTTAGCAAAGATCGGAAGAGCGTAGTGTAG

Probel4 (SEQ ID NO 93)
GCGTTCAGAGTGGCAGTCGAGATCACCCATCAGAATTTAATTCCAGATCGGAAGAGCGTAGTGTAG
Probel5 (SEQ ID NO 94)
GCGTTCAGAGTGGCAGTCGAGATCACCTTAGCACCTATGTAATGAGATCGGAAGAGCGTAGTGTAG
Probel6 (SEQ ID NO 95)
GCGTTCAGAGTGGCAGTCGAGATCACGTGCGAGCGCAAGTCAATAGATCGGAAGAGCGTAGTGTAG
Probel7 (SEQ ID NO 96)
GCGTTCAGAGTGGCAGTCGAGATCACGAACTTGGGAGATACTATAGATCGGAAGAGCGTAGTGTAG
Probel8 (SEQ ID NO 97)
GCGTTCAGAGTGGCAGTCGAGATCACTGCGGATTACAGGCGCACAGATCGGAAGAGCGTAGTGTAG
Probel9 (SEQ ID NO 98)
GCGTTCAGAGTGGCAGTCGAGATCACCTACCTAAAGAGTGGCGCAGATCGGAAGAGCGTAGTGTAG
Probe20 (SEQ ID NO 99)
GCGTTCAGAGTGGCAGTCGAGATCACAAGCCAATCACTTGCATAAGATCGGAAGAGCGTAGTGTAG
Probe21 (SEQ ID NO 100)
GCGTTCAGAGTGGCAGTCGAGATCACCGTATAAACGTGGCTTGGAGATCGGAAGAGCGTAGTGTAG
Probe22 (SEQ ID NO 101)
GCGTTCAGAGTGGCAGTCGAGATCACGTTATACAGCAATCAGGTAGATCGGAAGAGCGTAGTGTAG
Probe23 (SEQ ID NO 102)
GCGTTCAGAGTGGCAGTCGAGATCACTAGAGGATAGATGCGCTGAGATCGGAAGAGCGTAGTGTAG
Probe24 (SEQ ID NO 103)
GCGTTCAGAGTGGCAGTCGAGATCACACTAGTCCTACGCGTGGAAGATCGGAAGAGCGTAGTGTAG
Probe25 (SEQ ID NO 104)
GCGTTCAGAGTGGCAGTCGAGATCACGCGCTACATACTTAGTCGAGATCGGAAGAGCGTAGTGTAG

Frame probe

Layoutl (SEQ ID NO: 105)
AAATTTCGTCTGCTATCGCGCTTCTGTACC

Capture probe

LP_Poly-dTVN (SEQ ID NO: 106)
GTGATCTCGACTGCCACTCTGAA I I I I I I I I I I I I I I I I
I I I I VN Amplification handle probe

A-handle (SEQ ID NO: 107)
ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Second strand synthesis and first PCR amplification
handles

A_primer (SEQ ID NO: 108)
ACACTCTTTCCCTACACGACGCTCTTCCGATCT

B_dt20VN_primer

(SEQ ID NO: 109) AGACGTGTGCTCTTCCGATC I I I I
I I I I I I I I I I I I I I I I VN Second PCR

A_primer (SEQ ID NO: 110)
ACACTCTTTCCCTACACGACGCTCTTCCGATCT

B_primer (SEQ ID NO: 111)
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Example 11

Template switching

TemplateswitchJongB

(SEQ ID NO: 112)
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATrGrGrG

Example 12

Spatial genomics

A_primer (SEQ ID NO: 113)
ACACTCTTTCCCTACACGACGCTCTTCCGATCT

B_A431_C h r2 + 2_F W_A

(SEQ ID NO: 114)
AGACGTGTGCTCTTCCGATCTTGGCTGCCTGAGGCAATG
B_A43 l_Ch r2 + 2_RE_A

(SEQ ID NO: 115)
AGACGTGTGCTCTTCCGATCTCTCGCTAACAAGCAGAGAGAAC
B_A431_C h r3 + 7_F W_B

(SEQ ID NO: 116)
AGACGTGTGCTCTTCCGATCTTGAGAACAAGGGGGAAGAG
B_A43 l_Ch r3 + 7_RE_B

(SEQ ID NO: 117)
AGACGTGTGCTCTTCCGATCTCGGTGAAACAAGCAGGTAAC
B_NT_1_FW (SEQ ID NO: 118)
AGACGTGTGCTCTTCCGATCTCATTCCCACACTCATCACAC
B_NT_1_RE (SEQ ID NO: 119)
AGACGTGTGCTCTTCCGATCTTCACACTGGAGAAAGACCC
B_NT_2_FW (SEQ ID NO: 120)
AGACGTGTGCTCTTCCGATCTGGGGTTCAGAGTGA
I I I I I CAG B_NT_2_RE (SEQ ID NO: 121)
AGACGTGTGCTCTTCCGATCTTCCGTTTTCTTTCAGTGCC

Espacenet

Claims: JP2014513523 (A) — 2014-06-05

METHOD AND PRODUCT FOR LOCALISED OR SPATIAL DETECTION OF NUCLEIC ACID IN A TISSUE SAMPLE

Claims not available for JP2014513523 (A)
Claims of corresponding document: WO2012140224 (A1)

A high quality text as facsimile in your desired language may be available amongst the following family members:

AU2012241730 (A1) CA2832678 (A1) CN103781918 (A) EP3677692 (A1) KR20140024378 (A) MX340330 (B) RU2013148909 (A) US2014066318 (A1) WO2012140224 (A1) CN106796056 (A) US2019017106 (A1) US2019024153 (A1) US2019024154 (A1) US2019264268 (A1)

- Original claims
- Claims tree

The EPO does not accept any responsibility for the accuracy of data and information originating from other authorities than the EPO; in particular, the EPO does not guarantee that they are complete, up-to-date or fit for specific purposes.

Claims

1. A method for localised detection of nucleic acid in a tissue sample comprising:
(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':
(i) a positional domain that corresponds to the position of the capture probe on the array, and
(ii) a capture domain;
(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing nucleic acid of the tissue sample to hybridise to the capture domain in said capture probes;
(c) generating DNA molecules from the captured nucleic acid molecules using said capture probes as extension or ligation primers, wherein said extended or ligated

DNA molecules are tagged by virtue of the positional domain;
(d) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying said tagged DNA;
(e) releasing at least part of the tagged DNA molecules and/or their complements or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof; and
(f) directly or indirectly analysing the sequence of the released DNA molecules.
2. The method of claim 1 , further comprising step (g) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (c).
3. The method of claim 1 being a method for determining and/or analysing a transcriptome of a tissue sample comprising: (a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a reverse transcriptase (RT) primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':
(i) a positional domain that corresponds to the position of the capture probe on the array; and
(ii) a capture domain;
(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing RNA of the tissue sample to hybridise to the capture domain in said capture probes;
(c) generating cDNA molecules from the captured RNA molecules using said capture probes as RT primers and optionally amplifying said cDNA molecules;
(d) releasing at least part of the cDNA molecules and optionally their amplicons from the surface of the array, wherein said released molecule may be a first strand and/or second strand cDNA molecule or an amplicon thereof and wherein said part includes the positional domain or a complement thereof;
(e) directly or indirectly analysing the sequence of the released DNA molecules; and optionally
(f) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (c).
4. The method of any one of claims 1 to 3, wherein the capture probes are DNA molecules.

5. The method of any one of claims 1 to 4, wherein the capture probes further comprise a universal domain which is 5' relative to the positional domain, wherein said universal domain comprises:
(i) an amplification domain, for amplifying the generated DNA molecules; and/or
(ii) a cleavage domain for releasing the generated DNA molecules from the surface of the array.
6. The method of any one of claims 1 to 5, wherein the positional domain of each species of capture probe comprises a unique barcode sequence.
7. The method of any one of claims 1 to 6, wherein the capture domain comprises a poly-T DNA oligonucleotide comprising at least 10 deoxythymidine residues and/or a random or degenerate oligonucleotide sequence.
8. The method of any one of claims 1 to 7, wherein the capture probes are directly immobilized on the array surface by their 5' end.
9. The method of any one of claims 1 to 7, wherein the capture probes are indirectly immobilized on the array surface by hybridization to a surface probe, wherein the capture domain of the capture probes comprises an upstream sequence that is capable of hybridizing to 5' end of surface probes that are immobilized on the array.
10. The method of claim 9, wherein the surface probes are immobilized to the array surface by their 3' end.
1 1. The method of claim 9 or 10, wherein the surface probes comprise a sequence that is complementary to:
(i) at least part of the capture domain;
(ii) the positional domain; and
(iii) at least part of the universal amplification domain.
12. The method of any one of claims 1 to 11 , wherein said complementary strand or said second strand cDNA molecule is generated before or after said tagged DNA molecules or said cDNA molecules are released from the surface of the array.
13. The method of any one of claims 1 to 12, wherein a strand displacement polymerase is used for the synthesis of the complementary strand of the second strand of cDNA.
14. The method of any one of claims 1 to 13, wherein random primers are used for complementary strand or second strand cDNA synthesis.
15. The method of claim 14, wherein the random primers comprise an amplification domain.
16. The method of claim 15, wherein the amplification domain of the random primers consists of a nucleotide sequence that is different to the amplification domain of the capture probe.

17. The method of any one of claims 1 to 16, wherein adaptors are ligated to one or both ends of the tagged molecules or their complements or the cDNA molecules.
18. The method of any one of claims 1 to 17, further comprising the step of amplifying the tagged molecules or cDNA molecules prior to sequence analysis.
19. The method of claim 18, wherein the amplification step is performed after the release of the tagged DNA or cDNA molecules from the substrate of the array, or wherein the amplification step is performed in situ on the array.
20. The method of claim 18 or 19, wherein the step of amplifying comprises a PCR.
21. The method of any one of claims 1 to 20, wherein the molecules are released from the surface of the array by:
(i) nucleic acid cleavage;
(ii) denaturation; and/or
(iii) physical means.
22. The method of claim 21 (i), wherein the molecules are released by enzymatic cleavage of a cleavage domain, which is located in the universal domain or positional domain of the capture probe.
23. The method of claim 21 (ii) or (iii), wherein the molecules are released by applying hot water or buffer to the array.
24. The method of any one of claims 1 to 23, further comprising a step of purifying the released molecules prior to sequencing.
25. The method of any one of claims 1 to 24, wherein the substrate is selected from the group consisting of glass, silicon, poly-L-lysine coated material, nitrocellulose, polystyrene, cyclic olefin copolymers (COCs), cyclic olefin polymers (COPs), polypropylene, polyethylene and polycarbonate.
26. The method of any one of claims 1 to 25, wherein the tissue sample is a tissue section. 27. The method of claim 26, wherein the tissue section is prepared using a fixed tissue, e.g. a formalin-fixed paraffin-embedded (FFPE) tissue, or deep-frozen tissue.
28. The method of any one of claims 1 to 27, further comprising a step of rehydrating the tissue sample after contacting the sample with the array and prior to step (c).
29. The method of any one of claims 1 to 28, wherein the tissue sample is from a plant, animal or fungus.
30. The method of any one of claims 1 to 29, further comprising a step of washing the array to remove residual tissue, wherein said step is prior to the release

of the molecules from the array. 31. The method of any one of claims 1 to 30, wherein the array comprises at least one positional marker to enable orientation of the tissue sample on the array.
32. The method of claim 31 , wherein the array comprises at least 10, 50, 100, 200, 500, 1000 or 2000 positional markers in distinct positions on the surface of the array.
33. The method of claim 31 or 32, wherein the positional markers are capable of hybridizing to a labelled, preferably fluorescently labelled, marker nucleic acid molecule.
34. The method of any one of claims 2 to 33, wherein the tissue sample is imaged using light, bright field, dark field, phase contrast, fluorescence, reflection, interference or confocal microscopy or a combination thereof.
35. The method of claim 34, wherein the tissue sample is imaged using fluorescence microscopy.
36. The method of any one of claims 1 to 35, wherein the sequence analysis step includes a step of sequencing, and preferably wherein the sequencing step comprises a sequencing reaction based on reversible dye-terminators.
37. A method for making or producing an array (i) for use in capturing nucleic acid from a tissue sample that is contacted with said array; or (ii) for use in localised detection of nucleic acid in a tissue sample, said method comprising immobilizing, directly or indirectly, multiple species of capture probe to an array substrate, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':
(i) a positional domain that corresponds to the position of the capture probe on the array; and
(ii) a capture domain.
38. The method of claim 37, wherein each species of capture probe occupies a distinct position on the array.
39. The method of claims 37 or 38, further comprising any one or more of the following steps:
(a) immobilizing directly or indirectly multiple surface probes to an array substrate, wherein the surface probes comprise:
(i) a domain capable of hybridizing to part of the capture domain oligonucleotide (a part not involved in capturing the nucleic acid); (ii) a complementary positional domain; and
(iii) a complementary universal domain;
(b) hybridizing to the surface probes immobilized on the array capture domain oligonucleotides and universal

domain oligonucleotides;
(c) extending the universal domain oligonucleotides, by templated polymerization, to generate the positional domain of the capture probe; and
(d) ligating the positional domain to the capture domain oligonucleotide to produce the capture oligonucleotide.
40. An array for use in (i) capturing nucleic acid from a tissue sample that is contacted with said array; or (ii) for use in the localised detection of nucleic acid in a tissue sample, comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as an extension or ligation primer, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':
(i) a positional domain that corresponds to the position of the capture probe on the array, and
(ii) a capture domain to capture nucleic acid of a tissue sample that is contacted with said array.
41. The method of any one of claims 37 to 39 or the array of claim 40, wherein the capture probes are as defined in any one of claims 4 to 9, the surface probes are as defined in claims 10 or 11 , the substrate is as defined in claim 25, the tissue sample is as defined in any one of claims 26, 27 or 28, the array is as defined in any one of claims 31 to 33 and the positional markers are as defined in claim 33.
42. The method of claim 1 or claim 2 being a method for localised detection of DNA in a tissue sample comprising:
(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3':
(i) a positional domain that corresponds to the position of the capture probe on the array, and
(ii) a capture domain;
(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample and allowing DNA of the tissue sample to hybridise to the capture domain in said capture probes;
(c) fragmenting DNA in said tissue sample, wherein said fragmentation is carried out before, during or after

contacting the array with the tissue sample in step (b);
(d) extending said capture probes in a primer extension reaction using the captured DNA fragments as templates to generate extended DNA molecules, or ligating the captured DNA fragments to the capture probes in a ligation reaction to generate ligated DNA molecules, wherein said extended or ligated DNA molecules are tagged by virtue of the positional domain;
(e) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying said tagged DNA;
(f) releasing at least part of the tagged DNA molecules and/or their complements and/or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof ;
(g) directly or indirectly analysing the sequence of (e.g. sequencing) the released DNA molecules; and optionally;
(h) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (d).
43. The method of claim 42 comprising:
(a) providing an array comprising a substrate on which multiple species of capture probes are directly or indirectly immobilized such that each species occupies a distinct position on the array and is oriented to have a free 3' end to enable said probe to function as a primer for a primer extension or ligation reaction, wherein each species of said capture probe comprises a nucleic acid molecule with 5' to 3': (i) a positional domain that corresponds to the position of the capture probe on the array, and
(ii) a capture domain;
(b) contacting said array with a tissue sample such that the position of a capture probe on the array may be correlated with a position in the tissue sample;
(c) fragmenting DNA in said tissue sample, wherein said fragmentation is carried out before, during or after contacting the array with the tissue sample in step (b);
(d) providing said DNA fragments with a binding domain which is capable of hybridising to said capture domain;
(e) allowing said DNA fragments to hybridise to the capture domain in said capture probes;
(f) extending said capture probes in a primer extension reaction using the captured DNA fragments as templates to generate extended DNA molecules, or ligating the captured DNA fragments to the capture probes in a ligation reaction to generate ligated DNA

molecules, wherein said extended or ligated DNA molecules are tagged by virtue of the positional domain;
(g) optionally generating a complementary strand of said tagged DNA and/or optionally amplifying the tagged DNA
(h) releasing at least part of the tagged DNA molecules and/or their complements and/or amplicons from the surface of the array, wherein said part includes the positional domain or a complement thereof;
(i) directly or indirectly analysing the sequence of (e.g. sequencing)the released DNA molecules; and optionally
(j) correlating said sequence analysis information with an image of said tissue sample, wherein the tissue sample is imaged before or after step (f).

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/395,534	08/06/2021	Evan R. Daugharthy	010498.01437\US	5147

22907 7590 11/10/2021

BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

EXAMINER
CENTRAL, DOCKET

ART UNIT	PAPER NUMBER
OPAP	

NOTIFICATION DATE	DELIVERY MODE
11/10/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

GPD@bannerwitcoff.com
eofficeaction@bannerwitcoff.com

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 17/395,534	**Applicant(s)** Daugharthy et al.	
	Examiner Dale A Hall	**Art Unit** OPET	**AIA (FITF) Status** Yes

1. THE REQUEST FILED 06 August 2021 IS **GRANTED** .

The above-identified application has met the requirements for prioritized examination

A. ☑ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination**. The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;

B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims** , or a multiple dependent claim;

C. filing a **request for continued examination** ;

D. filing a notice of appeal;

E. filing a request for suspension of action;

F. mailing of a notice of allowance;

G. mailing of a final Office action;

H. completion of examination as defined in 37 CFR 41.102; or

I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Dale Hall at (571)272-3586. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/Michelle R. Eason/ Lead Paralegal Specialist	

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	10370698		2019-08-06	Nolan et al.	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20030165852	A1	2003-09-04	Schueler et al.	
	2	20160002704	A1	2016-01-07	DIEHL et al.	

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	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	DIRKS et al. "Triggered amplificaiton by hybridization chain reaction" PNAS; October 26, 2004; Vol. 101, No. 43, pp. 15275-15278	
	2	LUBECK et al. "Single cell systems biology by super-resolution imaging and combinatorial labeling" Nature Methods; 9 (7); pp. 743-748; 2012	

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Examiner Signature		Date Considered	

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-11-23
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	44360683
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	24-NOV-2021
Filing Date:	06-AUG-2021
Time Stamp:	08:19:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	DIRKS_et_al_PNAS_2004_21602172.pdf	297384 ef2ad138d0ac51c488c78a187f374cf5eb347dd2	no	4

Warnings:

Information:					
2	Non Patent Literature	LUBECK_et_al_Nature_Methods_2012_21602182.pdf	1140387 ff0dfc8d69a69a300a73c438c49cd32a624971f6	no	18
Warnings:					
Information:					
3	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21602540.pdf	1034437 cc93e07782577727 4da7f0a8a3c2199a4b931954	no	4
Warnings:					
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		Total Files Size (in bytes):	2472208		

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/395,534	08/06/2021	Evan R. Daugharthy	010498.01437\US

CONFIRMATION NO. 5147

22907
BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

PUBLICATION NOTICE

OC000000130049885

Title: Method and Apparatus for Volumetric Imaging

Publication No.US-2021-0363579-A1
Publication Date:11/25/2021

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20170262984	A1	2017-09-14	Barnes et al.	
	2	20200034347	A1	2020-01-30	SELLY	
	3	20100049448	A1	2010-02-25	Doyle et al.	

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	1							

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

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	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-11-30
Name/Print	John P. Iwanicki	Registration Number	34,628

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	44397244
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	30-NOV-2021
Filing Date:	06-AUG-2021
Time Stamp:	16:01:09
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21611906.pdf	1034211 63e8ee6360dd6983d5483fc773eea0ca413db6aa	no	4
Warnings:					

Information:	
Total Files Size (in bytes):	1034211

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/395,534	08/06/2021	Evan R. Daugharthy	010498.01437\US	5147

22907 7590 12/14/2021

BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

EXAMINER	
THOMAS, DAVID C	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
12/14/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

GPD@bannerwitcoff.com
eofficeaction@bannerwitcoff.com

Office Action Summary	Application No. 17/395,534	Applicant(s) Daugharthy et al.	
	Examiner DAVID C THOMAS	Art Unit 1637	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-8 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☐ Claim(s) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 1-8 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Election/Restrictions

2. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-7, drawn to a method of analyzing a plurality of nucleic acids within a three dimensional polymerized matrix, classified in CPC C12Q 1/6869, CPC C12Q 1/6841 and CPC C12Q 2565/601.

II. Claim 8, drawn to an automated sequencing and volumetric imaging device comprising a multi axis state or positioning system, a heating or cooling apparatus, a fluidics dispenser, a pump, an optical assembly, one or more detectors, and one or more microprocessors, classified in CPC C12M 3/00 and CPC B01L 7/52.

3. The inventions are distinct, each from the other because of the following reasons:

4. Inventions in Groups I and II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product (MPEP § 806.05(h)). In the instant case Group II, drawn

to an automated sequencing and volumetric imaging device, can be used in the method of Group I but can also be used in other processes that comprise detection or analysis of nucleic acids, including probe hybridization methods, including for applications of sequencing analysis, as well as methods of nucleic acid sequence detection comprising signal amplification processes rather than nucleic acid amplification, wherein amplified signals may be detected optically.

5. Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and examination burden if restriction were not required because one or more of the following reasons apply:

(a) the inventions have acquired a separate status in the art in view of their different classification;

(b) the inventions have acquired a separate status in the art due to their recognized divergent subject matter;

(c) the inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);

(d) the prior art applicable to one invention would not likely be applicable to another invention;

(e) the inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

6. The examiner has required restriction between product and process claims. Where applicant elects claims directed to the product, and a product claim is subsequently found allowable, withdrawn process claims that depend from or otherwise include all the limitations of the allowable product claim will be rejoined in accordance with the provisions of MPEP § 821.04. **Process claims that depend from or otherwise include all the limitations of the patentable product** will be entered as a matter of right if the amendment is presented prior to final rejection or allowance, whichever is earlier. Amendments submitted after final rejection are governed by 37 CFR 1.116; amendments submitted after allowance are governed by 37 CFR 1.312.

In the event of rejoinder, the requirement for restriction between the product claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103, and 112. Until an elected product claim is found allowable, an otherwise proper restriction requirement between product claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowed product claim will not be rejoined. See "Guidance on Treatment of Product and Process Claims in light of In re Ochiai, In re Brouwer and 35 U.S.C. § 103(b)," 1184 O.G. 86 (March 26, 1996). Additionally, in order to retain the right to rejoinder in accordance with the above policy, Applicant is advised that the process claims should be amended during prosecution either to maintain dependency on the product claims or to otherwise include the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder.**

Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

7. **Applicant is advised that the reply to this requirement to be complete must include (i) an election of a invention to be examined** even though the requirement may be traversed (37 CFR 1.143) **and (ii) identification of the claims encompassing the elected invention**.

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable

over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

8. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Correspondence

9. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David C. Thomas whose telephone number is 571-272-3320 and whose fax number is 571-273-3320. The examiner can normally be reached on 5 days, 9-5:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/David C Thomas/
Primary Examiner, Art Unit 1637

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COMMISSIONER FOR PATENTS
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ALEXANDRIA VA 22313-1451

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BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

Courtesy Reminder for
Application Serial No: 17/395,534

Attorney Docket No: 010498.01437\US
Customer Number: 22907
Date of Electronic Notification: 12/14/2021

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

An email notification regarding the correspondence was sent to the following email address(es) associated with your customer number:

eofficeaction@bannerwitcoff.com
GPD@bannerwitcoff.com

To view your correspondence online or update your email addresses, please visit us anytime at **https://ppair-my.uspto.gov/pair/PrivatePair.**
If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov or call 1-866-217-9197.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

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U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20020029979	A1	2002-03-14	Freund et al.	
	2	20050064435	A1	2005-03-24	Su et al.	

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2010104533	WO	A2	2010-09-16	Ikonisys, Inc		

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	CHEN et al. "Functional organization of the human 4D Nucleome" PNAS, Vol. 112, No. 26, June 15, 2015, pp. 8002-8007	
	2	JARVIUS et al. "Digital quantification using amplified single-molecule detection" Nature Methods, Vol. 3, No. 9, September 2006, pp. 725-727	

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2022-01-17
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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Electronic Acknowledgement Receipt

EFS ID:	44813123
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	24-JAN-2022
Filing Date:	06-AUG-2021
Time Stamp:	11:55:35
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Foreign Reference	WO2010104533A2_21674410.pdf	2663024 1d08933d6331e80dc12b330ad87f29634d480053	no	32
Warnings:					

Information:					
2	Non Patent Literature	CHEN_et_al_PNAS_2015_21674393.PDF	988638 89d640a8aa2a8e46965c18602cdc029cfe311370	no	6
Warnings:					
Information:					
3	Non Patent Literature	JARVIUS_et_al_Nature_Methods_2006_21674420.PDF	307695 73231476b352bf2bc108edd94da5233be51ceba4	no	3
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21691065.pdf	1034446 96e9bcd734d18faa7469bf5e2940937e1ef51d15	no	4
Warnings:					
Information:					
		Total Files Size (in bytes):	4993803		

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New Applications Under 35 U.S.C. 111

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If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Espacenet

Bibliographic data: WO2010104533 (A2) — 2010-09-16

AUTOMATED METHOD FOR DETECTING CANCERS AND HIGH GRADE HYPERPLASIAS

Inventor(s): TAFAS TRIANTAFYLLOS [US]; TSIPOURAS PETROS [US]; KILPATRICK MICHAEL [US]; SARKIS GARY [US] ± (TAFAS, TRIANTAFYLLOS, ; TSIPOURAS, PETROS, ; KILPATRICK, MICHAEL, ; SARKIS, GARY)

Applicant(s): IKONISYS INC [US]; TAFAS TRIANTAFYLLOS [US]; TSIPOURAS PETROS [US]; KILPATRICK MICHAEL [US]; SARKIS GARY [US] ± (IKONISYS, INC, ; TAFAS, TRIANTAFYLLOS, ; TSIPOURAS, PETROS, ; KILPATRICK, MICHAEL, ; SARKIS, GARY)

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(71) Applicant *(for all designated States except US)*: **IKONISYS, INC.** [US/US]; 5 Science Park, New Haven, CT 06511 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **TAFAS, Triantafyllos** [US/US]; 9 Clemens Road, Rocky Hill, CT 06067 (US). **TSIPOURAS, Petros** [US/US]; 74 Island Avenue, Madison, CT 06443 (US). **KILPATRICK, Michael** [GB/US]; 25 Beverly Road, West Hartford, CT 06119 (US). **SARKIS, Gary** [US/US]; 4766 Durham Road, Guilford, CA 06437 (US).

(74) Agents: **MOORE, Steven, J.** et al.; Kelley Drye & Warren LLP, 400 Atlantic Street, 13th Floor, Stamford, CT 06901-3229 (US).

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BACKGROUND OF THE INVENTION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This PCT application claims the benefit of priority to U.S. Patent Application No. 12/400,975, filed March 10, 2009, which is a continuation-in-part application of U.S. Patent Application No. 11/925,123, filed October 26, 2007 which is a continuation application of U.S. Patent Application No.11/924,293, filed October 25, 2007, which claims the benefit of priority of U. S. Provisional Application No. 60/862,974, filed October 25, 2006. This reference and all additional references cited in this specification, and their references, are incorporated by reference herein where appropriate for teachings of additional or alternative details, features, and/or technical background.

Field of the Invention

[0002] The present invention generally relates to an automated method for detecting cancer, and dysplasias, particularly high grade dysplasias, in an individual. There is presented in one aspect a method for monitoring the effectiveness of treatment protocols in the treatment of one or more cancers.

Description of the Related Art

[0003] Many methods are known to aid in the microscopic analysis of samples. For example, without limitation, it is known that certain dyes have an affinity for certain cellular or subcellular structures. Such dyes may therefore be used to aid in analysis by helping to further elucidate such structures. Binding of dyes to such structures may be identified and analyzed using various techniques of microscopic detection.

[0004] Fluorescence microscopy of cells and tissues is well known in the art. Methods have been developed to image fluorescent cells in a microscope and extract information about the spatial distribution and temporal changes occurring in these cells. Some of these methods and their applications are described in an article by Taylor, *et al.* in American Scientist 80 (1992), p. 322 - 335. These methods have been designed and optimized for the preparation of a few specimens for high spatial and temporal resolution imaging measurements of distribution,

amount and biochemical environment of the fluorescent reporter molecules in the cells. Detection of fluorescent signals may be by way of an epifluorescent microscope which uses emitted fluorescent light to form an image (whereas a conventional reflecting microscope uses scattered illumination light to form an image). The excitation light of a epifluorescence microscope is used to excite a fluorescent tag in the sample causing the fluorescent tag to emit fluorescent light. The advantage of an epifluorescence microscope is that the sample may be prepared such that the fluorescent molecules are preferentially attached to the biological structures of interest thereby allowing identification of such biological structures of interest.

[0005] Automated methods of conducting microscopic analysis of biological samples enhance diagnostic procedures and optimize the throughput of samples in a microscope-based diagnostic facility. Various co-owned U. S. patent applications, described more fully below, disclose aspects and embodiments of apparatuses and methods for automated microscopic analysis. These include an integrated robotic microscope system, a dynamic automated microscope operation and slide scanning system, various interchangeable objective lenses, filters, and similar elements for use in an automated microscope system, an automated microscope stage for use in an automated microscope system, an automated microscope slide cassette and slide handling system for use in an automated microscope system, an automated microscope slide loading and unloading mechanism for use in an automated microscope system, automated methods that employ computer-resident programs to drive the microscopic detection of fluorescent signals from a biological sample, useable to drive an automated microscope system, automatic operation of a microscope using computer-resident programs to drive the microscope in conducting a FISH assay for image processing.

[0006] The acronym "FISH" (fluorescence in situ hybridization) references a technique that uses fluorescent tags or labels that emit a characteristic light or color when illuminated by a light source, such as an ultraviolet or visible light source, to detect chromosomal structure. FISH uses fluorescent probes which bind only to those parts of a chromosome with which they show a high degree of sequence similarity. Such probes may be directed to specific chromosomes and specific chromosome regions. The probe has to be long enough to hybridize specifically to its target (and not to similar sequences in the genome), but not too large to impede the hybridization process, and it should be tagged directly with fluorophores. This can be done in various ways, for example nick translation and PCR using tagged nucleotides. If signal amplification is necessary to exceed the detection threshold of the microscope (which depends

on many factors such as probe labelling efficiency, the kind of probe and the fluorescent dye), probes labeled with haptens such biotin or digoxygenin are used, and specific fluorescent tagged antibodies or streptavidin are bound to the hapten molecules, thus amplifying the fluorescence. The FISH technique may be used for identifying chromosomal abnormalities and gene mapping.

[0007] A commonly studied mechanism for gene overexpression in cancer cells is generally referred to as gene amplification. This is a process whereby a gene is duplicated within the chromosomes of an ancestral cell into multiple copies. The process involves unscheduled replications of the region of the chromosome comprising the gene, followed by recombination of the replicated segments back into the chromosome (Alitalo K. et al. (1986), Adv. Cancer Res. 47:235-281). As a result, 50 or more copies of the gene may be produced. The duplicated region is sometimes referred to as an "amplicon". The level of expression of the gene (that is, the amount of messenger RNA produced) escalates in the transformed cell in the same proportion as the number of copies of the gene that are made (Alitalo et al.).

[0008] Work with other oncogenes, particularly those described for neuroblastoma, suggests that gene duplication of the proto-oncogene is an event involved in the more malignant forms of cancer, and could act as a predictor of clinical outcome (reviewed by Schwab M. et al. (1990), Genes Chromosomes Cancer 1:181-193; and Alitalo et al.). In breast cancer, duplication of the erbB2 gene has been reported as correlating both with reoccurrence of the disease and decreased survival times (Slamon D. J. et al. (1987), Science 235:178-182.). There is some evidence that erbB2 helps identify tumors that are responsive to adjuvant chemotherapy with cyclophosphamide, doxorubicin, and fluorouracil (Muss et al. N Engl J Med. 1994 330(18):1260-6).

[0009] Only a proportion of the genes that can undergo gene duplication in breast cancer have been identified. First, chromosome abnormalities, such as double minute (DM) chromosomes and homogeneously stained regions (HSRs), are abundant in cancer cells. HSRs are chromosomal regions that appear in karyotype analysis with intermediate density Giemsa staining throughout their length, rather than with the normal pattern of alternating dark and light bands. They correspond to multiple gene repeats. HSRs are particularly abundant in breast cancers, showing up in 60-65% of tumors surveyed (Dutrillaux B. et al. (1990), Cancer Genet Cytogenet 49:203-217.; Zafrani B. et al. (1992), Hum Pathol 23:542-547). When such regions are checked by in situ hybridization with probes for any of 16 known human oncogenes, including erbB2 and myc, only a proportion of tumors show any hybridization to HSR regions. Furthermore, only a proportion of the HSRs within each karyotype are implicated.

[00010] Second, comparative genomic hybridization (CGH) has revealed the presence of copy number increases in tumors, even in chromosomal regions outside of HSRs. CGH is a new method in which whole chromosome spreads are stained simultaneously with DNA fragments from normal cells and from cancer cells, using two different fluorochromes. The images are computer-processed for the fluorescence ratio, revealing chromosomal regions that have undergone amplification or deletion in the cancer cells (Kallioniemi A. et al. (1992), Science 258:818-821.). This method was recently applied to 15 breast cancer cell lines (Kallioniemi A. et al. (1994), Proc. Natl. Acad. Sci. USA 91:2156-2160.). DNA sequence copy number increases were detected in all 23 chromosome pairs.

[00011] So, C-K, et al. (Clinical Cancer Research 10: 19-27, 2004) found internal tandem duplication of cyclic AMP response element binding protein (CBP), a nuclear transcriptional coactivator protein, in esophageal squamous cell carcinoma samples from Linzhou (Linxian), China. So et al. show internal tandem duplication of the CBP gene is a frequent genetic event in human squamous cell carcinoma.

[00012] The human epidermal growth factor receptor 2 (HER-2)/neu (c-erbB-2) gene is localized to chromosome 17q and encodes a transmembrane tyrosine kinase receptor protein that is a member of the epidermal growth factor receptor (EGFR) or HER family (Ross, JS, et al., The Oncologist, Vol. 8, No. 4, 307–325, August 2003). The HER-2 gene is amplified in a fraction, perhaps 25%, of human breast cancers.

[00013] Fluorescence in situ hybridization (FISH) is commonly used for the detection of chromosomal abnormalities including sequence alterations such as single nucleotide polymorphisms or mutations found in oncogenes.

[00014] A number of methods and kits have been disclosed for screening of cancer and dysplasias, such as high grade dysplasias.

[00015] For example, ProVysion Multi-color Probe Set manufactured by Abbott Molecular is designed to detect and quantify chromosome 8, the lipoprotein lipase (LPL) gene located at 8p22, and the C-MYC gene located at the 8q24 region. Gain of 8q24 and 8p21-22 (LPL) and loss of heterozygosity are two genetic alterations that have been observed in abnormal samples. The ProVysion Multi-color Probe Set consists of three probes with three separate fluorophore labels. The multicolor probe set design is said to permit simultaneous analysis of the three genomic markers within a single cell, CEP® 8 probe labeled with SpectrumAqua, LSI LPL labeled with SpectrumOrange, and LSI C-MYC labeled with SpectrumGreen. The CEP 8 alpha

satellite DNA probe hybridizes to the centromere region of chromosome 8 (8p11.1-q11.1) and provides a mechanism for the identification of copy number of chromosome 8. The LSI LPL hybridizes to the LPL gene at 8p22 and is approximately 170 kb in size. The LSI C-MYC Probe (an approximately 750 kb probe) hybridizes to the C-MYC gene located at 8q24. The manufacturer asserts that in a normal cell hybridized with the ProVysion Multi-color Probe Set, the expected pattern is the two orange, two green and two aqua (2O2G2A) signal pattern, while in an abnormal cell, combinations of copies of the three probe signals may be observed. The test kit indicates that copy numbers of more or less than two of any probe indicates chromosome or gene gain or loss, respectively. Less than two copies of the LSI LPL or multiple copies of the LSI C-MYC Probe relative to CEP 8 copy number indicates loss of the LPL region and gain of the C-MYC region, respectively, relative to the chromosome 8 copy number.

[00016] U.S. Patent Publication Nos. 2004/028107 and 2005/0026190 to Vysis, Inc. assert methods of using probes and probe sets for the detection of high grade dysplasia and carcinoma in cervical cells. The methods entail hybridizing one or more chromosomal probes to a biological sample and detecting the hybridization pattern of the chromosomal probes to determine whether the subject has high grade dysplasia or carcinoma. The methods encompass the use of a set of one or more probes demonstrating a vector value of about 60 or less wherein the vector value is calculated by Vector = $[(100\text{-specificity})^2 + (100\text{-sensitivity})^2]^{1/2}$. The chromosomal probes may comprise probes for specific loci, such as 8q24, 3q36, Xp22, and CEP 15, or probes, for example, substantially complmentary to full coding sequence for each of HPV-16, HPV-18, HPV-30, HPV-45, HPV-51, and HPV-58. The biological sample screened may be pre-screened for the presence of a cell cycle protein, such as p16 or Cyclin E, or a cell proliferation marker, such as protein Ki67 or PCNA.

[00017] U.S. Patent Publication 2006/0063194 to Abbott Molecular also discloses probe sets and methods of using probes and probe sets for the detection of cancer, particularly lung cancer. Locus specific probes and chromosome enumeration probes are used in conjunction, and the hybridization pattern of the same used to determine whether the subject has lung cancer. Chromsomal compositions are specified, for example, a probe set for determining lung cancer may comprise a 5p15 locus specific probe, a 8q24 locus specific probe, a chromosome 6 enumeration probe and a 7p12 locus specifice probe.

[00018] Diagnostic FISH light dot counting has been conventionally performed manually, by a skilled microscopist. In addition to correctly identifying the dot and its color,

other size and shape characteristics must be categorized to correctly identify the chromosomal condition. The analysis is made more difficult by the time constraints imposed by the phenomena. The microscopist, therefore, must be trained to perform the examination. Even under the best conditions, the process has proven to be tedious, lengthily and subject to human error.

[00019] The application of automated microscopy has the potential to overcome many of the shortcomings of the manual approach. The automatic microscope can reliably identify the fluorescent dots in a sample, accurately determine their color, categorize them based on shape and size, and perform the summary analysis necessary to determine the presence or absence of the targeted condition without the inevitable subjective factors introduced by a human operator all in a timely manner.

[00020] It should be noted that kits for the detection of cancers are typically designed to provide only a positive or negative answer – one has a particular cancer or not. While such tests may indicate the need for interventional therapy, such as chemotherapy, they are not designed to lead one in the direction of the most appropriate interventional therapy. Rather, cancer patients are often subjected to multiple therapies, and the effectiveness of therapies determined by snap-shots of the cancer status at points in time after start of the therapy. Such snap-shots may entail for example, MRI and CAT scans of the body to determine the growth or shrinkage of tumors. As such snap-shot methods may entail considerable economic costs, as well as risks in themselves (e.g., radiation exposure), such snap-shots may be taken at considerably longer intervals than might be desired given the need for rapid intervention into resolving the disease state. As set forth below, the present inventors have also recognized that the use of automated microscopy may also be used advantageously to determine not only whether a person is inflicted with a particular cancer/hyperplasia, but also as a monitoring tool for the determination of the efficacy of different interventional therapeutic approaches to the treatment of cancer/high grade hyperplasia. In one embodiment, the monitoring of therapeutic efficacy is by means of monitoring cancer/hyperplastic cells in the systemic circulation (including the vasculature and lymph system) with a decrease in number of abnormal cells associated with the cancer/hyperplasia being taken as an indication of therapeutic success, and the degree of reduction in such cells being used as a guage of the efficacy of one therapy against another therapy.

[00021] There remains a need in the field for the automated imaging and analysis of images arising from cancer tissue samples treated with detectably labeled probes, including fluorescently labeled probes. Additionally there remains a need for convenient, rapid, hands-free automated fluorescence microscopy of such labeled samples.

SUMMARY OF THE INVENTION

[00022] Various embodiments are disclosed herein.

[00023] In one embodiment, an automated method of screening for the presence and/or extent of a pathology in a subject, the pathology characterized by an abnormal chromosomal component in a cell of the subject, comprising the steps of

a) contacting a biological sample comprising cell nuclei from said subject with one or more distinguishable labeled probes directed to at least one chromosomal sequence that characterizes the abnormality under conditions that promote hybridization of the one or more probes to the at least one sequence;

b) automatically obtaining a representation of the one or more distinguishable labels hybridized to the chromosomal sequences;

c) automatically analyzing the distribution and intensity of binding of the one or more labels in the representation to determine the presence and/or extent of an abnormal chromosomal component; and

d) automatically reporting results of the analysis of step c);

wherein steps b) – d) are carried out without intervention by a human

[00024] In various further embodiments of the method of screening an automated microscope system carries out one, and usually all, of the steps of automatically obtaining a representation, automatically analyzing binding, and automatically reporting results. In this method obtaining the representation and performing automated image analysis identifies nucleic acid properties characteristic of a pathology. Various targeted chromosomal abnormalities may include a single nucleotide polymorphism (SNP), a mutated sequence, or a duplicated gene or portion thereof. In one embodiment, the targeted chromosomal locus or loci is within an 800 kb region surrounding the TERC and PIK3A sequences. Chromosomal targets for a probe may include a centromere, or a target sequence of human chromosome 3 or human chromosome 7,

and all or part of a TERC gene. In one embodiment the targeted chromosomal locus or loci is the PIK3A sequences. The targeted chromosomal locus or loci may also be the surrounding 5' and 3' regions of either the TERC and/or PIK3A or a portion thereof. In additional embodiments a control probe is directed to the centromeric region of chromosome 7 and/or chromosome 3. In such embodiment the control count is 2 signals per nucleus for chromosome 7 and/or 3, and a count of 4, preferably 5 or greater of a probe directed to targeted chromosomal abnormalities is indicative of high grade displasia or cancer.

[00025] In additional embodiments various reference probes directed to a chromosomal locus known not to be abnormal or a reference stain may be used such that the representing and analysis steps are referenced to the reference probe or stain.

[00026] In an additional embodiment an automated method of screening for an abnormality related to a cancer, a high grade hyperplasia or a high grade dysplasia in a subject, comprising the steps of:

a) obtaining a biological sample comprising nuclei from the subject;
b) contacting the nuclei in the sample with a first probe bearing a first detectable label directed to a chromosomal sequence related to the abnormality under conditions that promote hybridization of the probes to targeted chromosomal loci;
c) contacting the sample under the hybridizing conditions with at least one of a detectably labeled reference probe directed to a chromosomal locus known not to be abnormal and a reference stain;
d) automatically imaging the labels bound to the chromosomal sequences, and imaging the stain if used;
e) automatically analyzing an image for the distribution and intensity of hybridized labels and stain if used; and
f) automatically reporting results of the analysis of step e);
wherein steps d)-f) are performed without intervention by a human;

thereby providing an assessment of the abnormality in the subject.

[00027] In an embodiment of this method of screening the nuclei are isolated from the sample, and the nuclei are deposited to form a layer of nuclei prior to the contacting step. In further embodiments an automated microscope performs at least one, and usually all, steps of automatic imaging, automatic analyzing, and automatic reporting of results. The single layer

nuclei preparation may be obtained by a number of methods known in the art, for example, by appropriate processing of thin sections from paraffin-embedded tumor tissue samples. A large variety of origins for the sample obtained from the subject is envisioned in this method. In additional embodiments of this method of screening an automated microscope is used at various stages of the method, including to automatically provide the images, to obtain the image the microscope automatically optimizes the field in which the image occurs, and to obtain images from two or more planes in a field of the sample to perform the automatic analysis of the image. In various embodiments the abnormality may be a cancer, a high grade hyperplasia or a high grade dysplasia. Various abnormalities targeted by the probe may include a single nucleotide polymorphism, a mutated sequence, or a duplicated gene or portion thereof. Additionally in certain embodiments the probe targets a centromere of chromosome 3 or a centromere of chromosome 7, or a sequence that includes the TERC gene or a portion thereof.

[00028] In still a further embodiment an automated method for monitoring the efficacy over time of a course of therapy in the treatment of a cancer or high grade hyperplasia in a patient is disclosed. This method includes the steps of:

(a) obtaining from the patient a fluid biological sample in which cells associated with the cancer or high grade hyperplasia are found;

(b) treating the fluid biological sample or a portion thereof with one or more detectably labeled chromosomal probes having a high degree of sequence similarity to one or more chromsomal loci associated with, or whose amplification is associated with, the cancer or high grade hyperplasia, wherein the treating is carried out under conditions sufficient to enable hybridization of the probes to chromosomes in the sample;

(c) automatically scanning the treated fluid biological sample and detecting the one or more labels bound to one or more chromosomal probes that are hybridized to any chromosomes in the sample;

(d) automatically detecting the number of cells associated with the chromosomes hybridized to said chromosomal probes; and

(e) automatically comparing the hybridization patterns of a label and cell number results provided in steps (c) and (d) at differing times in the therapeutic treatment course, thereby evaluating the efficacy of the therapy in the treatment of the cancer or high grade hyperplasia.

[00029] In various embodiments the fluid biological sample includes one or more of blood, lymph, urine, an effusion fluid, an epithelial scraping, a lavage fluid, in a biological fluid, one or more biopsy material, surgical resection specimem a smear (e.g., from the uterine cervix), a buccal smear, aspiration fluid, saliva, sputum and tissue.

[00030] In an embodiment the monitoring is performed at intervals of 1 day, or longer. In further embodiments of this method for monitoring efficacy an automated microscope system performs at least one of the automatic scanning and the automatic detection uses an automated microscope system, as well as automatically optimizes the field scanning the sample, and further scans two or more planes in a field of the sample. In various embodiments the automated microscope system operates without intervention by a human. In still additional embodiments a probe targets at least one of a single nucleotide polymorphism (SNP), a mutated sequence, a duplicated or amplified gene or portion thereof, a centromere of chromosome 3, a centromere of chromosome 7, and a sequence comprising the TERC gene or a portion thereof.

[00031] In still further embodiments a method for the automated high throughput characterization of a chromosomal abnormality is disclosed. This method includes the steps of:

a) providing at least one microscope slide comprising a biological sample thereon, wherein the sample is suspected of harboring the chromosomal abnormality and wherein the sample has been hybridized to at least one detectably labeled probe specific for detection of the abnormality;

b) installing the at least one sample-bearing slide in a means for automated, reversible, placement of the slide on the stage of an automated microscope;

c) causing the placement means automatically and reversibly to place a sample-bearing slide to be reversibly placed on the microscope stage;

d) causing the microscope automatically to obtain at least one image of the specimen wherein the image comprises a representation of a labeled probe hybridized to a chromosome;

e) causing the microscope automatically to analyze the image in order to characterize the abnormality;

f) automatically reporting the results of the analysis of step (e); and

g) automatically repeating steps (c)-(f).

[00032] In various embodiments the automated microscope operates without intervention by a human. In additional embodiments of this high throughput method the automatic microscope obtains an image automatically by optimizing the field in which the image occurs, and obtains images from two or more planes in a field of nuclei. The biological sample may originate in any of various tissues and biological fluids. Furthermore the abnormality may be a cancer, a high grade hyperplasia or a high grade dysplasia. Various abnormalities targeted by the probe used in the high throughput method may include at least one of a single nucleotide polymorphism (SNP), a mutated sequence, a duplicated or amplified gene or portion thereof, a centromere of chromosome 3, a centromere of chromosome 7, and a sequence comprising the TERC gene or a portion thereof.

[00033] A further embodiment discloses a method that includes in order: (a) hybridizing to a biological sample one or more chromosomal probes having a high degree of sequence similarity to one or more portions of chromsomic material under conditions sufficient to enable hybridization of the probes to chromosomes in the sample (if any), the probes characterized in being tagged with one or more tags detectable by a detector; (b) automatically scanning the biological sample and detecting by a detector the one or more tag(s) associated with the one or more chromosomal probes that is hybridized to any chromosomes in the sample; and (c) automatically reporting chromosomes if any in the sample which are tagged with hybridized probe and the particular probes associated with the chromosome.

[00034] In various embodiments of the methods disclosed herein the centromeric probe may be directed to chromosomes known to house loci the replication of which, or the existence of which, are associated with a particular cancer state. For example, the centromeric probe may be direct to chromosome 3 and/or chromosome 7. The locus specific probe may be for single copy sequences may likewise hybridize with loci associated with cancer, such as loci on the q arm of chromosome 3. The probe itself may advantageously have a high degree of sequence similarity to one or more portions of chromosomal material associated with a locus associated with, or the amplification of which is associated with, particular cancer(s)/hyperplasias under conditions sufficient to enable hybridization of the probes to chromosomes in the sample. The probe, for example, may be a contig consisting of four overlapping BAC clones containing the TERC gene at chromosomal location 3q26. Additional centromeric or locus specific probes may be added to a probe mixture. The nuclear staining may be by way of counterstain process. The nuclear stain may be, for example, DAPI. In

automatically scanning the sample, the sample may be loaded onto an automated microscope which automatically moves from one field of view to another. The microscope may be programmed or otherwise operationally configured to allow monitoring of a number of signal channels. For example, an automated microscope may scan in DAPI and other fluroescence channels (to enumerate, for example, signals for chromosome 3, locus on 3q, and other centromeric or locus specific signal). The scanned nuclei may be automatically recorded by the automated microscope, and/or may be presented to a cytogeneticist and/or pathologist, or other health care provider. Presentation may be in numerous fashions, such as in a sorted manner with the ones with the abnormal counts presented first (e.g., counts not equal to 2 of the 3q being present first). Different cancers may be detected, such as cervical cancer (using, for example, the centromeric probe for chromsome 3 and/or chromosome 7 and a locus specific probe for single copy sequences on the q arm of chromosome 3 comprising a contig consisiting of four overlappling BAC clones containg the TERC gene at chromosomal location 3q26 or a portion thereof, a DAPI nuclear counterstain, and then enumerating signals for chromosome 3, locus on 3q, and finding abnormal counts of not equal of 2 of the 3q related signals).

[00035] Automatic scanning in such embodiments may be performed, for example, by an automated microscope wherein the biological specimen is placed on slides which are manually or automatically loaded onto the microscope stage, and the slide automatically scanned. Automated microscopes that may find employment in such system are such as described in other of applicant's patent applications (see below). Scanning may also be made of other substrates onto/into which the biological sample is placed. Scanning may comprise scanning the biological sample in one plane, or in more than one plane, such as, for example, two, three or more planes. By scanning in multiple planes, detection of abnormal cells, which may be rare in terms of total number of cells in the sample, may be significantly improved. The probes may make use of FISH probes in which the fluorescent signal is picked up by the detector. The probes may produce a signal with or without another input signal, e.g. they may be radioactive, or fluoresce when impinged by an activating signal (such as an appropriate wavelength of light or other electromagnetic radiation). The probes may be directed to different replication associated cancer loci, and may comprise different fluorescent tags so as to produce different signals. The detector may be selected in accordance with signal(s) which are to be produced by the tags, e.g. a fluorescence detector for detecting fluorescent tags, with the detector operatively configured to permit detection of the particular fluorescent signals produced by the fluorescent tags. Reporting may include a simple report of the particular tags associated with the particular

chromosome and/or may comprise an automatic diagnostic (indicating the type of cancer associated with the particular hybridization pattern of the chromosomes). The vector value as compared to normal specimens may be selected to be less than a particular threshold, such as less than about 60, less than about 40, less than about 30, less than about 20, less than about 10, or less than about 0.500. A useful system may comprise automatic scanning and detection in multiple signal channels at once, or in a relatively short period of time (e.g., less than 1 minute) from one another. The system may be operatively configured to process each of the multiple signals in real time, simultaneously or concurrently (or a mix of the same), to allow for quick detection of chromosomal regions, and/or regional replications, which are indicative of one or more particular cancer/hyperplasia.

BRIEF DESCRIPTION OF FIGURES

[00036] Fig. 1 is a flow chart representation of a method for providing a diagnosis of cervical cancer.

DETAILED DESCRIPTION OF THE INVENTION

[00037] As used herein, "tag" and "label" relate synonymously to a moiety conjugated to a probe to render the probe detectable by a particular detection method and modality.

[00038] As used herein "probe" relates generally to a substance specifically designed to bind to a cellular target, and not to bind significantly to cellular moieties or structures not intended to be a target. In several embodiments a probe may be a nucleic acid, polynucleotide or oligonucleotide whose sequence is sufficiently complementary to a target sequence in a cellular chromosome or other nucleic acid to hybridize to the latter structure under appropriate conditions. In various additional embodiments a probe may be an antibody or a portion thereof bearing a specificity determining binding site that specifically targets a cellular structure.

[00039] As used herein "representation" relates generally to any visual, graphical, numerical, or similar assembly of information that characterizes a result obtained using a particular detection method to examine a biological sample. By way of nonlimiting example, a representation includes an image of a microscopic field that includes at least a portion of a biological sample, an image further modified for example by computer driven means to convey information by attaching color values to particular features in a field, a graphical presentation characterizing particular features derived from an image of a sample, and a table of values or verbal entries characterizing features derived from an image.

[00040] As used herein "target", "targeted", "targeting" and similar words or phrases relate generally to a cellular structure to which a probe is specifically directed. A target is any structure or component that is a member of a specific binding pair constituted of the probe and the target. The probe and target have high specificity and affinity for binding to each other, and low specificity and low affinity for a probe, or for a target, respectively, not intended to be recognized. For a probe that includes a nucleic acid or at least a specific sequence of bases, a target is a complementary sequence found in chromosomal or nucleic acid components of a cell. For a probe that is an antibody or specific binding fragment thereof, a target may be an antigenic or hapten structure found in a cell. In this framework, a probe is a "targeting" moiety, and the target structure is "targeted" by the probe.

[00041] There are provided herein systems and methods for detecting and monitoring cancers and hyperplasias, particularly high grade hyperplasias, employing automated detection of signals.

[00042] In a representative embodiment, a biological sample is interrogated with one or more chromosomal probes having a detectable tag. The chromosomal probes may be selected and/or configured to have a high degree of sequence similarity to one or more portions of chromosomal material which is indicative of an element associated with a cancer or hyperplasia such as a high grade hyperplasia. The probes may be selected such that they associate with regions on the chromosome which are indicative of a cancer/hyperplasia or the amplification of which is associated with a cancer/hyperplasia. For example, multiple replications of a particular loci on a chromosome may be indicative of a cancer/hyperplasia. The tag on the probes advantageously is detectable either directly or indirectly (e.g. by binding of another detectable molecule to a portion of the tag). In one case, the tag is fluorescent, such as in FISH (fluorescent in situ hybridization). To promote hybridization between the tagged probe and the loci of interest on the chromosomal material, hybridization should be conducted under conditions sufficient for hybridization. In such embodiment, the sample is automatically scanned using a detector that can detect the tags. Automatic scanning may be by means of an automated microscope which is operatively configured to search a sample through multiple fields of view without the need for human intervention. The ability to associate the tags with particular chromosomes enables one to determine whether the hybridization profile is indicative of a cancer or hyperplasia, such as a high grade hyperplasia. Such association permits a determination of whether a cancer/hyperplasia is likely there. Optionally, the system of such embodiment may

include a means, for example software, hardware, or a software/hardware combination, for automatically reporting chromosomes in the sample which are tagged with the hybridized probe and the particular probes that are associated with the chromosome. Automatic diagnosis based upon the hybridization may also be provided as part of the automated microscope system.

[00043] In another representative embodiment, there is provided a method and system for monitoring the efficacy of a therapy to treat a cancer or hyperplasia, such as a high grade hyperplasia. The monitoring can be conducted over time in order to trace the effect of the therapeutic regimen as the patient is being treated. In such embodiment, a readily available fluid sample, such as blood, lymph, and effusion fluid, a lavage fluid, or an aspiration fluid, `is taken from a patient under therapy for treating the cancer and/or hyperplasia. Any sample so obtained has in it nucleated cells, including cells suspected of harboring a detectable chromosomal abnormality characteristic of a cancer or high grade hyperplasia. The sample is then treated with chromosomal probes that hybridize with specific loci or positions in the chromosomal material, for example to detect amplification associated with an abnormal sample, comprised by the fluid sample. Optionally multiple probes directed to different loci two or more of which are associated with a particular cancer/hyperplasia may be used. Use of such combinations may improve the efficiency of the detection of the cancer/hyperplasia. Such multiple probes are advantageously tagged with different tags, such as different fluorescent tags. The tags are selected to be readable by the detector associated with an automated scanning device, such as an automated microscope, which is operatively configured to repeatedly view discrete areas of the sample without human intervention. By detecting the tags associated with one or more chromosomal probes that are hybridized to chromosomes in the sample, one can determine if a hybridization pattern indicative of a cancer/hyperplasia is seen. Improvement may be had by automatically detecting the number of cells associated with the chromosomes hybridized by the chromosomal probes. That is, by judging whether the number of cells in the fluid indicative of an abnormal chromosomal complement is lower or higher than the number of cells seen at an earlier time, one may decide whether the therapy being used is being effective in the treatment of the cancer/hyperplasia being treated. The efficacy of a particular defined therapy on a particular cancer is thus based on changes in the number of cells detected in the biological sample over time. For example, if less cells are seen after treatment than before, it may be determined that the therapy is working. Different therapy may also be compared by the degree of reduction seen in such circulating abnormal cells.

[00044] In a variant of a method provided a method and system for monitoring the efficacy of a therapy to treat a cancer or hyperplasia, a patient may provide samples independently of visits to a medical or hospital facility. For example, a patient may be provided with a kit, a system, or similar equipment for obtaining a sample of blood for subsequent analysis by methods described herein. In such nonlimiting examples, a small volume of sample blood, such as one drop or a few drops, are harvested, optionally treated to prevent clotting, optionally disposed on a slide, or otherwise maintained in a state suitable for subsequent analysis. The scheduling of accumulating such samples may include daily sampling, or sampling every other day, or twice weekly, or weekly, or biweekly, or monthly, or at even greater intervals. Samples may be stored in dessicated chambers, and may be refrigerated or frozen while awaiting shipment or transfer, and subsequent analysis.

[00045] There is also described in a representative embodiment, a system/method that can be used for detection of cancers/hyperplasias, such as high degree hyperplasias that are related with the amplification of chromosome 3q. In an embodiment method, a single layer preparation of nuclei for interphase FISH hybridization is made. For example, the layer of nuclei may be obtained following appropriate processing of thin sections from paraffin-embedded tumor tissue samples to provide a nuclear smear. The nuclear smear is then stained using a centromeric probe for chromosome 3 or chromosome 7. Subsequently, previously, or concurrently, the nuclei smear is also stained with a locus specific probe for single copy sequences on the q arm of chromosome 3 which are indicative of a cancer/hyperplasia state of interest. For example, the probe can be a contig consisting of four overlapping BAC clones containing the TERC gene at chromosomal location 3q26 or a portion thereof. Other centromeric or locus specific probes can be added to the probe mixture. In one advantageous aspect, each probe is labeled with a different fluorochrome to allow for easier detection of distinct signals. Optionally the smear may be counterstained with a nuclear stain, such as DAPI. The stained smear is then applied to an automated scanning device, such as an automated microscope, and automatically scanned in DAPI and as many fluorescence channels as needed to enumerate signals for chromosome 3, locus on 3q and any other centromeric or locus specific signal. The scanned nuclei may be presented to a health care profession, such as a cytogeneticist or pathologist for review thereof. The presentation to the health care profession may be in a sorted manner, for example with the nuclei with abnormal counts (e.g., not equal to 2) of the 3q related signals being presented first. Alternatively, or in conjunction, the system may be operatively configured (e.g. by means of a program) to analyze the scanned nuclei based on pre-

programmed algorithms (for example) and to provide an automated diagnostic indication to the health care provider. Such test may be use for the detection of a number of cancers, including cervical cancer.

[00046] Automated apparatuses and methods for carrying out the microscopic analysis of biological samples enhance diagnostic procedures and optimize the throughput of samples in a microscope-based diagnostic facility. A robotic microscope system is described in co-owned U. S. Patent Application 11833203 filed August 2, 2007. Among its disclosures, an integrated microscope system displaceable along a second surface is provided. The integrated microscope system includes an automated robotic microscope system housed in a light-tight enclosure. In this system, the automated robotic microscope system includes (i) a microscope having a stage; (ii) at least one specimen slide positionable on the stage; (iii) a light source that illuminates the slide; (iv) an image capture device that captures an image of the specimen; and (v)electrical, electronic and/or computer-driven means communicating with and controlling positioning of said specimen slide, said light source, and said image capture device. Furthermore, in this system the light-tight enclosure includes at least one shelf interior to said enclosure, wherein said automated robotic microscope system is positioned on a shelf; and a viewing monitor capable of displaying images or representations of a microscopic field being viewed or analyzed that is disposed in a surface of said enclosure viewable from a location exterior to the enclosure.

[00047] Referring to Fig. 1, an embodiment of an automated method of screening a biological sample for cervical cancer cells is presented. A slide containing the biological sample comprising nuclei from a subject is obtained (step 10). The biological sample is initially contacted (step 20) with a first nuclear centromeric probe bearing a first detectable label directed to chromosome 7. The biological sample is then contacted (step 30) with a second nuclear non-centromeric probe bearing a second detectable label directed to single copy sequences on the q arm of chromosome 3 wherein the single copy sequences comprises a contig sequence of four overlapping BAC clones containing at least a portion of the TERC gene at chromosomal location 3q26, and counterstained with DAPI. The first and second probes are contacted under conditions that promote hybridization of each said first and second probe to the respective chromosomal loci. The DAPI stained areas, comprising nuclei, are then located (step 40). The presence of first and second probes are determined (step 50). The labels of the first nuclear centromeric probe and the second nuclear non-centromeric probe, within the area of each nucleus are quantified (step 60). .Two labels of the first probe should be detected (step 70) within each nucleus while greater than or equal to 4 labels of the second probe are a positive indication (step 90) of cervical

cancer cells. If less than 4 second probe labels are found (step 80), a negative indication can be concluded.

[00048] A dynamic automated microscope operation and slide scanning system is described in co-owned U. S. Patent Application 11833594 filed August 3, 2007. Embodiments disclosed include an automated microscope and method for dynamically scanning a specimen mounted on a microscope slide using a dynamic scanning microscope incorporating a microscope slide stage, at least one source of illumination energy, at least one electronic imaging device, at least one interchangeable component carousel and a synchronization controller. An exemplary automated microscope has the ability to significantly reduce the time required to perform an examination, reduce vibration reaching the system, and to provide diagnostic results. During the imaging process, the stage and color filter wheel are in constant motion rather than stationary as in previous approaches. Real time position sensors on each of the moving sub-systems accurately telemeter the instant position of the stage mounted slide and the color filter wheel. The color filter wheel rotates at a sufficient speed to allow the capture of images, at each of the filter wavelengths, at each imaging location and focal plane.

[00049] Interchangeable objective lenses, filters, and similar elements for use in an automated microscope system are described in co-owned U. S. Patent Application 11833154 filed August 2, 2007. This application generally relates to remotely operated or robotically controlled microscopes, and specifically to the mechanization of a means for automatically interchanging objective lens assemblies, filters and/or other optical components. An apparatus for interchanging optical components in an optical path is disclosed, which includes a control motor having a rotatable motor shaft; a support structure supporting the control motor; a planar base defined by a periphery that is generally symmetric about a central point on the planar base, the planar base including a plurality of mounting fixtures housing a plurality of optical components equi-angularly placed at a same distance from the base center, and a mechanism that causes generally symmetric rotation of the planar base about its center, so that a particular optical component of choice is positioned in the optical beam.

[00050] An automated microscope stage for use in an automated microscope system is described in co-owned U. S. Patent Application 11833183 filed August 2, 2007. This application generally relates to a microscope stage that is adjustably moveable along the optic axis of the microscope. For example, a microscope slide mount is disclosed that is adjustable along a direction of the optic axis of the microscope, including a base plate; a microscope stage assembly movably mounted on said base plate operably configured to permit displacement of the assembly

along the direction of the optic axis; and a microscope slide holding means fixed to said microscope stage assembly.

[00051] An automated microscope slide cassette and slide handling system for use in an automated microscope system is disclosed in co-owned U. S. Patent Application 11833517 filed August 3, 2007. This application discloses a mechanism for removing and replacing a slide housed in a cassette defining a plurality of slots configured for holding slides in spaced parallel configuration.

[00052] An automated microscope slide loading and unloading mechanism for use in an automated microscope system is described in co-owned U. S. Patent Application 11833428 filed August 3, 2007. An exemplary embodiment discloses a microscope slide manipulation device which includes: a base structure; a sleeve defining a through-void, the sleeve having a first end and a second end, the second end fastened to the base, and the sleeve being oriented perpendicular to the base; a longitudinal shaft symmetric about an imaginary longitudinal axis in part positioned in the sleeve through-void in a manner to permit axial and longitudinal movement of the longitudinal shaft in the sleeve through-void, the longitudinal shaft having a shaft first end and a shaft second end, the shaft second end positioned within the sleeve through-void and the shaft first end projecting beyond the sleeve first end and including a parallel track structure in a plane to the sleeve imaginary longitudinal axis; a plate slideably positioned between the parallel track structures on the sleeve first end, the plate having a first plate end and a second plate end, one of the first plate end or second plate end having a two-pronged forked configuration defining a void area between each prong that corresponds to the width of a microscope slide, and wherein the fork has a gripping structure operatively configured to permit gripping of a microscope slide along its edges.

[00053] Automated methods that employ computer-resident programs to drive the microscopic detection of fluorescent signals from a biological sample, useable to drive an automated microscope system, are disclosed in co-owned U. S. Patent Application 11833849 filed August 3, 2007. An exemplary method of microscopic analysis, adaptable for high throughput analysis of multiple samples, disclosed therein includes steps of providing an automated microscope comprising a slide stage, at least one objective lens, image capturing means, programmable means for operating the microscope according to a protocol, and programmable means for providing an analytical outcome; providing a microscope slide containing a sample and interrogatable data thereon, wherein the interrogatable data provide

information related to a protocol for analysis of said sample; interrogating the data; positioning the slide on the slide stage; causing the microscope to analyze the sample in accordance with the analytical protocol encoded in the interrogatable data; and causing the microscope to provide an analytical outcome representing the sample. Automatic operation of a microscope using computer-resident programs to drive the microscope in conducting a FISH assay for image processing is described in co-owned U. S. Patent Application 11833204 filed August 2, 2007. Embodiments are disclosed which perform various image processing functions that may be employed to implement an automated fluorescence *in situ* hybridization method. The embodiments include an auto-exposure method for acceptably imaging all regions of the sample over an intensity range exceeding the dynamic range of the digital electronics; a method for enumeration of fluorescence *in situ* hybridization objects-of-interest which locates targets within the sample; nuclei identification which is a method for classifying and characterizing the objects-of-interest enumerated; segmenting nuclei which, is a method for defining the shape of an identified object of interest. Embodiments of the method are useful to characterize cell nuclei, or to enumerate a chromosome.

[00054] The automated microscope system described in the preceding paragraphs operates under control of computer-resident and computer-implemented instructions. Accordingly the system permits automated detection and analysis of samples without human intervention. The automated slide cassette and automated slide loading and unloading mechanism permit unattended high throughput analysis of a plurality of samples.

[00055] Methods disclosed herein are directed toward automating the detection and analysis of tissue specimens whose cells are suspected of harboring genes that have undergone somatic gene duplication or gene amplification during carcinogenesis. The methods afford computer driven image accumulation, and computer driven analysis of images obtained, as well as reporting results of such analyses in a variety of formats in an automated procedure that frees the methods from human intervention to a significant extent. Reports may be presented, by way of nonlimiting example, in the form of charts, tables, images of representations of a field on a slide, and the like. Reports are in digital formats as files or records, and as such are conveniently disseminated to local or remote locations for review. Because of the use of automated fluorescence microscopy, such as a system including components and software that is referenced herein, rapid, convenient, and accurate screening of tissue samples is afforded. These methods, and the automated microscope system employed in implementing them, are particularly well suited for use in high throughput analysis of a plurality of tissue samples.

[00056] Tissue samples may be derived from medical or surgical procedures that yield specimens from suspect tissues or organs, including by way of nonlimiting example scrapings from epithelial surfaces, surgical excision of epithelial tissues, various biopsies, and surgically resected tissues and organs. In nonlimiting embodiments, such samples are fixed and embedded in a supporting material, and tissue slices thereof are prepared in a microtome or similar instrument. The tissue slices are mounted on microscope slides. Additionally samples for analysis may originate from a biopsy, blood, lymph, urine, an effusion fluid, a biological fluid, a lavage fluid, aspiration fluid, sputum, and a tissue.

[00057] In various embodiments a slide-mounted tissue slice is then treated with a generic fluorescent dye that stains chromosomes or nucleic acids with a fluorescent probe having a particular emission color isolatable by a suitable optical filter. A nonlimiting example of a generic dye is 4',6-diamidino-2-phenylindole (DAPI). Staining with DAPI affords a means of identifying the location of nuclei, or of chromosomes, for the computer driven process of image capture for further capture of images from FISH probes.

[00058] The tissue specimen is hybridized to a fluorescently labeled FISH probe whose nucleotide sequence is constructed specifically to target a gene sequence, or a segment or portion of a gene sequence, that is specific for an oncogene sought to be targeted. The various fluorescent labels used in the probes are optically isolatable by the use of suitable filters and related optical components. The specificity of the nucleotide sequence ensures that all, or most, chromosomes in a specimen having the target sequence are in fact hybridized to the probe, while non-target sequences remain unhybridized. Hybridization is caused to proceed by heating sufficiently to denature the target sequence, thereby exposing single stranded DNA complementary to the probe. The process then continues by annealing the probe to the exposed single strand, thus labeling the sequence with the fluorescent label. A worker of skill in the field of the invention knows specific conditions of solution ionic strength, buffer composition, temperature, and the like, to achieve the required hybridization. Following annealing the excess probe is rinsed away.

[00059] The slide bearing the hybridized specimen is inserted into a slide-loading cassette that is a component of the automated microscope system. The system is set into operation, at which point the slide is caused to be transported from the cassette and placed on the stage of the microscope. In many embodiments each slide may bear a code interrogatable by the automated microscope that may include information such as a specimen identification, and the identities of any generic chromosome dye, and the various fluorescent labels on the FISH probes,

used with the specimen in question. Such information guides the automated microscope in selection of appropriate optical filters and related optical elements for use throughout the image accumulation process.

[00060] The automated scanning device, such as an automated microscope, may be configured to scan the biological sample in one plane, or in more than one plane, such as, for example, two, three or more planes. By scanning in multiple planes, detection of abnormal cells, which may be rare in terms of total number of cells in the sample, may be significantly improved.

[00061] In an embodiment, the probes may make use of FISH probes in which the fluorescent signal is detected by the detector. It should be understood that the probes may produce a signal with or without another input signal, e.g. they may be radioactive, or fluoresce when stimulated by an activating signal (such as an appropriate wavelength of light or other electromagnetic radiation). The probes may be directed to different replication associated cancer/hyperplasia loci, particular loci associated with a cancer/hyperplasia. Different fluorescent tags may be associated with probes to different loci so as to produce different signals.

[00062] The detector may be selected in accordance with signal(s) which are to be produced by the tags, e.g. a fluorescence detector for detecting fluorescent tags, with the detector operatively configured to permit detection of the particular fluorescent signals produced by the fluorescent tags.

[00063] Automated analysis may begin by directing the use of a low magnification of the microscope, using at least the generic dye, and possibly the probe labels, to identify regions within the specimen for imaging at a higher magnification. When the computer software identifies regions of interest at low magnification, it may direct the automated microscope to interchange objective lenses and/or filters, and any other optical components, for suitable image analysis of identified loci at higher magnification based on emitted light originating from one or another of a fluorescent label used in a probe. The computer software may then use features in an image, by way of nonlimiting example, the intensity and number of FISH-labeled spots, to enumerate such spots arising within single nuclei. Such an enumeration may provide a resulting indication of the extent of gene amplification in cells of the tissue in the specimen being analyzed.

[00064] Reporting may include a simple report of the particular tags associated with the particular chromosome and/or may comprise an automatic diagnostic (indicating the type of cancer associated with the particular hybridization pattern of the chromosomes). In certain

embodiments the automated microscope system automatically generates a report detailing the findings obtained in the various images, fields and representations obtained during operation. Such reports may make use of, or may reference, historical information, or patient information, already resident in a memory device associated with the automated microscope.

[00065] A useful system may comprise automatic scanning and detection in multiple signal channels at once, or in a relatively short period of time (e.g., less than 1 minute) from one another. The system may be operatively configured to process each of the multiple signals in real time, simultaneously or concurrently (or a mix of the same), to allow for quick detection of chromosomal regions, and/or regional replications, which are indicative of one or more particular cancer.

[00066] The vector value as compared to normal specimens may be selected to be less than a particular threshold, such as less than about 60, or less than about 40, or less than about 30, or less than about 20, or less than about 10, or less than about 3, or less than about 1, or less than about 0.500.

[00067] In a nonlimiting example of an analysis procedure, the automated method may involve steps such as the following:

1. A microscopic specimen is deposited by layering on a slide a thin section from a paraffin embedded tissue.

2. The tissue section is stained using fluorescence in situ hybridization (FISH) probes for targeted chromosomal loci or sequences.

3. Following FISH probe treatment the slide is scanned using a desktop scanner at a resolution that may be set at 100, or 200, or 300, or 400 dots per inch, or more, and the scanned image is processed in order to identify an area that has been marked by a pathologist for attention. The digitized information about this area is passed to an automated fluorescence microscope, such as an Ikoniscope™ microscope system (Ikonisys, Inc., New Haven, CT).

4. The slide is loaded in the automated microscope.

5. Automated scanning begins by using a low magnification, such as 2X, or 4X , or 5X, or 10X magnification, or a similar low magnification, for analysis using the DAPI channel, by which the instrument detects the regions of the slide that contain nuclei. Typically, scanning is done within the marked area in step (3).

6. Then, using a higher magnification, such as 10X, or 15X, or 20X, or 40X, or even greater magnification, the microscope system scans the regions identified in the previous step. Scanning is performed in the DAPI channel for the detection of nuclei and then in a channel directed to a wavelength of light in the range emitted by the fluorescent label used in the probe, such as an orange channel, for the enumeration of, for example, orange signals from a FISH probe with a label that emits orange radiation, and in a green channel for the enumeration of signals from a FISH probe with a label that emits green radiation . These provide features of interest, such as nuclei, for further characterization.

7. The positions of features of interest are recorded for subsequent scanning and verification of signal count in a highest magnification, such as a 100X magnification.

8. The automated microscope presents all images collected during 20X and 100X scanning to the pathologist for review and also offers the possibility for subsequent rescanning of the slides if the pathologist requires review in high magnification of another slide area.

STATEMENT REGARDING PREFERRED EMBODIMENTS

[00068] While the invention has been described with respect to preferred embodiments, those skilled in the art will readily appreciate that various changes and/or modifications can be made to the invention without departing from the spirit or scope of the invention as defined by the appended claims. All documents cited herein are incorporated by reference herein where appropriate for teachings of additional or alternative details, features and/or technical background.

WHAT IS CLAIMED IS:

1. A method of screening for the presence and/or extent of a pathology in a subject, the pathology characterized by an abnormal chromosomal component in a cell of the subject, comprising the steps of

a) contacting a biological sample comprising cell nuclei from said subject with one or more distinguishable labeled probes directed to at least one chromosomal sequence that characterizes the abnormality under conditions that promote hybridization of the one or more probes to the at least one sequence;

b) automatically obtaining a representation of the one or more distinguishable labels hybridized to the chromosomal sequences;

c) automatically analyzing the distribution and intensity of binding of the one or more labels in the representation to determine the presence and/or extent of an abnormal chromosomal component; and

d) automatically reporting results of the analysis of step c);

wherein steps b) – d) are carried out without intervention by a human.

2. The method described in claim 1 wherein an automated microscope system carries out steps b)-d).

3. The method describes in claim 1 wherein a probe targets at least one of a single nucleotide polymorphism (SNP), a mutated single or multi-base nucleotide sequence, a duplicated or amplified gene or portion thereof within an 800 kb chromosome region surrounding the TERC and PIK3A loci, a centromere of chromosome 3, a centromere of chromosome 7, and a sequence comprising the TERC, PIK3A genes, the surrounding 5' and 3' regions of either gene or a portion of the genes thereof.

4. The method described in claim 1 further comprising contacting the sample under the hybridizing conditions with a tagged reference probe directed to a chromosomal locus known not to be abnormal, or contacting the sample with a reference stain, and referencing the representing and analysis steps to the reference probe or stain.

5. An method of screening for an abnormality related to a cancer, a high grade hyperplasia or a high grade dysplasia in a subject, comprising the steps of:

a) obtaining a biological sample comprising nuclei from the subject;

b) contacting the biological sample with a first probe bearing a first detectable label directed to a chromosomal sequence related to the abnormality under conditions that promote hybridization of the probes to targeted chromosomal loci;

c) contacting the sample under the hybridizing conditions with at least one of a detectably labeled reference probe directed to a chromosomal locus known not to be abnormal and further contacting the sample with a nuclear reference stain;

d) automatically finding areas in the sample having the reference stain and imaging the labels bound to the chromosomal sequences;

e) automatically analyzing said label image for the distribution and intensity of hybridized labels ; and

f) automatically reporting results of the analysis of step e);

wherein steps d)-f) are performed without intervention by a human.

6. The method described in claim 5 wherein an automated microscope performs steps d)-f).

7. The method described in claim 5 wherein prior to the contacting step the nuclei are isolated from the sample and are deposited to form a layer.

8. The method described in claim 5 wherein the sample comprises one or more of a biopsy, surgical resection specimen, blood, lymph, urine, an effusion fluid, a biological fluid, an epithelial scraping, a smear from the uterine cervix, a buccal smear, a lavage fluid, aspiration fluid, saliva, sputum, and a tissue.

9. The method described in claim 5 wherein the first and second labels are fluorescent labels.

10. The method described in claim 5 wherein the first probe targets at least one of a single nucleotide polymorphism (SNP), a mutated sequence, a duplicated or amplified gene or portion thereof, a centromere of chromosome 3, a centromere of chromosome 7, and a sequence comprising the TERC gene or a portion thereof.

11. An automated method for monitoring the efficacy over time of a course of therapy in the treatment of a cancer or high grade hyperplasia in a patient, said method comprising the steps of:

(a) obtaining from the patient a fluid biological sample in which cells associated with the cancer or high grade hyperplasia are found;

(b) treating said fluid biological sample or a portion thereof with one or more detectably labeled chromosomal probes having a high degree of sequence similarity to one or more chromosomal loci associated with, or whose amplification is associated with, said cancer or high grade hyperplasia, under conditions sufficient to enable hybridization of said probes to chromosomes in the sample;

(c) automatically scanning by way of a robotic microscope said treated fluid biological sample and detecting said one or more labels bound to any chromosomes in said sample;

(d) automatically detecting by way of a robotic microscope the number of cells associated with said chromosomes hybridized to said chromosomal probes; and

(e) automatically comparing the hybridization patterns of a label and cell number results provided in steps (c) and (d) at differing times in the therapeutic treatment course, thereby evaluating the efficacy of the therapy in the treatment of the cancer or high grade hyperplasia.

12. The method of claim 11 wherein monitoring is performed at intervals of 1 day or greater.

13. The method described in claim 11 wherein the fluid biological sample comprises one or more of blood, lymph, urine, an effusion fluid, an epithelial scraping, a smear from the uterine cervix, a buccal smear, a lavage fluid, saliva, aspiration fluid, and sputum.

14. The method described in claim 11 wherein an automated microscope system performs steps (c)-(e) without intervention by a human.

15. The method described in claim 14 wherein the automated microscope system automatically optimizes the field scanning the sample.

16. The method described in claim 14 wherein the automated microscope scans two or more planes in a field of the sample.

17. The method described in claim 11 wherein a probe targets at least one of a single nucleotide polymorphism (SNP), a mutated single or multi-base nucleotide sequence, a duplicated or amplified gene or portion thereof within an 800 kb chromosome region surrounding the TERC and PIK3A loci, a centromere of chromosome 3, a centromere of chromosome 7, and a sequence comprising the TERC, PIK3A genes, the surrounding 5' and 3' regions of either gene or a portion of the genes thereof.

18. The method described in claim 11 wherein a patient obtains samples without assistance from another human.

19. An automated method of screening for cervical cancer cells, comprising the steps of:

(a) obtaining a slide containing a biological sample comprising nuclei from a subject, said biological sample having been contacted with a first nuclear centromeric probe bearing a first detectable label directed either to chromosome 7, and a second nuclear non-centromeric probe bearing a second detectable label directed to single copy sequences on the q arm of chromosome 3 wherein said single copy sequences comprises a contig sequence of four overlapping BAC clones containing at least a portion of the TERC gene at chromosomal location 3q26, and counterstained with DAPI, wherein said first and second probes are contacted under conditions that promote hybridization of each said first and second probe to the respective chromosomal loci;

(b) searching said slide for DAPI stained areas within said biological sample;

(c) searching said DAPI stained areas for the presence of label of said first nuclear centromeric probe and label of said second nuclear non-centromeric probe;

(d) quantitatively determining the number of said first nuclear centromeric probe and and second nuclear non-centromeric probe per nucleus in each DAPI stained area within said biological sample;

(e) designating the biological sample as comprising a potential for progression to high hyperplasia and invasive cervical cancer when the number of said first probe per within the DAPI is about 2 per nucleus and the number of said second nuclear non-centromeric probe per nucleus is within the DAPI stained area is equal to or greater than 4.

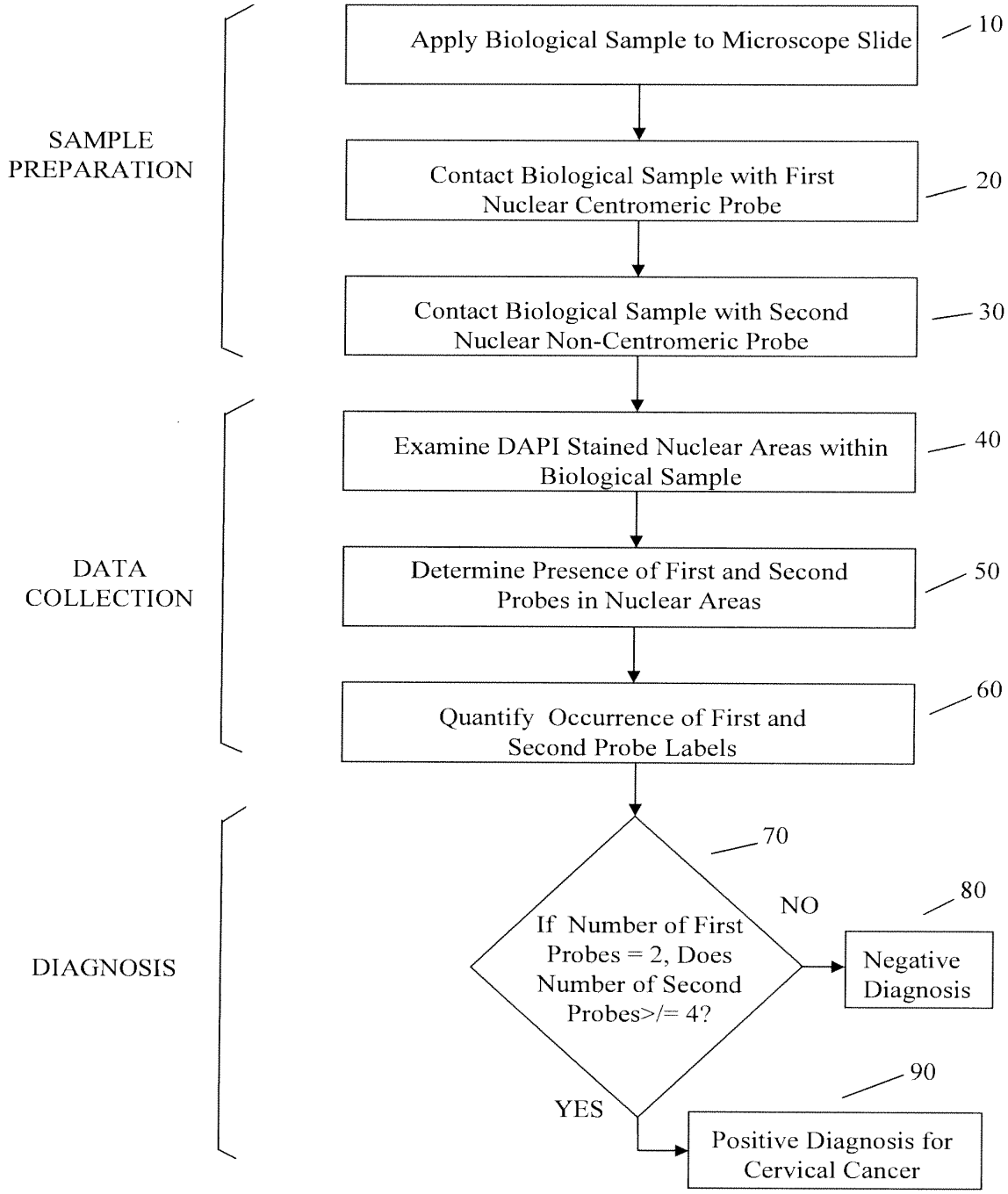

SAMPLE PREPARATION
DATA COLLECTION
DIAGNOSIS
Apply Biological Sample to Microscope Slide
10
Contact Biological Sample with First Nuclear Centromeric Probe
20
Contact Biological Sample with Second Nuclear Non-Centromeric Probe
30
Examine DAPI Stained Nuclear Areas within Biological Sample
40
Determine Presence of First and Second Probes in Nuclear Areas
50
Quantify Occurrence of First and Second Probe Labels
60
70
If Number of First Probes = 2, Does Number of Second Probes>/= 4?
NO
80
Negative Diagnosis
YES
90
Positive Diagnosis for Cervical Cancer

FIG. 1

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT
(PCT Article 17(2)(a), Rules 13*ter*.1(c) and (d) and 39)

Applicant's or agent's file reference	**IMPORTANT DECLARATION**	Date of mailing (*day/month/year*)
IK-167-P-US-C-CIP		12 JULY 2010 (12.07.2010)
International application No.	International filing date (*day/month/year*)	(Earlist) Priority date *(day/month/year)*
PCT/US2009/063141	**03 NOVEMBER 2009 (03.11.2009)**	10 MARCH 2009 (10.03.2009)

International Patent Classification (IPC) or both national classification and IPC

C12Q 1/68(2006.01)i, G01N 33/50(2006.01)i

Applicant

IKONISYS, INC. et al

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☒ The subject matter of the international application relates to:
   - a. ☐ scientific theories.
   - b. ☐ mathematical theories.
   - c. ☐ plant varieties.
   - d. ☐ animal varieties.
   - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
   - f. ☐ schemes, rules or methods of doing business.
   - g. ☐ schemes, rules or methods of performing purely mental acts.
   - h. ☐ schemes, rules or methods of playing games.
   - i. ☒ methods for treatment of the human body by surgery or therapy.
   - j. ☐ methods for treatment of the animal body by surgery or therapy.
   - k. ☒ diagnostic methods practised on the human or animal body.
   - l. ☐ mere presentation of information.
   - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.
2. ☒ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

   ☐ the description ☒ the claims ☐ the drawings
3. ☐ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:
   - ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   - ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   - ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b)
4. Further coments:

Name and mailing address of ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro,
Seo-gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

Lee, Jun Hyuk

Telephone No. 82-42-481-8115

Form PCT/ISA/203 (July 2009)

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	6232067		2001-05-15	Hunkapiller et al.	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20070190543	A1	2007-08-16	Livak	

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

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EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2022-01-26
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	44847143
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	27-JAN-2022
Filing Date:	06-AUG-2021
Time Stamp:	09:10:04
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21708460.pdf	1034146 b25c64dc309aa23fe9571f5d22b795861a58a0be	no	4
Warnings:					

Information:	
Total Files Size (in bytes):	1034146

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Patent Application Fee Transmittal

Application Number:	17395534
Filing Date:	06-Aug-2021
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Filer:	John P. Iwanicki/Jennifer Hazzard
Attorney Docket Number:	010498.01437\US

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1202	10	100	1000
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1000**

Electronic Acknowledgement Receipt

EFS ID:	44983958
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	14-FEB-2022
Filing Date:	06-AUG-2021
Time Stamp:	07:54:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1000
RAM confirmation Number	E20222D854572619
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Response to Election / Restriction Filed	Response_to_RR_of_12-14-21_21738119.pdf	26081 e2058a484a684c2307eee353bb7cc62cfa6ae3fd	no	6
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38288 448e34ce1841e680ef8b7b8fc05b03bbd6eb697f	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	64369		

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PATENT
ATTORNEY DOCKET NO. 010498.01437

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Evan R. Daugharthy	)	Examiner: David C. Thomas
	)	
Serial No.: 17/395,534	)	Art Unit: 1637
	)	
Filed: August 6, 2021	)	Conf. No.: 5147
	)	
Title: Method and Apparatus for Volumetric Imaging of a Three-Dimensional Nucleic Acid Containing Matrix	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO RESTRICTION REQUIREMENT

Dear Commissioner:

This is in response to the Restriction Requirement dated December 14, 2021. Please amend the above-referenced application as follows.

Claims begin at page 2 of this paper.

Remarks begin at page 6 of this paper.

In the Claims:

1. – 8. (Cancelled)

9. (New) A device, comprising:

(a) a stage, comprising:

(i) a sample holder, wherein said sample holder is configured to receive a solid substrate comprising a biological sample,

(ii) a heating or cooling apparatus programmable for a time and temperature parameter, wherein said heating or cooling apparatus is operationally connected to said sample holder, and

(iii) a multi-axis motion control system programmable for XY motion control of said stage;

(b) a fluidics system configured to dispense one or more reagents to said sample holder, wherein said fluidics system comprises:

(i) one or more reservoirs for containing said one or more reagents;

(ii) a fluidics dispenser configured to dispense said one or more reagents into said sample holder, wherein said fluidics dispenser is fluidically connected to said one or more reservoirs,

(iii) a pump that facilitates the dispensation of a programmed volume of fluid from at least one reservoir of said one or more reservoirs to said sample holder, and

(c) an optical system configured for volumetric imaging of said biological sample, wherein said optical system comprises:

(i) an objective,

(ii) a Z-axis motion control system operatively connected to one or more components of said optical system, wherein said Z-axis motion control system is configured for Z motion of an objective focal plane through said biological sample,

(iii) a light source configured to deliver light to said biological sample, and

(iv) one or more detectors positioned in light receiving communication with said sample holder, wherein said one or more detectors receives light intensity signals which are processed into a three-dimensional volumetric image of said biological sample.

10. (New) The device of claim 9, wherein said solid substrate is a flow cell.

11. (New) The device of claim 10, wherein said flow cell is a closed flow cell.

12. (New) The device of claim 11, wherein said closed flow cell comprises a fluid inlet and a fluid outlet, and wherein said fluid inlet comprises said fluidics dispenser.

13. (New) The device of claim 10, wherein said flow cell is an open flow cell.

14. (New) The device of claim 9, wherein said objective is a water immersion objective.

15. (New) The device of claim 9, wherein said objective is a water dipping objective.

16. (New) The device of claim 9, wherein said heating or said cooling apparatus comprises a heat sink or a Peltier element.

17. (New) The device of claim 9, wherein said heating or said cooling apparatus comprises a temperature sensor for providing temperature feedback to a thermal control system.

18. (New) The device of claim 9, wherein said multi-axis motion control system is programmable for XYZ motion control of said stage.

19. (New) The device of claim 9, wherein said pump is a syringe pump.

20. (New) The device of claim 9, wherein said fluidics system further comprises one or more valves downstream of said one or more reservoirs and upstream of said fluidics dispenser, wherein, upon actuation, said one or more valves facilitate dispensation of said programmed volume of fluid to said sample holder.

21. (New) The device of claim 20, wherein said one or more valves comprise a rotary valve.

22. (New) The device of claim 20, wherein said one or more valves comprise a solenoid valve or a microfluidic valve.

23. (New) The device of claim 13, wherein said fluidics system further comprises an aspiration system configured to remove liquid from said open flow cell, wherein said aspiration system comprises an aspiration tube that removes liquid from said open flow cell and a pump or vacuum source operatively coupled to said aspiration tube.

24. (New) The device of claim 23, wherein said aspiration tube is operatively coupled to a motor, wherein said motor is configured to adjust a motion axis of said aspiration tube to control an interface between said aspiration tube and said flow cell.

25. (New) The device of claim 24, wherein said motor is a stepper motor.

26. (New) The device of claim 9, wherein said Z-axis motion control system comprises a Z-axis linear motor configured for Z motion of said objective focal plane through said biological sample.

27. (New) The device of claim 26, further comprising a linear encoder configured to provide Z motion feedback to said Z-axis linear motor.

28. (New) The device of claim 9, further comprising an objective wetting apparatus.

29. (New) The device of claim 9, wherein said light source comprises one or more light emitting diodes (LEDs).

30. (New) The device of claim 9, said light source comprises one or more lasers.

31. (New) The device of claim 9, wherein said optical system further comprises one or more emission filters and one or more excitation filters for directing certain wavelengths of light within said optical system.

32. (New) The device of claim 31, wherein said one or more excitation filters are configured to provide light having a wavelength of about 480 nm, about 530 nm, about 590 nm, or about 640 nm to said biological sample.

33. (New) The device of claim 31, wherein said one or more emission filters are configured to provide light having a wavelength of about 510 nm, about 570 nm, about 620 nm, or about 680 nm to said one or more detectors.

34. (New) The device of claim 9, wherein said optical system is mechanically, electrically, or electromechanically coupled with a controller system configured to synchronize one or more imaging events within said device.

35. (New) The device of claim 9, where said device comprises a vibration control rigid foundation.

36. (New) The device of claim 9, further comprising, one or more microprocessors with software for automating or controlling said dispensation of said one or more reagents into said sample holder.

37. (New) The device of claim 9, further comprising, one or more microprocessors with software for automating or controlling said time and temperature parameter of said heating or cooling apparatus.

38. (New) The device of claim 9, further comprising, one or more microprocessors with software for automating or controlling image detection and acquisition of said optical system.

REMARKS

At page two of the Restriction Requirement, the Examiner has required restriction among

Group I, claims 1-7 drawn to a method of analyzing a plurality of nucleic acids within a three dimensional polymerized matrix; and

Group II, claim 8, drawn to an automated sequencing and volumetric imaging device comprising a multi axis state or positioning system, a heating or cooling apparatus, a fluidics dispenser, a pump, an optical assembly, or or more detectors, and one or more microprocessors.

Applicant has cancelled claims 1-8 thereby mooting the Restriction Requirement.

Notwithstanding, solely for the purpose of being responsive to the Restriction Requirement, Applicant elects with traverse the invention of Group II.

Respectfully submitted,

Dated: February 14, 2022

/John P. Iwanicki/

John P. Iwanicki, Reg. No. 34,628
BANNER & WITCOFF, LTD.
28 State Street, Suite 1800
Boston, MA 02109
(617) 720-9640

PTO/SB/07 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET Substitute for Form PTO-1360 (For use with Form PTO/SB/06)	Application Number 17/395,534	Filing Date 08/06/2021	☐ To be Mailed	
	Applicant(s) Daugharthy et al.	Examiner THOMAS, DAVID C	AIA (FITF) Status Yes	Page 1 of 1

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SEC. AMENDMENT		*			*		*	
	Indep	Depend	Indep	Depend	Indep	Depend		Indep	Depend	Indep	Depend	Indep	Depend
1							51						
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47							97						
48							98						
49							99						
50							100						
Total Indep							Total Indep						
Total Depend							Total Depend						
Total Claims							Total Claims						

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2

Part of Paper No 20220221 .

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 17/395,534	Filing Date 08/06/2021	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $480 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/VICTOR BARLOW/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	5635352		1997-06-03	Urdea et al.	

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

	1		

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2022-02-28
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	45099994
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	28-FEB-2022
Filing Date:	06-AUG-2021
Time Stamp:	12:32:19
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21763357.pdf	1034114 be38753c3dfe9eac7ac7147a713a456f5966ab21	no	4
Warnings:					

Information:	
Total Files Size (in bytes):	1034114

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-22)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

U.S.PATENTS Remove

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Patent citation information please click the Add button. Add

U.S.PATENT APPLICATION PUBLICATIONS Remove

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20080180790	A1	2008-07-31	Tafas et al.	

If you wish to add additional U.S. Published Application citation information please click the Add button. Add

FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1							

If you wish to add additional Foreign Patent Document citation information please click the Add button Add

NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

	1		

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

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That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2022-03-01
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

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The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these record s.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

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Electronic Acknowledgement Receipt

EFS ID:	45115712
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	01-MAR-2022
Filing Date:	06-AUG-2021
Time Stamp:	13:57:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Information_Disclosure_Statement_Fillable_PDF_21766526.pdf	1263131 3f6ac7137c32922e59eee185a1e45917f0488305	no	4
Warnings:					

Information:	
Total Files Size (in bytes):	1263131

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New Applications Under 35 U.S.C. 111

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National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/395,534	08/06/2021	Evan R. Daugharthy	010498.01437\US	5147

22907 7590 03/04/2022

BANNER & WITCOFF, LTD.
1100 13th STREET, N.W.
SUITE 1200
WASHINGTON, DC 20005-4051

EXAMINER	
THOMAS, DAVID C	

ART UNIT	PAPER NUMBER
1637	

NOTIFICATION DATE	DELIVERY MODE
03/04/2022	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

GPD@bannerwitcoff.com
eofficeaction@bannerwitcoff.com

Office Action Summary	Application No. 17/395,534	Applicant(s) Daugharthy et al.	
	Examiner DAVID C THOMAS	Art Unit 1637	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 14 February 2022.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 9-38 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 9-38 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date See Continuation Sheet.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

Continuation of Attachment(s) 2) Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date: 01/27/2022, 01/24/2022, 11/30/2021, 11/24/2021, 11/04/2021, 11/01/2021, 10/04/2021, 09/21/2021, 08/06/2021, 08/06/2021, 08/06/2021, 08/06/2021, 08/06/2021, 02/28/2022

DETAILED ACTION

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA. This application is a Continuation of U.S. Application No. 16/386,337, now U.S. Patent No. 11,118,220, which is a Continuation of U.S. Application No. 15/969,118, now U.S. Patent No. 10,266,888, which claims benefit of U.S. Provisional Application 62/250,182, filed November 3, 2015. Claims 9-38 will be examined on the merits. Claims 1-8 have been canceled. No claims have been withdrawn from consideration.

Double Patenting

Nonstatutory Obviousness-type Double Patenting Rejections

2. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

With respect to the non-statutory double patenting rejection(s) made in this Office action, note as follows. The use of the terminology “defined in 35 U.S.C. §154 to §156 and §173” in a terminal disclaimer can result in the terminal disclaimer being found improper. To address this, note that a proper terminal disclaimer need only disclaim the patent’s remaining “full statutory term” as defined in 35 U.S.C., without specifying 35

U.S.C. 154 and 173. This is so, because the "full statutory term" inherently is a statutorily defined item.

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

3. Claims 9-38 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-14 of U.S. Patent No. 11,118,220 in view of Callahan et al. (U.S. Patent Pub. No. 2011/0216953). Although the conflicting claims are not identical, they are not patentably distinct from each other because both sets of claims are drawn to a device or system for nucleic acid analysis comprising a stage comprising a sample holder, a heating or cooling apparatus (see claim 8 of the '220 patent), a multi-axis motion control system (see claims 2, 5 and 7 of the '220 patent), a fluidics system comprising a fluidics dispenser, an optical system comprising an objective (see claim 3 of the '220 patent), a Z-axis motion control system and one or more detectors. The claims of the instant application differ from the claims of U.S. Patent No. 11,118,220 in that the claims of U.S. Patent No. 11,118,220 do not expressly teach a fluidics system comprising one or more reservoirs for containing one or more reagents wherein the fluidics dispenser is fluidically connected to the one or more reservoirs, and a pump that facilitates the dispensation of a programmed volume of fluid from at least one reservoir to the sample holder, and also does not teach an optical system comprising a light source configured to deliver light to a biological sample. However, Callahan teaches devices and systems for performing visual servoing optical microscopy for close monitoring of hundreds of individual living cells

over time (see Abstract and paragraphs 12 and 13). In addition, Callahan teaches that the device comprises computer-controlled syringe pumps for applying computer-controlled stimulations, perturbations, and interrogations of the cells, wherein the pumps deliver various probes and solutions of chemical and biological agents to the cells, wherein the agents are stored in inflow fluid or multi-well fluid reservoirs and delivered through fluid channels (see paragraphs 56 and 95, claim 20 and Figure 1). Callahan further teaches that the device comprises an optical reader instrument that comprises a fluorescence microscope comprising a computer-controlled x,y,z-stage, one or more objectives, a white light source with an excitation filter wheel, and a detector (paragraph 109). Therefore, it would have been prima facie obvious to one of ordinary skill in the art before the effective filing date of the claimed invention to modify the claims of U.S. Patent No. 11,118,220 with the teaching of Callahan to obtain the claimed invention since the skilled artisan would have been motivated with a reasonable expectation of success to combine the device comprising a means for reagent delivery to a biological sample within a microscopy device, and an optical reader system comprising a light source, as taught by Callahan in a system for nucleic acid analysis according to the claims of U.S. Patent No. 11,118,220.

4. Claims 9-38 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 18-30 of U.S. Patent No. 10,266,888 (cited on IDS of 08/06/2021, 26-page document) in view of Callahan et al. (U.S. Patent Pub. No. 2011/0216953). Although the conflicting claims are not identical, they are not patentably distinct from each other because both sets of claims are drawn

to a device or system for nucleic acid analysis comprising a stage comprising a sample holder, a heating or cooling apparatus (see claim 25 of the '888 patent), a multi-axis motion control system (see claims 19, 21 and 22 of the '888 patent), a fluidics system comprising a fluidics dispenser, an optical system comprising an objective (see claim 20 of the '888 patent), a Z-axis motion control system and one or more detectors. The claims of the instant application differ from the claims of U.S. Patent No. 10,266,888 in that the claims of U.S. Patent No. 10,266,888 do not expressly teach a fluidics system comprising one or more reservoirs for containing one or more reagents wherein the fluidics dispenser is fluidically connected to the one or more reservoirs, and a pump that facilitates the dispensation of a programmed volume of fluid from at least one reservoir to the sample holder, and also does not teach an optical system comprising a light source configured to deliver light to a biological sample. However, Callahan teaches devices and systems for performing visual servoing optical microscopy for close monitoring of hundreds of individual living cells over time (see Abstract and paragraphs 12 and 13). In addition, Callahan teaches that the device comprises computer-controlled syringe pumps for applying computer-controlled stimulations, perturbations, and interrogations of the cells, wherein the pumps deliver various probes and solutions of chemical and biological agents to the cells, wherein the agents are stored in inflow fluid or multi-well fluid reservoirs and delivered through fluid channels (see paragraphs 56 and 95, claim 20 and Figure 1). Callahan further teaches that the device comprises an optical reader instrument that comprises a fluorescence microscope comprising a computer-controlled x,y,z-stage, one or more objectives, a white light source with an excitation filter wheel, and a detector (paragraph 109). Therefore, it would have been

prima facie obvious to one of ordinary skill in the art before the effective filing date of the claimed invention to modify the claims of U.S. Patent No. 10,266,888 with the teaching of Callahan to obtain the claimed invention since the skilled artisan would have been motivated with a reasonable expectation of success to combine the device comprising a means for reagent delivery to a biological sample within a microscopy device, and an optical reader system comprising a light source, as taught by Callahan in a system for nucleic acid analysis according to the claims of U.S. Patent No. 10,266,888.

Claim Objections

5. Claim 30 is objected to because of the following informalities: Claim 30 recites "The device of claim 9, said light source comprises one or more lasers". The claim lacks a connecting language term such as "wherein" or "further comprising". Appropriate correction is required.

Claim Rejections - 35 USC § 103

6. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

7. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have

> been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

8. This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

9. Claim 9 and 14-38 is/are rejected under 35 U.S.C. 103 as being unpatentable over Keller et al. (U.S. Patent Pub. No. 2015/0098126) in view of Callahan et al. (U.S. Patent Pub. No. 2011/0216953).

With regards to claim 9, Keller teaches a device (a microscope system is provided for imaging a live biological specimen, see Abstract and paragraph 26), comprising:

(a) a stage (a specimen chamber, part 168, houses the specimen holder, part 166, and also houses a multi-stage adapter module for connecting the specimen holder to the specimen positioning system, paragraphs 153, 154 and 159 and Figure 5B), comprising:

(i) a sample holder, wherein said sample holder is configured to receive a solid substrate comprising a biological sample (the specimen holder, part 166, may comprise a glass capillary, part 200, containing the specimen, part 101, that is mounted to a solid base, part 201, paragraphs 28, 153 and 154 and Figure 5B)

(ii) a heating or cooling apparatus programmable for a time and temperature parameter, wherein said heating or cooling apparatus is operationally connected to said sample holder (the specimen holder and specimen chamber may be connected to a thermoelectric cooling device, paragraph 155), and

(iii) a multi-axis motion control system programmable for XY motion control of said stage (the stage connected to a positioning system may be set up to translate the holder along three orthogonal directions, such as the x, y and z axes, paragraph 154);

(b) a fluidics system configured to dispense one or more reagents to said sample holder, wherein said fluidics system comprises:

(iii) a pump that facilitates the dispensation of a programmed volume of fluid from at least one reservoir of said one or more reservoirs to said sample holder (the chamber has inlet and outlet valves connected to a perfusion system operated by dual-channel 12-roller pump that is connected to the specimen chamber by a clear, flexible tubing for providing temperature control, pH control, and maintaining oxygen concentration and levels of sugars and amino acids in the surrounding medium of the specimen, paragraphs 159 and 161), and

(c) an optical system configured for volumetric imaging of said biological sample (the microscope system may be configured for performing volumetric imaging of a biological specimen, see paragraphs 139 and 386), wherein said optical system comprises:

(i) an objective (the microscope system comprises one or more microscope objectives placed between a light source unit and the detection unit, including

illumination objectives, part 143, and detection objectives, part 150 and 152, paragraphs 10, 11, 29, 142 and 146, and Figures, 3, 4 and 5A),

(ii) a Z-axis motion control system operatively connected to one or more components of said optical system, wherein said Z-axis motion control system is configured for Z motion of an objective focal plane through said biological sample (the optical scanner device may deflect a light beam along the x-axis or another axis, and when performing volumetric imaging without moving the specimen, the light beam may be deflected along the z axis, paragraphs 106, 139 and 386),

(iii) a light source configured to deliver light to said biological sample (light sources may include a single light source, or two separate light sources, including lasers, paragraphs 128 and 129 and Figures 3 and 5A, part 122), and

(iv) one or more detectors positioned in light receiving communication with said sample holder, wherein said one or more detectors receives light intensity signals which are processed into a three-dimensional volumetric image of said biological sample (cameras are positioned to receive light after it is emitted from the specimen, collected by a detection objective, parts 150 and 152, passes through filter, parts 154 and 156, and onto the sensor of the camera, part 106 and 108, paragraph 146 and Figure 3; the system is capable of performing volumetric imaging in the x, y and z dimensions, paragraphs 106, 139 and 386).

With regards to claims 14 and 15, Keller teaches a device wherein said objective is a water immersion objective or a water dipping objective (the detection objectives may be high numerical aperture water-dipping objectives, paragraphs 148 and 159).

With regards to claim 16, Keller teaches a device wherein said heating or said cooling apparatus comprises a heat sink or a Peltier element (the specimen holder and specimen chamber may be connected to a thermoelectric cooling device such as in integrated Peltier cooler, heater, or thermoelectric heat pump to maintain the specimen at a particular temperature, paragraphs 155 and 161).

With regards to claim 17, Keller teaches a device wherein said heating or said cooling apparatus comprises a temperature sensor for providing temperature feedback to a thermal control system (a temperature control system is thermally connected to the specimen chamber, part 168, to permit temperature regulation of the chamber and the environment around the specimen, paragraph 161).

With regards to claim 18, Keller teaches a device wherein said multi-axis motion control system is programmable for XYZ motion control of said stage (the stage connected to a positioning system may be set up to translate the holder along three orthogonal directions, such as the x, y and z axes, wherein the positioning system may be a high performance stepper and servo motion controller connected to an interface within the electronics controller, paragraphs 154 and 157).

With regards to claims 20 and 21, Keller teaches a device wherein said fluidics system further comprises one or more valves downstream of said one or more reservoirs and upstream of said fluidics dispenser, wherein, upon actuation, said one or more valves facilitate dispensation of said programmed volume of fluid to said sample holder, wherein said one or more valves comprise a rotary valve (the specimen chamber, part 168, has inlet and outlet valves connected to the perfusion system to control the environment around the specimen, paragraphs 159 and 161).

With regards to claims 26 and 27, Keller teaches a device wherein said Z-axis motion control system comprises a Z-axis linear motor configured for Z motion of said objective focal plane through said biological sample, and further comprising a linear encoder configured to provide Z motion feedback to said Z-axis linear motor (the relative placement of the specimen and light sheets may be modified by moving the specimen along the z axis in steps while maintaining the light sheet and detection systems stationary, or moving the light sheets and the detection systems in synchrony along the z axis, while maintaining the specimen stationary, wherein linear motors are used to move the specimen, and actuators are used to control the light sheets and the detection systems, paragraphs 199 and 120).

With regards to claim 28, Keller teaches a device further comprising an objective wetting apparatus (see paragraphs 148 and 159 regarding water-dipping objectives).

With regards to claims 29 and 30, Keller teaches a device wherein said light source comprises one or more light emitting diodes (LEDs), or wherein said light source comprises one or more lasers (the light sources may include a laser, such as a single-pulsed Ti:Sapphire laser that is split into two beams for a two-photon excitation arrangement, or wherein the light source may comprise up to six laser diodes and diode pumped solid state lasers, paragraphs 128-130).

With regards to claim 31, Keller teaches a device wherein said optical system further comprises one or more emission filters and one or more excitation filters for directing certain wavelengths of light within said optical system (light beams that are output from light sources may be directed through optical (excitation) filters, and may be mounted on a filter wheel, paragraph 143; fluorescence light emitted from a specimen

and collected by objectives, then passes through a filter (emission) which rejects light of wavelengths outside a wavelength band centered around the wavelength to be detected by the detector, paragraph 146 and Figure 3).

With regards to claims 32 and 33, Keller teaches a device wherein said one or more excitation filters are configured to provide light having a wavelength of about 480 nm, about 530 nm, about 590 nm, or about 640 nm to said biological sample, wherein said one or more emission filters are configured to provide light having a wavelength of about 510 nm, about 570 nm, about 620 nm, or about 680 nm to said one or more detectors (light from a light source such as a laser may be output at wavelengths from 405 to 685 nm, paragraph 132; after passing through an optical filter, paragraph 143, the excitation light sheets excite fluorophores within the specimen to higher energy levels, and the emitted light is collected by objectives, then passes through a filter (emission) which rejects light of wavelengths outside a wavelength band centered around the wavelength to be detected by the detector, paragraphs 109, 146, 270 and 271 and Figure 3).

With regards to claim 34, Keller teaches a device wherein said optical system is mechanically, electrically, or electromechanically coupled with a controller system configured to synchronize one or more imaging events within said device (the microscope system may be operated through an electronics controller computational controller, paragraphs 57 and 123 and Figures 6 and 20).

With regards to claim 38, Keller teaches a device further comprising, one or more microprocessors with software for automating or controlling image detection and acquisition of said optical system (data acquisition for the microscope system occurs at

speeds that match the time-scales of the fastest processes of interest and with minimal time shifts between complementary views, wherein the system comprise an electronics controller and computational system to provide synchronized control of all mechanical components within the microscope with millisecond precision over long periods of time, and provides a robust pipeline for simultaneous high-speed image acquisition with a plurality of detectors, paragraphs 44 and 123).

However, with regards to claim 1, Keller does not teach (i) one or more reservoirs for containing said one or more reagents and (ii) a fluidics dispenser configured to dispense said one or more reagents into said sample holder, wherein said fluidics dispenser is fluidically connected to said one or more reservoirs. In addition, Keller does not teach a device wherein said pump is a syringe pump (claim 19), device wherein said one or more valves comprise a solenoid valve or a microfluidic valve (claim 22), or wherein said fluidics system further comprises an aspiration system configured to remove liquid from said open flow cell, wherein said aspiration system comprises an aspiration tube that removes liquid from said open flow cell and a pump or vacuum source operatively coupled to said aspiration tube, wherein said aspiration tube is operatively coupled to a motor, wherein said motor is configured to adjust a motion axis of said aspiration tube to control an interface between said aspiration tube and said flow cell (claims 23 and 24). In addition, Keller fails to teach a device wherein said motor is a stepper motor (claim 25), or comprises a vibration control rigid foundation (claim 35), and lastly, does not teach a device further comprising, one or more microprocessors with software for automating or controlling for automating or controlling said dispensation of said one or more reagents into said sample holder

(claim 36), or said time and temperature parameter of said heating or cooling apparatus (claim 37).

Callahan teaches devices and systems for performing visual servoing optical microscopy for close monitoring of hundreds of individual living cells over time (see Abstract and paragraphs 12 and 13).

Specifically with regards to claims 1 and 19, Callahan teaches that the device comprises computer-controlled syringe pumps for applying computer-controlled stimulations, perturbations, and interrogations of the cells, wherein the pumps deliver various probes and solutions of chemical and biological agents to the cells, wherein the agents are stored in inflow fluid or multi-well fluid reservoirs and delivered through fluid channels (see paragraphs 56 and 95, claim 20 and Figure 1).

With regards to claim 22, Callahan teaches a device wherein said one or more valves comprise a solenoid valve or a microfluidic valve (the device may comprise a control system for fluid delivery using a combination of micro-values and micropumps, or using microfluidic valves, paragraph 97 and claim 20).

With regards to claims 23 and 24, Callahan teaches a device wherein said fluidics system further comprises an aspiration system configured to remove liquid from said open flow cell, wherein said aspiration system comprises an aspiration tube that removes liquid from said open flow cell and a pump or vacuum source operatively coupled to said aspiration tube, wherein said aspiration tube is operatively coupled to a motor, wherein said motor is configured to adjust a motion axis of said aspiration tube to control an interface between said aspiration tube and said flow cell (the device may

comprise a vacuum-powered aspirator to remove excess liquid form a microincubation chamber, paragraphs 205 and 206 and claim 20).

With regards to claim 25, Callahan teaches a device wherein said motor is a stepper motor (motors such as stepping motors may be provided for functions such as z-axis positioning, and a computer controlled XYZ microcapillary positioner or robotic arm, paragraphs 81 and 251).

With regards to claim 35, Callahan teaches a device where said device comprises a vibration control rigid foundation (the device comprises an optical platform comprising a fluorescence microscope, wherein the microscope has sufficient weight to be stable one the camera and microscope peripherals are mounted on the microscope, wherein the optical platform is relatively stable and vibration-free, paragraphs 233 and 242).

With regards to claims 36 and 37, Callahan teaches a device further comprising, one or more microprocessors with software for automating or controlling said dispensation of said one or more reagents into said sample holder, and further comprising, one or more microprocessors with software for automating or controlling said time and temperature parameter of said heating or cooling apparatus (the device is a computer-controlled system for controlling delivery of fluorescent probes and solutions of chemical and biological agents to the cells, and also for controlling the temperature of the cell vessel, paragraph 56).

It would have been prima facie obvious to one having ordinary skill in the art before the effective filing date of the claimed invention to combine the devices and systems of Keller and Callahan since both references teach microscopy-based devices

for analyzing biological specimens such as cells, wherein the devices each comprise an optical instrument that comprises a fluorescence microscope comprising a sample holder to receiving a biological sample, a computer-controlled x,y,z-stage to allow for volumetric imaging of the specimen, a heating/cooling system to maintain the environmental conditions around the specimen, a fluidics system, and an optical system comprising one or more objectives, one or more light sources with an excitation filter wheel and one or more emission filters, and a detector. In addition, Callahan teaches that the device may comprise a fluidics system for delivery of reagents such as probes or chemical agents to the cell specimen sample, wherein the reagents are stored in reservoirs and delivered to the specimen by a syringe pump, wherein the fluidics system is controlled by a computer system (see paragraph 56). Thus, an ordinary practitioner would have been motivated to combine the devices and systems of Keller and Callahan since Callahan provides a fluidics control system that may be readily accommodated in the device of Keller for maintaining a live specimen over a period of time during optical analysis. While Keller also teaches the use of a pump perfusion system for maintaining oxygen, pH and temperature levels around the specimen, the computer-controlled system of Callahan enables a user to perform additional analytical operations on a specimen, including stimulations, perturbations and interrogations using the computer-controlled syringe pumps for delivery of a wide variety of reagents to the specimen (see paragraph 56, lines 1-7). The user can take advantage of the computer's ability to keep track of vast numbers of previous combinations of such stimuli and how they were applied and what the responses were, for both the current experiment as well as to refer to a database of previous cell stimulations and responses (paragraph 56, lines 14-24).

10. Claim 10-13 is/are rejected under 35 U.S.C. 103 as being unpatentable over Keller et al. (U.S. Patent Pub. No. 2015/0098126) in view of Callahan et al. (U.S. Patent Pub. No. 2011/0216953) as applied to claims 9 and 14-38 above, and further in view of Frisen et al. (U.S. Patent Pub. No. 2019/0203275).

Keller and Callahan together teach the limitations of claims 9 and 14-38, as discussed above. However, neither Keller nor Callahan teach a device wherein said solid substrate is a flow cell, including an open or closed flow cell, or wherein said closed flow cell comprises a fluid inlet and a fluid outlet, and wherein said fluid inlet comprises said fluidics dispenser.

Frisen teaches compositions, apparatus and methods for preserving spatial information when performing multiplex nucleic acid analyses of biological specimens, including attaching oligonucleotides such as primers to a solid support, binding probes comprising barcode and target capture sequences to the primers and amplifying the probes to generate clusters having copies of the barcodes and target capture sequences on the support, contacting a biological specimen to the support and capturing nucleic acid sequences of the specimen and tagging nucleic acids of the specimen with the barcode sequences (see Abstract and paragraph 8). Frisen further teaches that captured biological specimens containing fluorescent labels may be imaged using a fluorescence microscope (see paragraphs 104-108 and Figure 5).

Specifically with regards to claims 10-13, Frisen teaches that solid supports for capturing and analyzing a biological specimen may be comprised within a flow cell, wherein the flow cell comprises a chamber where a reaction may be carried out, an inlet

for delivering reagents to the chamber, and an outlet for removing reagents from the chamber, and may also include one or more transparent surfaces allowing optical detection of biological specimens (paragraphs 36 and 42 and Figures 3 and 5). Frisen further teaches that flow cells may be in an open configuration to expose a solid support inside or to remove the solid support from the flow, such as to allow convenient delivery of a biological specimen to the solid support, and the flow cell may be subsequently closed to proceed with further analysis (see paragraph 91).

It would have been prima facie obvious to one having ordinary skill in the art before the effective filing date of the claimed invention to combine the devices and systems of Keller and Callahan with the compositions, apparatus and methods of Frisen since Frisen, like Keller and Callahan, teaches imaging of biological specimens using an optical system such as a fluorescence microscope, wherein reagents may be delivered to the specimen for maintaining optimal conditions around the specimen or for performing further analysis of the specimen. In addition, Frisen teaches that specimens may be captured on a solid support within a flow cell to allow both performing analysis of the captured specimen as well as imaging the specimen (see Figures 3 and 5). Thus, an ordinary practitioner would have been motivated to combine the devices and systems of Keller and Callahan with the compositions, apparatus and methods of Frisen since Frisen provides an additional means for capturing or containing a biological sample or specimen within an optical system or device that is readily adaptable to the devices of Keller and Callahan. A flow cell provides a convenient apparatus for use in methods of analyzing a biological specimen, as it is convenient for housing a support that may be treated with multiple fluidic reagents such as the repeated fluidic deliveries

used for some nucleic acid sequencing or nucleic acid hybridization protocols (Frisen, paragraph 91, lines 1-11), and furthermore, may comprise one or more transparent surfaces allowing optical detection and imaging of the biological specimen (paragraphs 36, lines 6-9).

Conclusion

11. Claims 9-38 are rejected on the grounds of nonstatutory obviousness-type double patenting. In addition, claims 9-38 are rejected over the prior art. No claims are free of the prior art.

Correspondence

12. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David C. Thomas whose telephone number is 571-272-3320 and whose fax number is 571-273-3320. The examiner can normally be reached on 5 days, 9-5:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on 571-272-0782. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/David C Thomas/
Primary Examiner, Art Unit 1637

Notice of References Cited	Application/Control No. 17/395,534	Applicant(s)/Patent Under Reexamination Daugharthy et al.	
	Examiner DAVID C THOMAS	Art Unit 1637	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-20150098126-A1	04-2015	Keller; Philipp Johannes	G02B21/367	359/385
*	B	US-20110216953-A1	09-2011	Callahan; Daniel E.	C12Q3/00	382/128
*	C	US-11118220-B2	09-2021	Daugharthy; Evan R.	C12Q1/6841	1/1
*	D	US-20190203275-A1	07-2019	FRISÉN; JONAS	C12N15/1065	1/1
	E					
	F					
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FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/395,534	Daugharthy et al.
	Examiner	**Art Unit**
	DAVID C THOMAS	1637

CPC - Searched*		
Symbol	**Date**	**Examiner**
C12Q 1/68	02/25/2022	DCT
C12Q 1/6841	02/25/2022	DCT
C12Q 1/6869	02/25/2022	DCT
C12Q 2565/518	02/25/2022	DCT
C12Q 2565/601	02/25/2022	DCT

CPC Combination Sets - Searched*		
Symbol	**Date**	**Examiner**

US Classification - Searched*			
Class	**Subclass**	**Date**	**Examiner**

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Search Notes		
Search Notes	**Date**	**Examiner**
EAST	12/09/2021	DCT
EAST	02/25/2022	DCT
EAST	03/01/2022	DCT
Google-search terms: optical device, fluorescence microscope, objective, pump, flow cell, xyz motion, volumetric imaging, fluidics dispenser, multi-axis motion control, detectors, filters, lasers, emission, excitation	02/25/2022	DCT
PALM, inventor name search	02/25/2022	DCT

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/395,534	Daugharthy et al.
	Examiner	Art Unit
	DAVID C THOMAS	1637

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

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	1	AL-ATTAR et al., Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs): The Hallmark of an Ingenious Antiviral Defense Mechanism in Prokaryotes, Bioi Chern. (20 11) vol. 392, Issue 4, pp. 277-289.	
	2	CARROLL, "A CRISPR Approach to Gene Targeting" 20(9) Molecular Therapy 1658-1660 (Sep. 2012).	
	3	JINEK , et al. 'RNA-programmed genome editing in human cells.' eLite 2013;2:e00471 . [retrieved 1-3, 6, 7, 10-12 on 06.03 .2014). Retrieved from the Internet. <URL: http://elife .elifesciences.org/content/2/e00471 >. entire document.	
	4	HALE et al., Essential Features and Rational Design of CRISPR RNAs That Function With the Cas RAMP Module Complex to Cleave RNAs, Molecular Cell, (20 12) vol. 45, Issue 3, 292-302.	
	5	HATOUM-ASLAN, et al. 'Mature clustered, regularly interspaced, short palindromic repeats RNA 5,9, 14 (crRNA) ength is measured by a ruler mechanism anchored at the precursor processing site.' Proceedings of the National Academy of Sciences. Vol. 108, No. 52. Pages 21218-21222. December 2011. entire document.	
	6	MAKAROVA et al., "Evolution and classification of the CRISPR-Cas systems" 9(6) Nature Reviews Microbiology 467-477 (1-23) (Jun. 2011).	
	7	RHO, Mina et al. 'Diverse CRISPRs Evolving in Human Microbiomes.' PLoS Genetics. Vol. 8, No. 6. 1-14 Pages 1-12. June 2012. entire document.	
	8	SONTHEIMER Erik, Project 7: Establishing RNA-Directed DNA Targeting in Eukaryotic Cells; Project dates: Nov. 16, 2011 to Dec. 31, 2012 (Feb. 4, 2012).	
	9	WIEDENHEFT eta!., "RNA -guided genetic silencing systems in bacteria and archaea" 482 Nature 331-338 (Feb. 16, 2012).	
	10	Liu et al, Cell-Penetrating Peptide-Mediated Delivery of TALEN Proteins via Bioconjugation for Genome Engineering, PLOS ONE, 2014, Vol9(1), pages 1-7.	
	11	Ramakrishna et al, Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA, Genome Res. published online April 2, 2014, pages 1-20 plus figures.	

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	12	The Delivery Problem, Nature Biotechnology, 2006, Vol 24(3), pages 305-306	
	13	Ansari et al, Riboactivators: Transcription activation by non-coding RNA, Grit Rev Biochem Mol Bioi. 2009 ; 44(1): 50-61.	
	14	Sapranauskas et al (Nucleic Acids Research, 2011, 39:9275-9282)	
	15	US Application No. 61/794,422, filed March 15, 2013, DAVIS, G. et al.	
	16	US Application No. 61/781,598, filed March 14, 2013, HAURWITZ, R.	
	17	GILBERT, Luke A., et al.,"CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes," CELL, vol. 154, no. 2, 1 July 2013 (2013-07-01), pages 442-451	
	18	MALI, P. et al., "Supplementary Materials for RNA-Guided Human Genome Engineering via Cas9," SCIENCE, vol. 339, no. 6121, 3 January 2013 (2013-01-03), pages 1-36	
	19	MAEDER, Morgan L., et al.," Robust, synergistic regulation of human gene expression using TALE activators," HHS PUBLIC ACCESS AUTHOR MANUSCRIPT, vol. 10, no. 3, 10 February 2013 (2013-02-10), pages 243-245	
	20	PEREZ-PINERA, Pablo, et al., "Synergistic and tunable human gene activation by combinations of synthetic transcription factors," NATURE METHODS, vol . 10. no. 3, 3 February 2013 (2013-02-03), pages 239-242	
	21	Preliminary Office Action issued by Brazilian Patent Office on April 7, 2020	X
	22	Official Notification issued May 24, 2020 for IL 242959	☒

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	23	2020 June 2 - (JP) Notice of Reasons for Rejection for App. No. 2019-039027	☒
	24	2020 July 3 - (AU) Examination Report for App. No. 20202039777	☐
	25	2020 Aug 19 - (MX) Office Action - App. No. MX/a/2015/016798	☒
	26	2020 Sept 10 - (CA) Office Action - App. No. 2,914,638	☐
	27	2020 Sept 21 - (NZ) First Examination Report - App. No. 715280	☐
	28	2020 Sept 21 - (NZ) First Examination Report - App. No. 753950	☐
	29	Fu et al., "Improving CRISPR-Cas nuclease specificity using truncated guide RNAs," Nature Biotechnology, Vol. 32, pp. 249-284 (January 26, 2014).	☐
	30	Lin et al., "CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences," Nucleic Acids Research, Vol. 42, No. 11, pp. 7473-7485 (May 16, 2014).	☐
	31	2020 Sept 21 - (NZ) First Examination Report - App. No. 753951	☐
	32	2020 Sept 25 - (RU) Office Action - App. No. 2019114706	☒
	33	DiCarlo et al., "Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems," Nucleic Acids Research, Vol. 41, No. 7, pp. 4336-4343 (2013).	☐

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	34	Gusev et al. "Rolling Circle Amplification: A New Approach to Increase Sensitivity for Immunohistochemistry and Flow Cyometry" American Journal of Pathology, Vol. 159, No. 1, July 2001, pp. 63-69	☐
	35	2020 Nov 10 - (US) Non-Final Office Action - Appl. No. 16/285,292	☐
	36	Pihlak et al. "Rapid genome sequencing with short universal tiling probes" Nature Biotechnology, Vol. 26, No. 6, June 2008, pp. 676-684	☐
	37	Lizardi "Next-generation sequencing-by-hybridization" Nature Biotechnology, Vol. 26, No. 6, June 2008, pp. 649-650	☐
	38	Mignardi et al. "Fourth-generation sequencing in the cell and the clinic" Genome Medicine, 2014, 6:31	☐
	39	2020 Dec 24 (US) - Notice of Allowance - App 16/393,215	☐
	40	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155459/http://www.polonator.org/index.htm; Wayback Machine (August 7, 2008) "Open, Affordable, Sequencing..."	☐
	41	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155600/http://www.polonator.org;/vision.aspx; Wayback Machine (August 7, 2008) "The Vision"	☐
	42	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155250/http://www.polonator.org/ecosystem; Wayback Machine (August 7, 2008) "The Polonator Ecosystem"	☐
	43	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155759/http://www.polonator.org/instrument; Wayback Machine (August 7, 2008) "Instrument Overview"	☐
	44	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155857/http://www.polonator.org; Wayback Machine (August 7, 2008) "Protocols"	☐

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	45	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133362/http://www.polonator.org/protocols/pet.aspx; Wayback Machine (September 5, 2008) "PET (Paired End-Tag) Genomic Shotgun Library Construction Protocol"	☐
	46	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133855/http://www.polonator.org/protocols/pcr.aspx; Wayback Machine (September 5, 2008) "Emulsion PCR Protocol"	☐
	47	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133913/http://www.polonator.org; Wayback Machine (September 5, 2008) "Emulsion Breaking Protocol"	☐
	48	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133837/http://www.polonator.org/protocols/beadenrichment.aspx; Wayback Machine (September 5, 2008) "Bead Enrichment Protocol"	☐
	49	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133372/http://www.polonator.org/protocols.beadcapping.aspx; Wayback Machine (September 5, 2008) "Bead Capping Protocol"	☐
	50	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133372/http://www.polonator.org/protocols/coverslip.aspx; Wayback Machine (September 5, 2008) "Coverslip Aminosilanation and Arraying Protocol"	☐

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	Attorney Docket Number	010498.01437\US

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Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-08-05
Name/Print	John P. Iwanicki	Registration Number	34,628

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Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

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/D.C.T/	1	H04-268359	JP	A	1992-09-24	Taiho Pharmaceutical Co Ltd		X

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
/D.C.T/	1	6232067		2001-05-15	Hunkapiller et al.	

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/D.C.T/	1	20070190543	A1	2007-08-16	Livak	

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Examiner Signature	/DAVID C THOMAS/	Date Considered	02/25/2022

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Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2022-01-26
Name/Print	John P. Iwanicki	Registration Number	34,628

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Art Unit	N/A
Examiner Name	Not Yet Assigned
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	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

/D.C.T/	1	Tillberg et al., "Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies," Nat Biotechnol., Vol. 34, No. 9, pp. 987-992 (2016).	

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Name/Print	John P. Iwanicki	Registration Number	34,628

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Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	10030261		2018-07-24	Frisen et al.	US counterpart to JP 2014-513523
	2	7771949		2010-08-10	Kramer	
	3	9017992		2015-04-28	Winther et al.	
	4	9896720		2018-02-20	Raj et al.	
	5	8946389		2015-02-03	Gao et al.	
	6	8658361		2014-02-25	Wu et al.	
	7	10465235		2019-11-05	Gullberg et al.	
	8	8013134		2011-09-06	Fredriksson	

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	1	20130323729	A1	2013-12-05	Landegren et al.	

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	1	2014-513523	JP	A	2014-06-05	Spatial Transcriptomics AB		X

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	1	GORANSSON et al. "A single molecule array for digital targeted molecular analyses" Nucleic Acids Research, 2009, Vol. 37, No. 1, e7, doi:10.1093/nar/gkn921	

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	1	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133800/http://www.polonator.org; Wayback Machine (September 5, 2008) "Polony Sequence by Ligation Protocol"	
	2	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080705172604/http://www.polonator.org; Wayback Machine (July 5, 2008) "Polony Sequence Protocols"	
	3	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133818/http://www.polonator.org; Wayback Machine (September 5, 2008) "Help Wanted"	
	4	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155529/http://www.polonator.org/software.aspx; Wayback Machine (August 7, 2008) "Software"	
	5	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155404/http://www.polonator.org; Wayback Machine (August 7, 2008) "Reagent Kits"	
	6	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133541/http://www.polonator.org/reagentkits/run.aspx; Wayback Machine (September 5, 2008) "Run Kits"	
	7	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133601/http://www.polonator.org/reagentkits.pairedtag.aspx; Wayback Machine (September 5, 2008) "Paired-Leg Library Kits"	
	8	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133741/http://www.polonator.org/reagentkits.emulsion.aspx; Wayback Machine (September 5, 2008) "Emulsion PCR/Bead Capping Kits"	
	9	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080905133702/http://www.polonator.org/reagentkits/enrichment.aspx; Wayback Machine (September 5, 2008) "Enrichment Kits"	
	10	Terry R, Porreca G, McCarthy K, Church GM. 2008. Polonator Instrument https://web.archive.org/web/20080807155316/http://www.polonator.org/flowcells.aspx; Wayback Machine (August 7, 2008) "Flow Cells"	
	11	Church GM. 2006. "Genomes for all" Sci Am 294: 46-54	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

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	12	de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. 2005. "Efficiency and power in genetic association studies" Nat Genet 37: 1217-23.	
	13	Dixon AL, Liang L, Moffatt MF, Chen W, Heath S, Wong KC, Taylor J, Burnett E, Gut I, Farrall M, Lathrop GM, Abecasis GR, Cookson WO. 2007. "A genome-wide association study of global gene expression" Nat Genet 39: 1202-7	
	14	Emilsson V, Thorleifsson G, Zhang B, Leonardson AS, Zink F, Zhu J, Carlson S, Helgason A, Walters GB, Gunnarsdottir S, Mouy M, Steinthorsdottir V, Eiriksdottir GH, Bjornsdottir G, Reynisdottir I, Gudbjartsson D, Helgadottir A, Jonasdottir A, Jonasdottir A, Styrkarsdottir U, Gretarsdottir S, Magnusson KP, Stefansson H, Fossdal R, Kristjansson K, Gislason HG, Stefansson T, Leifsson BG, Thorsteinsdottir U, Lamb JR, Gulcher JR, Reitman ML, Kong A, Schadt EE, Stefansson K. 2008; "Genetics of gene expression and its effect on disease" Nature 452: 423-8.	
	15	Risch N, Merikangas K. 1996. "The future of genetic studies of complex human diseases" Science 273: 1516-7	
	16	Schadt EE, Monks SA, Drake TA, Lusis AJ, Che N, Colinayo V, Ruff TG, Milligan SB, Lamb JR, Cavet G, Linsley PS, Mao M, Stoughton RB, Friend SH. 2003. "Genetics of gene expression surveyed in maize, mouse and man" Nature 422: 297-302	
	17	Altshuler D, Daly MJ, Lander ES. 2008. "Genetic mapping in human disease" Science 322: 881-8	
	18	Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M. 2009. "Mapping complex disease traits with global gene expression" Nat Rev Genet 10: 184-94	
	19	International HapMap C. 2005. "A haplotype map of the human genome" Nature 437: 1299-320. PMC ID: PMC1880871	
	20	Klein RJ. 2007. "Power analysis for genome-wide association studies" BMC Genet 8: 58. PMC ID: PMC2042984	
	21	Kwan T, Benovoy D, Dias C, Gurd S, Provencher C, Beaulieu P, Hudson TJ, Sladek R, Majewski J. 2008. "Genome-wide analysis of transcript isoform variation in humans" Nat Genet 40: 225-31	
	22	McCarroll SA. 2008. "Extending genome-wide association studies to copy-number variation" Hum Mol Genet 17: R135-42	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

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	23	Morley M, Molony CM, Weber TM, Devlin JL, Ewens KG, Spielman RS, Cheung VG. 2004. "Genetic analysis of genome-wide variation in human gene expression" Nature 430: 743-7
	24	Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, Sherry S, Mullikin JC, Mortimore BJ, Willey DL, Hunt SE, Cole CG, Coggill PC, Rice CM, Ning Z, Rogers J, Bentley DR, Kwok PY, Mardis ER, Yeh RT, Schultz B, Cook L, Davenport R, Dante M, Fulton L, Hillier L, Waterston RH, McPherson JD, Gilman B, Schaffner S, Van Etten WJ, Reich D, Higgins J, Daly MJ, Blumenstiel B, Baldwin J, Stange-Thomann N, Zody MC, Linton L, Lander ES, Altshuler D, International SNPMWG. 2001. "A map of human genome sequence variation containing 1.42 million +
	25	Schadt EE, Molony C, Chudin E, Hao K, Yang X, Lum PY, Kasarskis A, Zhang B, Wang S, Suver C, Zhu J, Millstein J, Sieberts S, Lamb J, GuhaThakurta D, Derry J, Storey JD, Avila-Campillo I, Kruger MJ, Johnson JM, Rohl CA, van Nas A, Mehrabian M, Drake TA, Lusis AJ, Smith RC, Guengerich FP, Strom SC, Schuetz E, Rushmore TH, Ulrich R. 2008. "Mapping the genetic architecture of gene expression in human liver" PLoS Biol 6: e107. PMC ID: PMC2365981
	26	Serre D, Gurd S, Ge B, Sladek R, Sinnett D, Harmsen E, Bibikova M, Chudin E, Barker DL, Dickinson T, Fan JB, Hudson TJ. 2008. "Differential allelic expression in the human genome: a robust approach to identify genetic and epigenetic cis-acting mechanisms regulating gene expression" PLoS Genet 4: e1000006. PMC ID: PMC2265535
	27	Ball MP, Li JB, Gao Y, Lee J, LeProust E, Park I-H, Xie B, Daley GQ, Church GM. 2009. "Targeted and whole-genome methylomics reveals gene-body signatures in human cell lines" Nat Biotechnol 27: 361-8
	28	Brenner S, Williams SR, Vermaas EH, Storck T, Moon K, McCollum C, Mao JI, Luo S, Kirchner JJ, Eletr S, DuBridge RB, Burcham T, Albrecht G. 2000. "In vitro cloning of complex mixtures of DNA on microbeads: physical separation of differentially expressed cDNAs" Proc Natl Acad Sci U S A 97: 1665-70. PMC ID: PMC26493
	29	Chiang DY, Getz G, Jaffe DB, O'Kelly MJ, Zhao X, Carter SL, Russ C, Nusbaum C, Meyerson M, Lander ES. 2009. "High-resolution mapping of copy-number alterations with massively parallel sequencing" Nat Methods 6: 99-103. PMC ID: PMC2630795
	30	Choy E, Yelensky R, Bonakdar S, Plenge RM, Saxena R, De Jager PL, Shaw SY, Wolfish CS, Slavik JM, Cotsapas C, Rivas M, Dermitzakis ET, Cahir-McFarland E, Kieff E, Hafler D, Daly MJ, Altshuler D. 2008. "Genetic analysis of human traits in vitro: drug response and gene expression in lymphoblastoid cell lines" PLoS Genet 4: e1000287. PMC ID: PMC2583954
	31	Christian AT, Pattee MS, Attix CM, Reed BE, Sorensen KJ, Tucker JD. 2001. "Detection of DNA point mutations and mRNA expression levels by rolling circle amplification in individual cells" Proc Natl Acad Sci U S A 98: 14238-43. PMC ID: PMC64666
	32	Church GM, Porreca GJ, Terry RC, Lares M. 2008. "High-Speed Imaging for DNA Sequencing" Biophotonics (<http://www.photonics.com/Content/ReadArticle.aspx?ArticleID=33989>)
	33	Deng J, Shoemaker R, Xie B, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K. 2009. "Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming" Nat Biotechnol 27: 353-60

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

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	34	Eberwine J, Kacharmina JE, Andrews C, Miyashiro K, McIntosh T, Becker K, Barrett T, Hinkle D, Dent G, Marciano P. 2001. "mRna expression analysis of tissue sections and single cells" J Neurosci 21: 8310-4	
	35	Kolb HC, Finn MG, B. SK. 2001. "Click Chemistry: Diverse Chemical Function from a Few Good Reactions" Angew. Chem. Int. 40: 2004-21	
	36	Kwiatkowski M, Fredriksson S, Isaksson A, Nilsson M, Landegren U. 1999. "Inversion of in situ synthesized oligonucleotides: improved reagents for hybridization and primer extension in DNA microarrays" Nucleic Acids Res 27: 4710-4. PMC ID: PMC148770	
	37	Li JB, Gao Y, Aach J, Zhang K, Kryukov GV, Xie B, Ahlford A, Yoon J-K, Rosenbaum AM, Wait-Zaranek A, LeProust E, Sunyaev S, Church GM. 2009. "Multiplex padlock capture and sequencing reveal human hypermutable CpG variations" Genome Res in press	
	38	Mitra RD, Butty VL, Shendure J, Williams BR, Housman DE, Church GM. 2003. "Digital genotyping and haplotyping with polymerase colonies" Proc Natl Acad Sci U S A 100: 5926-31. PMC ID: PMC156303	
	39	Pan X, Urban AE, Palejev D, Schulz V, Grubert F, Hu Y, Snyder M, Weissman SM. 2008. "A procedure for highly specific, sensitive, and unbiased whole-genome amplification" Proc Natl Acad Sci U S A 105: 15499-504. PMC ID: PMC2563063	
	40	Stougaard M, Lohmann JS, Zajac M, Hamilton-Dutoit S, Koch J. 2007. "In situ detection of non-polyadenylated RNA molecules using Turtle Probes and target primed rolling circle PRINS" BMC Biotechnol 7: 69. PMC ID: PMC2203993	
	41	Wang Z, Gerstein M, Snyder M. 2009. "RNA-Seq: a revolutionary tool for transcriptomics" Nat Rev Genet 10: 57-63	
	42	Wu J, Zhang S, Meng Q, Cao H, Li Z, Li X, Shi S, Kim DH, Bi L, Turro NJ, Ju J. 2007. "3'-O-modified nucleotides as reversible terminators for pyrosequencing" Proc Natl Acad Sci U S A 104: 16462-7. PMC ID: PMC2034218	
	43	Zhang K, Li JB, Gao Y, Egli D, Xie B, Deng J, Li Z, Lee J, Aach J, Leproust E, Eggan K, Church GM. 2009. "Digital RNA Allelotyping Reveals Tissue-specific and Allele-specific Gene Expression in Human" (submitted to Nature Methods)	
	44	Bakal C, Aach J, Church G, Perrimon N. 2007. "Quantitative morphological signatures define local signaling networks regulating cell morphology" Science 316: 1753-6	

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	45	Bang D, Church GM. 2008. "Gene synthesis by circular assembly amplification" Nat Methods 5: 37-9
	46	Bell J. 2004. "Predicting disease using genomics" Nature 429: 453-6
	47	Eid J, Fehr A, Gray J, Luong K, Lyle J, Otto G, Peluso P, Rank D, Baybayan P, Bettman B, Bibillo A, Bjornson K, Chaudhuri B, Christians F, Cicero R, Clark S, Dalal R, Dewinter A, Dixon J, Foquet M, Gaertner A, Hardenbol P, Heiner C, Hester K, Holden D, Kearns G, Kong X, Kuse R, Lacroix Y, Lin S, Lundquist P, Ma C, Marks P, Maxham M, Murphy D, Park I, Pham T, Phillips M, Roy J, Sebra R, Shen G, Sorenson J, Tomaney A, Travers K, Trulson M, Vieceli J, Wegener J, Wu D, Yang A, Zaccarin D, Zhao P, Zhong F, Korlach J, Turner S. 2009. "Real-time DNA sequencing
	48	Harris TD, Buzby PR, Babcock H, Beer E, Bowers J, Braslavsky I, Causey M, Colonell J, Dimeo J, Efcavitch JW, Giladi E, Gill J, Healy J, Jarosz M, Lapen D, Moulton K, Quake SR, Steinmann K, Thayer E, Tyurina A, Ward R, Weiss H, Xie Z. 2008. "Single-molecule DNA sequencing of a viral genome" Science 320: 106-9
	49	Kim JB, Porreca GJ, Song L, Greenway SC, Gorham JM, Church GM, Seidman CE, Seidman JG. 2007. "Polony multiplex analysis of gene expression (PMAGE) in mouse hypertrophic cardiomyopathy" Science 316: 1481-4
	50	Kurimoto K, Yabuta Y, Ohinata Y, Saitou M. 2007. "Global single-cell cDNA amplification to provide a template for representative high-density oligonucleotide microarray analysis" Nat Protoc 2: 739-52

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-08-05
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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S2	1	("10266888").PN.	USPAT; USOCR	OR	OFF	2021/12/09 18:58
S3	1	("20210363579").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2021/12/09 19:00
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S19	1	("20140220587").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2022/02/25 17:28

S20	1,058	c12q 1/68	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:29
S21	76	c12q 1/6841	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:29
S22	877	c12q 1/6869	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:30
S23	32	c12q 2565/518	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:30
S24	18	c12q 2565/601	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:32
S25	3,940	volumetric imaging	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:40
S26	1	S25 and c12q 1/68	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:40
S27	2	S25 and c12q 1/6869	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:40
S28	27,090	3d imaging	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:41
S29	9	S28 and c12q 1/68	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:41
S30	17	S28 and c12q 1/6869	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:43
S31	0	S25 and biolgical sample	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:46

S32	230	S25 and biological sample	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:46
S33	19,040,946	S32 and device or apparatus	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:47
S34	221	S32 and device	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:48
S35	176	S34 and light source	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:48
S36	39	S35 and xyz	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:48
S37	24	S36 and pump	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:49
S38	13	S37 and reagent$1	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:49
S39	13	S38 and detector	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 17:49
S40	44	S35 and pump	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 18:23
S41	9	S40 and pump fluid	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2022/02/25 18:24
S42	5,101	S28 and biological	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 18:26
S43	95	S42 and pump fluid	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 18:27

S44	66	S43 and light source	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 18:27
S45	424	imaging biological specimen	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 18:55
S46	0	S27 and reagent pump	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 18:55
S47	2	S27 and reagent pump	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2022/02/25 18:55
S48	0	S42 and pump reagent	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/02/25 18:56
S49	47	S42 and pump reagent	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2022/02/25 18:56
S50	39	S49 and light source	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	WITH	ON	2022/02/25 18:57
S51	1	("20150098126").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2022/02/25 19:13
S52	1	("20150098126").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2022/02/25 19:13
S53	1	("20110216953").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2022/02/25 19:14
S54	1	("5635352").PN.	USPAT; USOCR	OR	OFF	2022/03/01 10:36
S55	424	imaging biological specimen	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/03/01 10:39
S56	11	S55 and flow cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/03/01 10:39
S57	2,522,352	imaging	US-PGPUB; USPAT; USOCR; FPRS; EPO;	NEAR	ON	2022/03/01 10:47

			JPO; DERWENT; IBM_TDB			
S58	9,453	S57 and biological specimen	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/03/01 10:47
S59	1,542	S58 and flow cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/03/01 10:47
S60	582	S59 and objective	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/03/01 10:47
S61	6	S60 and open flow cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/03/01 10:48
S62	13	S58 and open flow cell	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	NEAR	ON	2022/03/01 10:48

3/1/2022 12:52:16 PM
C:\Users\dthomas1\Documents\EAST\Workspaces\17395534.wsp

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
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/D.C.T/	1	20170262984	A1	2017-09-14	Barnes et al.	
/D.C.T/	2	20200034347	A1	2020-01-30	SELLY	
/D.C.T/	3	20100049448	A1	2010-02-25	Doyle et al.	

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	1							

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	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
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EXAMINER SIGNATURE

Examiner Signature	/DAVID C THOMAS/	Date Considered	02/25/2022

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[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-11-30
Name/Print	John P. Iwanicki	Registration Number	34,628

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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Doc description: Information Disclosure Statement (IDS) Filed

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First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

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/D.C.T/	1	5635352		1997-06-03	Urdea et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

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EXAMINER SIGNATURE

Examiner Signature	/DAVID C THOMAS/	Date Considered	03/01/2022

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2022-02-28
Name/Print	John P. Iwanicki	Registration Number	34,628

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	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	7473767		2009-01-06	Dimitrov	
	2	5563056		1996-10-08	Swan et al.	
	3	7323305		2008-01-29	Leamon et al.	
	4	9217151		2015-12-22	Yin et al.	
	5	8551710		2013-10-08	Bernitz et al.	
	6	4123610		1978-10-31	Summerton et al.	
	7	4981985		1991-01-01	Kaplan et al.	
	8	5830708	A	1998-11-03	Naughton	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	9	5594235	A	1997-01-14	Lee	
	10	4844617	A	1989-07-04	Kelderman et al.	
	11	8501459		2013-08-06	Chen et al.	
	12	5151189		1992-09-29	Hu et al.	
	13	6083726		2000-07-04	Mills, Jr. et al.	
	14	7745129	B1	2010-06-29	Schatz	
	15	5871921		1999-02-16	Landegren et al.	
	16	6632655		2003-10-14	Mehta et al.	
	17	10494662		2019-12-03	Church et al.	
	18	10138509		2018-11-27	Church et al.	
	19	5834758		1998-11-10	Trulson et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	20	10266888		2019-04-23	Daugharthy et al.	
	21	8697359		2014-04-15	Zhang	
	22	9257135		2016-02-09	Ong et al.	
	23	9267135		2016-02-23	Church et al.	
	24	8993233		2015-03-31	Zhang et al.	
	25	8999641		2015-04-07	Zhang et al.	
	26	8932814		2015-01-13	Cong et al.	
	27	8871445		2014-10-28	Cong et al.	
	28	8906616		2014-12-09	Zhang et al.	
	29	10494667		2019-12-03	Chee	
	30	8460865		2013-06-11	Chee et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	31	8268554		2012-09-18	Schallmeiner	
	32	8124751		2012-02-28	Pierce et al.	
	33	9201063		2015-12-01	Sood et al.	
	34	10267808		2019-04-23	Cai	
	35	6534266		2003-03-18	Singer	
	36	5695940		1997-12-09	Drmanac et al.	
	37	7255994		2007-08-14	Lao	
	38	6306597		2001-10-23	Macevicz	
	39	7906285		2011-03-15	Drmanac	
	40	7427479		2008-09-23	Karger et al.	
	41	10227639		2019-03-12	Levner et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	42	4886741		1989-12-12	Schwartz	
	43	10545075		2020-01-28	Deisseroth et al.	
	44	8329404		2012-12-11	McKernan et al.	
	45	6586176		2003-07-01	Trnovsky et al.	
	46	6068979		2000-05-30	Akhavan-Tafti	
	47	11021737		2021-06-01	Church et al.	
	48	9371598		2016-06-21	Chee	
	49	10501791		2019-12-10	Church et al.	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20110092376	A1	2011-04-21	Colston, JR. et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	2	20140087378	A1	2014-03-27	Chatre et al.	
	3	20060292611	A1	2006-12-28	Berka et al.	
	4	20100047924	A1	2010-02-25	WEBSTER et al.	
	5	20040248144	A1	2004-12-09	Mir	
	6	20100268478	A1	2010-10-21	Andregg et al.	
	7	20090246879	A1	2009-10-01	Drmanac et al.	
	8	20050147981	A1	2005-07-07	Yamakawa et al.	
	9	20120122712	A1	2012-05-17	Goldstein	
	10	20070117109	A1	2007-05-24	Rothemund	
	11	20100009868	A1	2010-01-14	Yan et al.	
	12	20050233318	A1	2005-10-20	Chee et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	13	20060177833	A1	2006-08-10	Brenner	
	14	20110086774	A1	2011-04-14	Dunaway	
	15	20110294135	A1	2011-12-01	Carlson	
	16	20090208965	A1	2009-08-20	Tafas et al.	
	17	20130245096	A1	2013-09-19	Abitbol	
	18	20060024711	A1	2006-02-02	Lapidus et al.	
	19	20070117177	A1	2007-05-24	LUO et al.	
	20	20120040397	A1	2012-02-16	Luo et al.	
	21	20020015952	A1	2002-02-07	Anderson et al.	
	22	20090005259	A1	2009-01-01	Ormanac	
	23	20060248349	A1	2006-11-02	Rathjen et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	24	20100223276	A1	2010-09-02	Al-Shameri et al.	
	25	20070087362	A1	2007-04-19	Church et al.	
	26	20140220587	A1	2014-08-07	Green, JR. et al.	
	27	20160265046	A1	2016-09-15	ZHANG et al.	
	28	20060234261	A1	2006-10-19	Pierce et al.	
	29	20150133319	A1	2015-05-14	Fu et al.	
	30	20170212983	A1	2017-07-27	CAI et al.	
	31	20070020650	A1	2007-01-25	Kahvejian	
	32	20110245111	A1	2011-10-06	Chee	
	33	20030148335	A1	2003-08-07	Shen et al.	
	34	20110257031	A1	2011-10-20	BODEAU et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	35	20040077014	A1	2004-04-22	Becker	
	36	20080176769	A1	2008-07-24	Rank et al.	
	37	20160024555	A1	2016-01-28	Church et al.	
	38	20130017229	A1	2013-01-17	Mooney et al.	
	39	20110020291	A1	2011-01-27	Banerjee et al.	
	40	20050106629	A1	2005-05-19	McGrath et al.	
	41	20040259190	A1	2004-12-23	Naughton	
	42	20020155989	A1	2002-10-24	Efimov et al.	
	43	20140049632	A1	2014-02-20	Hemmer	
	44	20060077536	A1	2006-04-13	Bromage et al.	
	45	20070206275	A1	2007-09-06	Hemmer et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	46	20020172950	A1	2002-11-21	Kenny et al.	
	47	20050191687	A1	2005-09-01	Wang et al.	
	48	20070231823	A1	2007-10-04	McKernan et al.	
	49	20080269068	A1	2008-10-30	CHURCH et al.	
	50	20090220968	A1	2009-09-03	Issadore et al.	
	51	20100151472	A1	2010-06-17	Nolan et al.	
	52	20180010166	A1	2018-01-11	Pierce et al.	
	53	20180282787	A1	2018-10-04	WALTER et al.	
	54	20120330636	A1	2012-12-27	Albou	
	55	20140087427	A1	2014-03-27	Bujnicki et al.	
	56	20080050718	A1	2008-02-28	Gesteland et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	57	20110104693	A1	2011-05-05	SELIGMANN	
	58	20060183107	A1	2006-08-17	Melkonyan et al.	
	59	20110033520	A1	2011-02-10	Mather et al.	
	60	20180051322	A1	2018-02-22	Church et al.	
	61	20070292877	A1	2007-12-20	Dimitrov	
	62	20100087325	A1	2010-04-08	Buermann	
	63	20140200146	A1	2014-07-17	Xie et al.	
	64	20140270435	A1	2014-09-18	DUNN	
	65	20160253584	A1	2016-09-01	Fodor et al.	
	66	20160289740	A1	2016-10-06	Fu et al.	
	67	20170176338	A1	2017-06-22	Wu et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
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	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	68	20100076057	A1	2010-03-25	Sontheimer et al.	
	69	20110189776	A1	2011-08-04	Terns et al.	
	70	20110223638	A1	2011-09-15	Wiedenheft et al.	
	71	20130130248	A1	2013-05-23	Haurwitz et al.	
	72	20130253040	A1	2013-09-26	Miller et al.	
	73	20140068797	A1	2014-03-06	Doudna et al.	
	74	20140179770	A1	2014-06-26	ZHANG et al.	
	75	20140273226	A1	2014-09-18	Wu	
	76	20140315985	A1	2014-10-23	May et al.	
	77	20140342457	A1	2014-11-20	MALI et al.	
	78	20140273233	A1	2014-09-18	Chen et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	79	20160024524	A1	2016-01-28	Joung et al.	
	80	20140310830	A1	2014-10-16	ZHANG et al.	
	81	20140179006	A1	2014-06-26	ZHANG	
	82	20150247150	A1	2015-09-03	Zhang et al.	
	83	20160153006	A1	2016-06-02	ZHANG et al.	
	84	20160355795	A1	2016-12-08	Ran et al.	
	85	20170191078	A1	2017-07-06	ZHANG et al.	
	86	20160340662	A1	2016-11-24	Zhang et al.	
	87	20160160210	A1	2016-06-09	Mali et al.	
	88	20110223585	A1	2011-09-15	GULLBERG et al.	
	89	20060228733	A1	2006-10-12	Pierce et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
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	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	90	20120252686	A1	2012-10-04	Umbarger et al.	
	91	20160358326	A1	2016-12-08	Sarachan et al.	
	92	20200090786	A1	2020-03-19	QUIROZ ZARATE et al.	
	93	20120126142	A1	2012-05-24	Matsui et al.	
	94	20140220578	A1	2014-08-07	Bohannon et al.	
	95	20060216339	A1	2006-09-28	Ambron et al.	
	96	20150004598	A1	2015-01-01	Gao et al.	
	97	20150267251	A1	2015-09-24	Cai et al.	

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FOREIGN PATENT DOCUMENTS Remove

Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2008069973	WO	A2	2008-06-12	Univ Columbia		

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
	First Named Inventor	Evan R. Daugharthy
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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	2	2009/046149	WO	A1	2009-04-09	Applied Biosystems		
	3	2007/121489	WO	A2	2007-10-25	Applera Corp		
	4	2010080134	WO	A1	2010-07-15	Harvard College		
	5	9746704	WO	A1	1997-12-11	Lynx Therapeutics, Inc		
	6	2012150035	WO	A1	2012-11-08	Cherkasov, Dmitry		
	7	2012/058638	WO	A2	2012-05-03	President And Fellows Of Harvard College,		
	8	2013/055995	WO	A2	2013-04-18	Harvard College		
	9	20080003402	KR	A	2008-01-07	Mycosol, Inc		
	10	2007086900	WO	A2	2007-08-02	Mycosol, Inc		
	11	2012005595	WO	A2	2012-01-12	De Laat, Wouter		
	12	2014/0163886	WO	A1	2014-10-09	Harvard College		

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	13	2014/182528	WO	A2	2014-11-13	California Inst Of Techn		
	14	2015/118029	WO	A1	2015-08-13	Olink AB		
	15	2011/143583	WO	A1	2011-11-17	Illumina, Inc		
	16	01/26708	WO	A1	2001-04-19	Univ Ohio State		
	17	01/37266	WO	A1	2001-05-25	Research Foundation of State University of New Yor		
	18	2004/104645	WO	A2	2004-12-02	Lucid, Inc.		
	19	2007/001986	WO	A2	2007-01-04	Luo, Yuling		
	20	2012/110899	WO	A2	2012-08-23	Bernitz, Mats		
	21	2015/127183	WO	A2	2015-08-27	Massachusetts Inst Technology		
	22	2008157696	WO	A2	2008-12-24	Kokoris, Mark		
	23	2016081740	WO	A1	2016-05-26	Nanostring Technologies, Inc		

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
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	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	24	2878671	EP	A1	2015-06-03	LSI Medience Corporation		
	25	98/56955	WO	A1	1998-12-17	Bioseparations, Inc.		
	26	2017/161251	WO	A1	2017-09-21	President and Fellows of Harvard College		
	27	2014/048083	WO	A1	2014-04-03	School Of Ophthalmology And Optometry Wenzhou Medi		
	28	2007076128	WO	A2	2007-07-05	Nanostring Technologies, Inc		
	29	2007/123744	WO	A2	2007-11-01	Solexa, Inc		
	30	2012-170337	JP	A	2012-09-10	Hitachi High Tech Corp		
	31	2009/046348	WO	A1	2009-04-09	Applied Biosystems		
	32	2010/087325	WO	A1	2010-08-05	Sharp KK		
	33	2007/149696	WO	A1	2007-12-27	Applera Corp		
	34	2006/138257	WO	A2	2006-12-28	Callida Genomics, Inc		

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	35	2008/108989	WO	A2	2008-09-12	Danisco		
	36	2010/054108	WO	A2	2010-05-14	University Of Georgia Research Foundation, Inc		
	37	2012/164565	WO	A1	2012-12-06	Yeda Res & Dev		
	38	2013/098244	WO	A1	2013-07-04	Univ Wageningen		
	39	2013176772	WO	A1	2013-11-28	Univ California		
	40	2013/142578	WO	A1	2013-09-26	Vilnius University,		
	41	2013/141680	WO	A1	2013-09-26	Univ Vilnius		
	42	2013/126794	WO	A1	2013-08-29	Hutchinson Fred Cancer Res		
	43	2011/143124	WO	A2	2011-11-17	Univ California		
	44	2014/022702	WO	A2	2014-02-06	Univ California		
	45	2014/113493	WO	A1	2014-07-24	Univ Emory		

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	46	2014/191518	WO	A1	2014-12-04	Cellectis		
	47	2014/150624	WO	A1	2014-09-25	Caribou Biosciences, Inc		
	48	2014/093694	WO	A1	2014-06-19	The Broad Institute, Inc		
	49	2014/099744	WO	A1	2014-06-26	Harvard College		
	50	2014/089290	WO	A1	2014-06-12	Sigma Aldrich Co LLC		

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Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T5
	1	Office Action issued for corresponding European Patent Application No. 12780609.9, dated September 23, 2015.	
	2	SEO, et al. Four-color DNA sequencing by synthesis on a chip using photocleavable fluorescent nucleotides. Proceeding of the National Academy of Sciences, 2005 April, 102 (17) 5926-5931.	
	3	BRENNER, et al. "Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays". Nature Biotechnology volume 18, pages 630-634 (2000) doi:10.1038/76469	
	4	2014 Dec 18 (PCT) International Preliminary Report - App PCT/US2013/044241	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /D.C.T/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	5	Shendure Jay et al., "Accurate multiplex polony sequencing of an evolved bacterial genome," Science, American Association for the Advancement of Science, Washington, DC; US, vol. 309, no. 5741, 1 September 2005, pages 1728-1732, XP002427180, ISSN: 0036-8075, DOI: 10.1126/SCIENCE.1117839	
	6	Extended European Seach Report issued in corresponding European Application No. 12860433.7, dated August 13, 2015.	
	7	International Search Report and Written Opinion issued in corresponding International Application No. PCT/US2012/071398, mailed April 8, 2013.	
	8	BENNER et al. "Gene Expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays". Nature Biotechnology, Vol. 18, pp. 630-634 (31 June 2000).	
	9	HAN et al. "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules". Nature Biotechnology, Vol. 19, 99. 631-635 (31 July 2001).	
	10	LEE, JH et al. Highly Multiplexed Subcellular RNA Sequencing In Situ. Science. 21 March 2014, Vol. 343, No. 6177; pages 1360-1363; abstract; page 1360, second column, second paragraph to third column, first paragraph; page 1361, first column, first paragraph; page 1363, first column, second paragraph to second column, first paragraph; DOI: 10.1126/science.1250212.	
	11	ASCANO, M et al. Identification Of RNA-Protein Interaction Networks Using PAR-CLIP. Wiley Interdiscip Rev RNA. March 2012, Vol. 3, No. 2; pages 159-177; page 3, third paragraph; page 16, figure 1; page 25, figure 6; DOI: 10.1002/wrna.1103.	
	12	GINART, P et al. RNA Sequencing In Situ. Nat Biotechnol. June 2014, Vol. 32, No. 6; pages 543-544; DOI: 10.1038/nbt.2921.	
	13	SALIBA, AE et al. Single-Cell RNA-Seq: Advances And Future Challenges. Nucleic Acids Res. 22 July 2014, Vol. 42, No. 14; pages 8845-8860; DOI: 10.1093/nar/gku555.	
	14	Eliscovich et al. mRNA on the move: The road to its biological destiny. Journal of Biological Chemistry, Vol. 288, No. 28, pages 20361-20368, July 2013, in press May 2013 (Year: 2013)	
	15	Weis et al. Protein targeting to subcellular organelles via mRNA localization. Biochimica et Biophysica Acta, Vol. 1833, pages 260- 273, 2013, available online April 2012 (Year: 2012)	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

	16	Jambhekar et al. Cis-acting determinants of asymmetric, cytoplasmic RNA transport. RNA, Vol. 13, pages 625-642, 2007 (Year: 2007)	
	17	Singer-Kruger et al. Here, there, everywhere. RNA Biology, Vol. 11, No. 8, pages 1031-1039, August 2014. (Year: 2014)	
	18	Matlin et al. Spatial expression of the genome: the signal hypothesis at forty. Nature Reviews. Molecular Cell Biology, Vol. 12, No. 5, pages 333-340, May 2011, Epub April 2011. (Year: 2011)	
	19	Polidoros et al. Rolling circle amplification-RACE: a method for simultaneous isolation of 5' and 3' cDNA ends from amplified cDNA templates. Bio Techniques, Vol. 41, No. 1, pages 35, 36, 38 and 40, July 2006, including page 1/1 of Supplementary Material. (Year: 2006)	
	20	Tsaftaris et al. Isolation of three homologous AP1-like MADS-box genes in crocus (Crocus sativus L.) and characterization of their expression. Plant Science, Vol. 166, No. 5, pages 1235-1243, May 2004. (Year: 2004)	
	21	Meeks et al. Characterization of genes encoding poly(A) polymerases in plants: Evidence for duplication and functional specialization. PLoS ONE, Vol. 4, No. 11, e8082, November 2009, printed as pages 1/10-10/10. (Year: 2009)	
	22	Kalivas et al. famRCA-RACE: A rolling circle amplification RACE for isolating a family of homologous cDNAs in one reaction ... Preparative Biochemistry and Biotechnology, Vol. 40, No. 3, pages 177-187, July 2010. (Year: 2010)	
	23	Thisse et al. "High-Resolution in situ hybridization to whole-mount zebrafish embryos" 2008. Nature Protocols. Vol.3 No.1 pp 59-69. Doi:10.1038/nprot.2007.514	
	24	Grompe (1993) Nature Genetics DOI: 10.1038/ng1093-111.	
	25	Thisse et al. 2008 Nature protocols vol.3 No 1 pp 59-69. Doi:10.1038/nprot.2007.514	
	26	Doillon et al. "Actin Filaments in Normal Dermis and During Wound Healing" The American Journal of Pathology, Vol 126 Issue 1 (1987): pages 164-170; page 164 col 1 para 1, page 170 col 1 para 2, fig. 4A-C	

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	27	International Search Report and Written Opinion based on PCT/US2018/027583 issued 29 June 2018	
	28	Soderberg, Ola et al.,"Direct observation of individual endogenous protein complexes in situ by proximity ligation," Nature Methods, December 2006, pp. 995-1000, vol. 3, no. 12, Nature Publishing Group	
	29	Schweitzer, Barry et al., "Immunoassays with rolling circle DNA amplification: A versatile platform for ultrasensitive antigen detection" PNAS, August 29, 2000, pp. 10113-10119, vol. 97, no. 18.	
	30	Cao, Yi et al.," In-situ immuno-PCR to detect antigens," The Lancet, September 16, 2000, pp. 1002-1003, vol. 356.	
	31	Sano, Takeshi et al. "Immuno-PCR: Very Sensitive Antigen Detection by Means of Specific Antibody-DNA Conjugates," Science, October 2, 1992, pp. 120-122, vol. 258	
	32	DASARI, Vivek et al., "Platform for Spatial Molecular Data by Vivek Dasari 1-7 Sig nature redacted Thesis Supervisor", 20 August 2015 (2015-08-20), XP055559164, Retreived from the Internet: URL:http://dspace.mit.edu/bitstream/handle/1721.1/107103/971494098-MIT.pdf?sequence=1 [retreived on 2019-02-20]	
	33	Extended European Search Report issued 13 MAY 2019 for EP Application No. 16862929.3	
	34	LEE, Je Hyuk et al., "Fluorescent in situ sequencing (FISSEQ) or RNA for gene expression profiling in intact cells and tissues", Nature Protocols, vol. 10, no. 3, 12 February 2015 (2015-02-12), pages 442-458. XP055272042, GB ISSN: 1754-2189, DOI: 10.1038/nprot.2014.191	
	35	Extended European Search Report issued May 21, 2019 for European Application No. 16862945.9	
	36	CHOI, Harry M.T. et al., "Next-Generation in Situ Hybridization Chain Reaction: Higher Gain, Lower Cost, Greater Durability" ACS NANO, vol. 8, no. 5, 27 May 2014 (2014-05-27), pages 4284-4294, XP055409053, US	
	37	RAVAN, Hadi, et al. "Isothermal RNA detection through the formation of DNA concatemers contiaining HRP-mimicking DNAzymes on the surface of gold nanoparticles", BIOSENSORS AND BIOELECTRONICS, ELSEVIER SCIENCE LTD. UK, AMSTERDAM, NL, vol. 80, 18 January 2016 (2016-01-18), pages 67-73, XP029441324	

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	38	Extended European Search Report issued for EP Application No. 17790240.0 on September 4, 2019	
	39	Brown et al., Review Article : In situ Hybridization with Riboprobes : An Overview for Veterinary Pathologists. Veterinary Pathology 35 : 159-167 (Year: 1998)	
	40	Choi et al.,Programmable in situ amplification for multiplexed imaging of mRNA expression. Nature Biotechnology 28 (11): 1208 (Year: 2010)	
	41	Choi & Love et al., Immuno-Hybridization Chain Reaction for Enhancing Detection of Individual Cytokine-Secreting Human Peripheral Mononuclear Cells. Analytical Chemistry 83 : 6890-6895 (Year: 2011)	
	42	Hansen et al., Sensitive ligand-based protein quantification using immuno-PCR: A critical review of single-probe and proximity ligation assays. Biotechniques 56:217-228 (Year: 2014)	
	43	Kuimelis et al., Cleavage properties of an oligonucleotide containing a bridged internucleotide 5 -phosphorothioate RNA linkage. Nucleic Acids Research 23 (23) : 4753-4760 (Year: 1999)	
	44	Mag et al., Synthesis and selective cleavage of an oligodeoxynucleotide containing a bridged internucleotide 5'-phosphorothioate linkage. Nucleic Acids Research 19(7): 1437 (Year: 1991)	
	45	Richardson et al., Experimental and Theoretical Studies of Light-to-Heat Conversion and Collective Heating Effects in Metal Nanoparticle Solutions. Nano Letters 9(3) : 1139-1146 (Year: 2009)	
	46	Song et al., Hybridization chain reaction-based aptameric system for the highly selective and sensitive detection of protein. Analyst 137: 1396 (Year: 2012)	
	47	Xiao et al., Single-step electronic detection of femtomolar DNA by target-induced strand displacement in an electrode-bound duplex. PNAS 103(45): 16677-16680 (Year: 2006)	
	48	Zhang et al., Dynamic DNA nanotechnology using strand-displacement reactions. Nature Chemistry 3 : 103-113 (Year: 2011)	

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	49	Zhao et al., An electrochemical aptasensor based on hybridization chain reaction with enzyme-signal amplification for interferon-gamma detection. Biosensors and Bioelectronics 36: 129-134 (Year: 2012)
	50	Srinivas et al., On the biophysics and kinetics of toehold-mediated DNA strand displacement. Nucleic Acids Research 41 (22) : 10641-10658 (Year: 2013)

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☒ A certification statement is not submitted herewith.

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-08-05
Name/Print	John P. Iwanicki	Registration Number	34,628

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	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	N/A
	Examiner Name	Not Yet Assigned
	Attorney Docket Number	010498.01437\US

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	1	7941279		2011-05-10	Hwang et al.	
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	3	8519115		2013-08-27	Webster et al.	
	4	8986926		2015-03-24	Ferree et al.	
	5	9371563		2016-06-21	Geiss et al.	
	6	9714446		2017-07-25	Webster et al.	
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	9	8431691		2013-04-30	McKernan et al.		
	10	6194148		2001-02-27	Hori et al.		

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	1	WANG et al. "Rapid and Sensitive Detection of Severe Acute Respiratory Syndrome Coronavirus by Rolling Circle Amplification" Journal of Clinical Microbiology, Vol. 43, No. 5, May 2005, pp. 2339-2344	

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Art Unit	1637
Examiner Name	D. C. Thomas
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/D.C.T/	1	DIRKS et al. "Triggered amplificaiton by hybridization chain reaction" PNAS; October 26, 2004; Vol. 101, No. 43, pp. 15275-15278	
/D.C.T/	2	LUBECK et al. "Single cell systems biology by super-resolution imaging and combinatorial labeling" Nature Methods; 9 (7); pp. 743-748; 2012	

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Examiner Signature	/DAVID C THOMAS/	Date Considered	02/25/2022

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

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Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-11-23
Name/Print	John P. Iwanicki	Registration Number	34,628

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Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

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/D.C.T/	1	20020029979	A1	2002-03-14	Freund et al.	
/D.C.T/	2	20050064435	A1	2005-03-24	Su et al.	

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/D.C.T/	1	2010104533	WO	A2	2010-09-16	Ikonisys, Inc		

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Examiner Name	Not Yet Assigned
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	1	Li JB, Levanon EY, Yoon J-K, Aach J, Xie B, LeProust E, Zhang K, Gao Y, G.M. C. 2009. "Genome-wide Identification of Human RNA Editing Sites by Parallel DNA Capturing and Sequencing" Science in press	
	2	Meng Q, Kim DH, Bai X, Bi L, Turro NJ, Ju J. 2006. "Design and synthesis of a photocleavable fluorescent nucleotide 3'-O-allyl-dGTP-PC-Bodipy-FL-510 as a reversible terminator for DNA sequencing by synthesis" J Org Chem 71: 3248-52	
	3	Mitra RD, Shendure J, Olejnik J, Edyta Krzymanska O, Church GM. 2003. "Fluorescent in situ sequencing on polymerase colonies" Anal Biochem 320: 55-65	
	4	Porreca GJ, Shendure J, Church GM. 2006. "Polony DNA sequencing" Curr Protoc Mol Biol Chapter 7: Unit 7 8	
	5	Porreca GJ, Zhang K, Li JB, Xie B, Austin D, Vassallo SL, LeProust EM, Peck BJ, Emig CJ, Dahl F, Gao Y, Church GM, Shendure J. 2007. "Multiplex amplification of large sets of human exons" Nat Methods 4: 931-6	
	6	Shendure J, Mitra RD, Varma C, Church GM. 2004. "Advanced sequencing technologies: methods and goals" Nat Rev Genet 5: 335-44	
	7	Shendure JA, Porreca GJ, Church GM. 2008. "Overview of DNA sequencing strategies" Curr Protoc Mol Biol Chapter 7: Unit 7 1	
	8	Tang F, Barbacioru C, Wang Y, Nordman E, Lee C, Xu N, Wang X, Bodeau J, Tuch BB, Siddiqui A, Lao K, Surani MA. 2009. "mRNA-Seq whole-transcriptome analysis of a single cell" Nat Methods 6: 377-82	
	9	Vigneault F, Sismour AM, Church GM. 2008." Efficient microRNA capture and bar-coding via enzymatic oligonucleotide adenylation" Nat Methods 5: 777-9	
	10	Zhang K, Martiny AC, Reppas NB, Barry KW, Malek J, Chisholm SW, Church GM. 2006. "Sequencing genomes from single cells by polymerase cloning" Nat Biotechnol 24: 680-6	
	11	Zhang K, Zhu J, Shendure J, Porreca GJ, Aach JD, Mitra RD, Church GM. 2006. "Long-range polony haplotyping of individual human chromosome molecules" Nat Genet 38: 382-7	

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	12	Church et al.; Center for Casual Consequences of Variation (CCV) "An NHGRI Center for Excellence in Genomic Science" https://web.archive.org/web/20110703211120/http://ccv.med.harvard.edu/; Wayback Machine (July 3, 2011)	
	13	Church et al.; Center for Casual Consequences of Variation (CCV) "Our four Specific Aims" https://web.archive.org/web/20110813071548//http://ccv.med.harvard.edu/specific_aims.htm; Wayback Machine (August 13, 2011)	
	14	Church; "Proposal for a Center for the determination of the Casual Transcriptional Consequences of Human Genetic Variation (CTCHGV)"" http://ccv.med.harvard.edu/CEGS09_Complete_Proposal_minus_Admin_Sections.09May21.final.pdf; Wayback Machine (August 13, 2011)	
	15	J. H. Lee, M.D. Ph.D. presentation entitled "Population-wide Tissue-specific Functional Analysis of Human iPS Cells Using Single-Cell In Situ Sequencing" George Church Laboratory, Wyss Institute for Biology Inspired Engineering, Harvard Medical School, Boston, January 10, 2010	
	16	2021 May 17 (US) Notice of Allowance - Appln No 17/122,168	
	17	2021 June 18 (US) Non-Final Office Action - Appln No 15/772,652	
	18	2021 May 8 - (CN) Office Action - App. No. 201680077501.7	X
	19	2021 July 2 - (US) Non-Final Office Action - Appln No 17/238,642	☐
	20	2021 July 21 (US) Non-Final Office Action - Appl No. 16/693,611	☐
	21	HO et al. "Sequencing by ligation variation with endonuclease V digestion and deoxyinosine-containing query oligonucleotides" BMC Genomics, 2011, 12:598	☐
	22	JIANG et al. "Solar thermal polymerase chain reaction for smartphone-assisted molecular diagnostics" Scientific Reports, 4:4137, 2014	☐

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	23	JU et al. "Four-color DNA sequencing by synthesis using cleavable fluorescent nucleotide reversible terminators" PNAS, December 26, 2006, vol. 103, no. 52, pp. 19635-19640	☐
	24	LUBECK et al. "Single cell in situ RNA profiling by sequential hybridization" Nature Methods, April 2014, 11(4), pp. 360-361	☐
	25	PARINOV et al. "DNA sequencing by hybridization to microchip octa- and decanucleotides extended by stacked pentanucleotides" Nucleic Acids Research, 1996, vol. 24, no. 15, pp. 2998-3004	☐
	26	SCHOUTEN et al. "Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification" Nucleic Acids Research, 2002, vol. 30, no. 12, e57	☐
	27	2021 July 23 (US) Non-Final Office Action - Appl No 16/170,751	☐

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Filing Date	
First Named Inventor	Evan R. Daugharthy
Art Unit	N/A
Examiner Name	Not Yet Assigned
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	1	2014/197568	WO	A2	2014-12-11	Harvard College		
	2	2014/093661	WO	A2	2014-06-19	The Broad Institute, Inc		
	3	2014/093595	WO	A1	2014-06-19	The Broad Institute, Inc		

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	4	2014/093622	WO	A2	2014-06-19	The Broad Institute, Inc		
	5	2014/065596	WO	A1	2014-05-01	Toolgen Inc		
	6	112015012375	BR	A2	2017-09-26	Sigma Aldrich Co LLC		X
	7	112015014425	BR	A2	2017-10-10	Harvard College		☒
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	11	112015008708	BR	A2	2017-09-26	Toolgen Incorporated		☒
	12	2891347	CA	A1	2014-06-12	Sigma Aldrich Co LLC		☐
	13	2014/144288	WO	A1	2014-09-18	Gen Hospital Corp		☐
	14	2003044229	WO	A1	2003-05-30	Gullberg, Mats		☐

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	15	1580283	CN	A	2005-02-16	Univ Tsinghua		☒
	16	1959384	CN	A	2007-05-09	Henan Provincial Bioengineerin		☒
	17	101553306	CN	A	2009-10-07	Clondiag GmbH		☒
	18	2013096851	WO	A1	2013-06-27	Harvard College		☐
	19	2016007839	WO	A1	2016-01-14	Harvard College		☐
	20	2017079406	WO	A1	2017-05-11	Harvard College		☐
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	1	WEIBRECHT, Irene et al., "Simultaneous Visualization of Both Signaling Cascade Activity and End-Point Gene Expression in Single Cells", PLOS ONE, vol. 6, no. 5, 25 May 2011 (2011-05-25)	

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	2	Larsson, Chatarina; Grundberg, Ida; Sbderberg, Ola; Nilsson, Mats: 11 In situ detection and genotyping of individual mRNA molecules", Nature Methods, vol . 7, no. 5 11 April 2010 (2010-04-11), pages 395-397, XP055035168, DOI: 10.1038/nmeth.1448 Retrieved from the Internet: URL:http://www.nature.com/nmeth/journal/v7/n5/pdf/nmeth.1448.pdf [retrieved on 2012-08-09] * the whole document *	
	3	NUOVO: "Co-labeling Using In Situ PCR: A Review "JOURNAL OF HISTOCHEMISTRY & CYTOCHEMISTRY, vol . 49, no. 11, 1 November 2001 (2001-11-01), pages 1329-1339, XP055164942, ISSN: 0022-1554, DOI: 10.1177/002215540104901101 * the whole document *	
	4	MITRA R. D. ET AL: 11 In situ localized amplification and contact replication of many individual DNA molecules 11 NUCLEIC ACIDS RESEARCH, INFORMATION RETRIEVAL LTD, GB, vol . 27, no. 24, 15 December 1999 (1999-12-15), page e34, XP002292358, ISSN: 0305-1048, DOI: 10.1093/NAR/27.24.E34 * abstract *	
	5	KE ET AL: 11 In situ sequencing for RNA analysis in preserved tissue and cells 11 NATURE METHODS, vol. 10, no. 9, 14 July 2013 (2013-07-14), pages 857-860, XP055163946, ISSN: 1548-7091, DOI: 10.1038/nmeth.2563 * the whole document *	
	6	LEE ET AL: "Highly Multiplexed Subcellular RNA Sequencing in Situ", SCIENCE, vol. 343, no. 6177, 27 February 2014 (2014-02-27), pages 1360-1363, XP055305772, us ISSN: 0036-8075, DOI: 10.1126/science.1250212	
	7	CLAUSSON ET AL: "Compaction of rolling circle amplification products increases signal integrity and signal-to-noise ratio", SCIENTIFIC REPORTS, vol. 5, 23 July 2015 (2015-07-23), page 12317, XP055305777, DOI: 10.1038/srep12317	
	8	NADJI et al.,"Photochemically and Photoenzymatically Cleavable DNA," J. Am. Chem. Soc. 1992, 114, 9266-9269.	
	9	Extended European Search Report and Written Opinion issued December 17, 2019 for EP 19180827.8	
	10	Supplementary European Search Report and Written Opinion issued March 18, 2020	
	11	CHEN et al., "Expansion microscopy," SCIENCE, Vol. 347, no. 6221, pages 543-548 (January 30, 2015)	
	12	CHOZINSKI et al., "Expansion microscopy with conventional antibodies and fluorescent proteins," NATURE METHODS, vol. 13, no. 6, pages 485-488 (June 1, 2016)	

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	13	CHEN et al., "Nanoscale imaging of RNA with expansion microscopy," NATURE METHODS, vol. 133, no. 8, pages 679-684 (August 1, 2016)	
	14	Supplementary European Search Report issued April 9, 2020 for EP 17847555	
	15	Amasino, "Acceleration of nucleic acid hybridization rate by polyethylene glycol," ANALYTICAL BIOCHEMISTRY, Vol. 152, No. 2, pages 304-307 (February 1, 1986).	
	16	Bouché et al., "The effect of spermidine on endonuclease inhibition by agarose contaminants," ANALYTICAL BIOCHEMISTRY, Vol. 115, No. 1, pages 42-45 (July 15, 1981).	
	17	Kuznetsova et al., "What Macromolecular Crowding Can Do to a Protein," Int. J. Mol. Sci., Vol. 15, No. 12, pages 23090-23140 (December 1, 2014).	
	18	Oupicky et al., "Laterally stabilized complexes of DNA with linear reducible polycations: Strategy for triggered intracellular actication of DNA delivery vectors," Journal of the American Chemical Society, Vol. 124, No. 1, pages 8-9 (January 9, 2002).	
	19	Nguyen, Son C., "Strategies for Studying Chromatin Regulation and Organization," Doctoral Dissertation, Harvard University (May 1, 2018); retrieved from https://dash.harvard.edu/bitstream/handle/1/33493431/NGUYEN-DISSERTATION-2016.pdf?sequence=4&isAllowed=y on April 8, 2020.	
	20	ZHOU et al. "In Situ Detection of Messenger RNA Using Digoxigenin-Labeled Oligonucleotides and Rolling Circle Amplification" Experimental and Molecular Pathology 70, 281-288 (2001)	
	21	2020 May 29 - Examination Report issued for EP 18173059.9	
	22	2020 June 1 - Examination Report issued for GB 1809029.0	
	23	Wright et al., "Dynamic closed-loop system for focus tracking using a spatial light modulator and a deformable membrane mirror," Optics Express, Vol. 14, No. 1, pp. 222-228 (January 9, 2006).	

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	24	Wang et al., "The method of axial drift compensation of laser differential confocal microscopy based on zero-tracking," Proc. of SPIE, Vol. 9618, 96180X (2015).	
	25	Ohata et al., "Confocal Imaging Analysis of Intracellular Ions in Mixed Cellular Systems or in Situ Using Two Types of Confocal Microscopic Systems," Methods in Enzymology, Vol. 307, pp. 425-441 (1999), particularly p. 437.	
	26	Supplemental Material for Schweitzer et al. (PNAS 2000; 97(18):10113-10119) (Year: 2000)	
	27	2020 Aug 03 - (US) Non-Final Office Action - US Appl. 16/157,243	
	28	2020 Aug 03 (US) Non-Final Office Action - Appl. No. 16/393,215	
	29	2020 Jul 02 - (US) Non-Final Office Action - US Appl. 16/255,920	
	30	2020 Aug 10 - (GB) Examination Report - GB App. No. 1809029.0	
	31	2020 Sept 24 - (US) Final Office Action - App. 15/772,652	
	32	Markaki et al. "Fluorescence In Situ Hybridization Applications for Super-Resolution 3D Structured Illumination Microscopy" Methods in Microbiology, January 2013	
	33	Achim et al. "High-throughput spatial mapping of single-cell RNA-seq data to tissue of origin" Nature Biotechnology, April 13, 2015	
	34	2020 Sept 25 - (US) Non-Final Office Action - Appl 16/386,337	

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	35	2020 Aug 25 - (JP) Notice of Reasons for Rejection - App. No. 2018-522985	X
	36	PI: Piezo Nano Positioning, 2008 (online), retrieved on 2020.08.12, pp. 1-6 <https://www.pi-usa.us/fileadmin/user_upload/pi_us/files/product_datasheets/N725_Piezo_Focus_Positioner.pdf>	☐
	37	2020 Sept 14 - (CA) Examination Report - App. No. 2,850,509	☐
	38	MALI, P. et al. RNA-Guided Human Genome Engineering Via Cas9. Science. 03 January 2013,Vol. 339; pages 823-826; abstract; page 823, second column, second to third paragraph; page 823, third column, second paragraph to third paragraph; figure 1; Supplementary material, page 4, first paragraph; page 7, first paragraph; Supplementary figures S1, S3. DOI: 10.1126/science.1232033.	☐
	39	TILEY, LS et al. The VP16 Transcription Activation Domain Is Functional When Targeted To A Promoter-Proximal RNA Sequence. Genes and Development. 1992. Vol. 6; pages 2077-2087; abstract; page 2077, first column, first paragraph.	☐
	40	TRAFTON, A. Editing The Genome With High Precision [online]. MIT News office. 03 January 2013 [retrieved on 2014-12-04). Retrieved from the Internet: <URL:http:/lnewsoffice. Trafton .edut20 13/editing-the-genome-with-high-precision-01 03 >;pages 1-3; page 3, third paragraph.	☐
	41	LEMAN, AR et al. The Replication Forie Understanding The Eukaryotic Replication Machinery And The Challenges To Genome Duplication. Genes. 29 January 2013. Vol. 4; pages 1-32; figure 1; DOI: 10.3390/genes4010001.	☐
	42	QI, L et al. Repurposing CRISPR As An RNA-Guided Platform For Sequence-Specific Control Of Gene Expression. Cell. 28 February 2013. Vol. 152; pages 1173-1183; figures 2, 4. DOI: 10.1 016/j.cell.2013.02.022.	☐
	43	GASIUNAS, G et aL Cas9-crRNA Ribonucleoprotein Complex Mediates Specific DNA Cleavage For Adaptive Immunity In Bacteria. PNAS. 04 September 2012. VoL 109, No. 39; pages E2579-E2586; page E2583, first column, first paragraph. DOI: 1 0.1073/pnas.1208507109.	☐
	44	CONG, Let al. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science. 03 January 2013, Vol. 339; pages 819-823; abstract; page 821, third column; page 822, first column, first paragraph; figure 4. DOI: 10.1126/science.1231143.	☐
	45	JINEK, M et al. A Programmable Dual-RNA-Guided DNA Endonuclease In Adaptive Bacterial Immunity. Science. 28 June 2012. Vol. 337; pages 816-821; DOI: 10.1126/science.1225829.	☐

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	46	CRISPR In The Lab: A Practical Guide [online]. Addgene. 04 September 2014. Retrieved on 2014-12-04. Retrieved from the Internet: <URL: https://www.addgene.org/CRISPR/guide/>.	☐
	47	CHENG, AW et al. Multiplexed Activation Of Endogenous Genes By CRISP R-on, An RNA-Guided Transcriptional Activator System. Cell Research. 27 August 2013. Vol. 23; pages 1163-1171. DOI: 10.1038/cr.2013.122.	☐
	48	MALI, P. et al. CAS9 Transcriptional Activators For Target Specificity Screening And Paired Nickases For Cooperative Genome Engineering. Nature Biotechnology. 01 August 2013. Vol.31; pages 833-838; entire document. DOI: 10.1038/nbt.2675. -	☐
	49	RAN, FA et al. Double Nicking By RNA-Guided CRISPR Cas9 For Enhanced Genome Editing Specificity. Cell. 12 September 2013. Vol. 154; pages 1380-1389. DOI: 10.1016/j.cell.2013.08.021.	☐
	50	International Search Report and Written Opinion issued in corresponding International Application No. PCT/US14/40868, mailed December 31, 2014.	☐

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See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

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SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2021-08-05
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APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $480 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	06/03/2022	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 36	Minus	** 30	= 6		x $100 =	600
	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	600

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/TINA J BARDEN/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc Code: DIST.E.FILE **Document Description: Electronic Terminal Disclaimer - Filed**	PTO/SB/26 U.S. Patent and Trademark Office Department of Commerce

Electronic Petition Request	**TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT**
Application Number	17395534
Filing Date	06-Aug-2021
First Named Inventor	Evan Daugharthy
Attorney Docket Number	010498.01437\US
Title of Invention	Method and Apparatus for Volumetric Imaging

☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action

☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

Owner	Percent Interest
President and Fellows of Harvard College	100%

The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

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as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:
- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

(●) Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request. ( ) I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.	
Applicant claims the following fee status: ( ) Small Entity ( ) Micro Entity (●) Regular Undiscounted I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.	
THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES I certify, in accordance with 37 CFR 1.4(d)(4) that I am: (●) An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application Registration Number 60023 ( ) A sole inventor ( ) A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application ( ) A joint inventor; all of whom are signing this request	
Signature	/Pei Wu/
Name	Pei Wu

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	17395534
Filing Date:	06-Aug-2021
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Filer:	Pei Wu/Jennifer Hazzard
Attorney Docket Number:	010498.01437\US

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
STATUTORY OR TERMINAL DISCLAIMER	1814	1	170	170
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**170**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 17395534

Filing Date: 06-Aug-2021

Applicant/Patent under Reexamination: Daugharthy

Electronic Terminal Disclaimer filed on June 3, 2022

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt	
EFS ID:	45867824
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	Pei Wu/Jennifer Hazzard
Filer Authorized By:	Pei Wu
Attorney Docket Number:	010498.01437\US
Receipt Date:	03-JUN-2022
Filing Date:	06-AUG-2021
Time Stamp:	11:00:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$170
RAM confirmation Number	E202263B00371300
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	41320 f01162911cce6e8483db2b0fc1f5e301caf06c78	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38281 3753f338955c52ab35a277b9e6509bd8833c0843	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	79601		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Patent Application Fee Transmittal

Application Number:	17395534
Filing Date:	06-Aug-2021
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Filer:	Pei Wu/Jennifer Hazzard
Attorney Docket Number:	010498.01437\US

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1202	6	100	600
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**600**

Electronic Acknowledgement Receipt	
EFS ID:	45867909
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	Pei Wu/Jennifer Hazzard
Filer Authorized By:	Pei Wu
Attorney Docket Number:	010498.01437\US
Receipt Date:	03-JUN-2022
Filing Date:	06-AUG-2021
Time Stamp:	11:05:16
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$600
RAM confirmation Number	E202263B05491389
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Amendment/Request for Reconsideration-After Non-Final Rejection	Amendment_and_Response_21932460.pdf	54005 1630c7827b49d9a1f2c67e04cfc7e45a96feeaa0	no	13
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38346 13b61eb5b130a93251f6d4174bc0d10394402ccd	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	92351		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

REMARKS

I. Status of the Application

Claims 9-38 were pending prior to entry of the amendments. Claim 18 has been cancelled herein without prejudice or disclaimer. Claims 9-17 and 19-38 have been amended. New claims 39-45 have been added. Support for the claim amendments can be found in the claims and specification of the originally filed application. No new matter is added by any of these amendments. Accordingly, upon entry, claims 9-17 and 19-45 will be pending.

Reconsideration and allowance of this application are respectfully requested in light of the abovementioned amendments and the following remarks.

II. Obviousness-type Double Patenting

Claims 9-38 stand rejected under obviousness-type double patenting over claims 18-30 of U.S. Patent No. 11,118,220 in view of U.S. Patent Publication No. 2011/0216953 (Callahan). Without conceding in the basis of rejection, Applicant submits a terminal disclaimer over this patent. In view of the terminal disclaimer, the Applicant believes that the rejection has been rendered moot. The Applicant respectfully requests that the rejection of claims 9-38 be withdrawn.

Claims 9-38 stand rejected under obviousness-type double patenting over claims 1-14 of U.S. Patent No. 10,266,888 in view of Callahan. Without conceding in the basis of rejection, Applicant submits a terminal disclaimer over this patent. In view of the terminal disclaimer, the Applicant believes that the rejection has been rendered moot. The Applicant respectfully requests that the rejection of claims 9-38 be withdrawn.

III. Claim Objections

Claim 30 stands objected for lacking a connecting term. Without conceding in the basis

of the objection, Applicant has amended claim 30 to recite "wherein said light source comprises one or more lasers." Applicant respectfully submits that the objection of claim 30 is overcome, and respectfully requests that the objection be withdrawn.

IV. Claim Rejections - 35 U.S.C. § 103

A. Claims 9 and 14-38 are not obvious over Keller in view of Callahan

Claims 9 and 14-38 stand rejected under 35 U.S.C. § 103 over U.S. Patent Publication No. 2015/0098126 (Keller) in view of Callahan.

Without conceding in the basis of rejection and solely to expedite prosecution of this application, Applicant has amended claim 9 to further clarify certain differences between the claimed subject matter and the cited art. Applicant submits that claim 9 is not obvious over the asserted combination of Keller and Callahan, because these references, alone or in any combination, do not meet all of the elements of claim 9.

Nothing in Keller teaches or discloses "at least one processor to perform a method comprising: determining a plurality of optically-encoded signals from a plurality of light signals, wherein said plurality of light signals is obtained from a plurality of read-out cycles, wherein said plurality of optically-encoded signals is associated with one or more nucleic acid molecules within said volume; and determining three-dimensional positional information of said one or more nucleic acid molecules using said plurality of optically-encoded signals," as is recited in claim 9.

Callahan does not cure the above-mentioned deficiencies of Keller.

Accordingly, Applicant respectfully requests that the § 103 rejection of claim 9 be withdrawn.

Claims 14-17 and 19-38 depend from and include all of the elements of claim 9, and recite additional elements of particular advantage and utility. The asserted combination of Keller

and Callahan does not meet all of the elements of claim 9, much less the combination of elements of claims 14-17 and 19-38. Accordingly, Applicant respectfully requests that the §103 rejections of claims 14-17 and 19-38 also be withdrawn.

Without conceding in the basis of rejection, Applicant submits that the § 103 rejection of claim 18 is moot in view of the cancellation of this claim.

B. Claims 10-13 are not obvious over Keller in view of Callahan and further in view of Frisen

Claims 10-13 stand rejected under 35 U.S.C. § 103 over Keller in view of Callahan and further in view of U.S. Patent Publication No. 2019/0203275 (Frisen).

Without conceding in the basis of rejection, Applicant submits that claim 103 is not obvious over the asserted combination of Keller, Callahan, and Frisen for at least the reason that this claim depends from and includes all of the elements of claim 9, and recite additional elements of particular advantage and utility. Keller does not teach or disclose all of the elements of claim 9, much less the combination of claims 10-13, and Callahan and Frisen do not cure the abovementioned deficiencies of Keller.

Accordingly, Applicant respectfully requests that the §103 rejection of claims 10-13 be withdrawn.

***

It shall be understood herein that any instance in which Applicant has addressed certain comments set forth by the Office shall not be construed as a concession to other comments or arguments advanced by the Office. Any circumstance in which Applicant has amended or canceled a claim also does not mean that Applicant concedes to the arguments or positions

advanced by the Office with respect to that claim or other claims pending herein.

V. Conclusion

Having addressed all outstanding issues, Applicant respectfully requests reconsideration and allowance of this case. To the extent the Examiner believes that it would facilitate allowance of the case, the Examiner is requested to telephone the undersigned at the number below. The Commissioner is authorized to apply any additional charges or credits to Deposit Account No. 19-0733.

Respectfully submitted,

Dated: June 3, 2022

/Pei Wu/

Pei Wu, Reg. No. 60,023
BANNER & WITCOFF, LTD.
28 State Street, Suite 1800
Boston, MA 02109
(617) 720-9655 (direct dial)

PATENT
ATTORNEY DOCKET NO. 010498.01437\US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Evan R. Daugharthy	)	Examiner: David C. Thomas
	)	
Serial No.: 17/395,534	)	Art Unit: 1637
	)	
Filed: August 6, 2021	)	Conf. No.: 5147
	)	
Title: Method and Apparatus for Volumetric Imaging of a Three-Dimensional Nucleic Acid Containing Matrix	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE

Dear Commissioner:

This is in response to the non-final office action dated March 4, 2022. Kindly reconsider the above-referenced application in view of the following amendments and remarks. The Commissioner is hereby authorized to charge such extension fee or credit any overpayment of fees for this submission to Deposit Account No. 19-0733.

Amendments to the Claims are reflected in the Listing of Claims, which begin at page 2 of this paper.

Remarks begin at page 10 of this paper.

Amendments to the Claims

The following claim listing replaces all prior claim listings in the subject application.

Listing of Claims:

1. – 8. (Cancelled)

9. (Currently Amended) A system~~device~~, comprising:

(a) a stage[[,]] comprising:

(i) a sample holder~~, wherein said sample holder is~~ configured to receive a [[solid]] substrate comprising a ~~biological~~ sample having a volume,

(ii) a temperature control ~~heating or cooling~~ apparatus ~~programmable for a time and temperature parameter, wherein said heating or cooling apparatus is~~operatively coupled ~~operationally connected~~ to said sample holder;[[,]] and

(iii) an XY multi-axis motion control system ~~programmable for XY motion control of said stage~~;

(b) a fluidics system comprising ~~configured to dispense one or more reagents to said sample holder, wherein said fluidics system comprises~~:

(i) one or more reservoirs configured to ~~for containing~~contain [[said]] one or more fluid reagents; and

(ii) a ~~fluidics~~ fluid dispenser operationally coupled to a pump, which pump is configured to ~~dispense~~ provide said one or more fluid reagents from said one or more reservoirs [[into]] to said sample holder;~~, wherein said fluidics dispenser is fluidically connected to said one or more reservoirs,~~

~~(iii) a pump that facilitates the dispensation of a programmed volume of fluid from at least one reservoir of said one or more reservoirs to said sample holder,~~ and

(c) an optical system ~~configured for volumetric imaging of said biological sample, wherein said optical system comprises~~comprising:

(i) an objective;[[,]]

(ii) ~~a Z-axis motion control system operatively connected to one or more components~~

~~of said optical system, wherein said Z-axis motion control system is configured for Z-motion of an objective focal plane through said biological sample,~~

~~(iii) a~~one or more light ~~source~~ sources, wherein each light source of said one or more light sources is configured to deliver light to said ~~biological sample~~volume;[[,]] and

[[(iv)]](iii) one or more detectors configured to receive light signals from said volume; and ~~positioned in light receiving communication with said sample holder, wherein said one or more detectors receives light intensity signals which are processed into a three-dimensional volumetric image of said biological sample~~

(d) a Z-axis motion control system configured for Z-motion of an objective focal plane through said volume; and

(e) a computing system comprising at least one computer readable storage medium having program instructions stored thereon, which program instructions are executable by at least one processor of said computing system to cause said at least one processor to perform a method comprising:

determining a plurality of optically-encoded signals from a plurality of light signals, wherein said plurality of light signals is obtained from a plurality of read-out cycles, wherein said plurality of optically-encoded signals is associated with one or more nucleic acid molecules within said volume; and

determining three-dimensional positional information of said one or more nucleic acid molecules using said plurality of optically-encoded signals.

10. (Currently Amended) The ~~device~~ system of claim 9, wherein said [[solid]] substrate comprises [[is]] a flow cell.

11. (Currently Amended) The system~~device~~ of claim 10, wherein said flow cell is a closed flow cell.

12. (Currently Amended) The system~~device~~ of claim 11, wherein said closed flow cell comprises a fluid inlet and a fluid outlet, and wherein said fluid inlet is fluidically coupled to ~~comprises~~ said ~~fluidics~~ fluid dispenser.

13. (Currently Amended) The system~~device~~ of claim 10, wherein said flow cell is an open flow cell.

14. (Currently Amended) The system~~device~~ of claim 9, wherein said objective is a water immersion objective.

15. (Currently Amended) The system~~device~~ of claim 9, wherein said objective is a water dipping objective.

16. (Currently Amended) The system~~device~~ of claim 9, wherein said ~~heating or said cooling~~temperature control apparatus comprises a heat sink or a Peltier element.

17. (Currently Amended) The system~~device~~ of claim 9, ~~wherein said heating or said cooling apparatus comprises a temperature sensor for providing temperature feedback to a~~further comprising thermal control system.

18. (Cancelled)

19. (Currently Amended) The system~~device~~ of claim 9, wherein said pump is a syringe pump.

20. (Currently Amended) The system~~device~~ of claim 9, wherein said fluidics system further comprises one or more valves downstream of said one or more reservoirs and upstream of said ~~fluidics~~ fluid dispenser, wherein, upon actuation, said one or more valves facilitate dispensation of ~~said programmed volume of~~ fluid to said sample holder.

21. (Currently Amended) The system~~device~~ of claim 20, wherein said one or more valves comprise a rotary valve.

22. (Currently Amended) The system~~device~~ of claim 20, wherein said one or more valves comprise a solenoid valve or a microfluidic valve.

23. (Currently Amended) The system~~device~~ of claim 13, wherein said fluidics system further comprises an aspiration system ~~configured to remove liquid from said open flow cell, wherein said aspiration system comprises~~ comprising: (1) an aspiration tube ~~that removes liquid~~configured to remove fluid from said open flow cell and a ~~pump or vacuum~~pressure source operatively coupled to said aspiration tube.

24. (Currently Amended) The system~~device~~ of claim 23, wherein said aspiration system further comprises a motor ~~tube is~~ operatively coupled to ~~a motor~~said aspiration tube, wherein said motor is configured to adjust a motion axis of said aspiration tube to control an interface between said aspiration tube and said open flow cell.

25. (Currently Amended) The system~~device~~ of claim 24, wherein said motor is a stepper motor.

26. (Currently Amended) The system~~device~~ of claim 9, wherein said Z-axis motion control system comprises a Z-axis linear motor configured for Z motion of said objective focal plane through said ~~biological sample~~volume.

27. (Currently Amended) The system~~device~~ of claim 26, further comprising a linear encoder configured to provide Z motion feedback to said Z-axis linear motor.

28. (Currently Amended) The system~~device~~ of claim 9, further comprising an objective wetting apparatus.

29. (Currently Amended) The system~~device~~ of claim 9, wherein said light source comprises one or more light emitting diodes (LEDs).

30. (Currently Amended) The systemdevice of claim 9, wherein said light source comprises one or more lasers.

31. (Currently Amended) The systemdevice of claim 9, wherein said optical system further comprises one or more emission filters and one or more excitation filters configured to selectively filter one or more for directing certain wavelengths of light from said one or more light sources to said volume within said optical system.

32. (Currently Amended) The systemdevice of claim 31, wherein at least one excitation filter of said one or more excitation filters [[are]] is configured to selectively filter provide light having a wavelength of about 480 nm, about 530 nm, about 590 nm, or about 640 nm from said one or more light sources to said biological samplevolume.

33. (Currently Amended) The systemdevice of claim [[31]]9, wherein said optical system further comprises one or more emission filters [[are]] configured to provide selectively filter one or more wavelengths of light emitted from said volume having a wavelength of about 510 nm, about 570 nm, about 620 nm, or about 680 nm to said one or more detectors.

34. (Currently Amended) The systemdevice of claim 9, wherein said optical system is mechanically, electrically, or electromechanically coupled [[with]] to a controller system configured to synchronize one or more imaging events within said systemdevice.

35. (Currently Amended) The systemdevice of claim 9, where said device comprisesfurther comprising a vibration control rigid foundation.

36. (Currently Amended) The systemdevice of claim 9, further comprising, one or more microprocessors with software for a fluidics controller operatively coupled to said fluid dispenser and configured to automate or control automating or controlling said dispensation of said one or more fluid reagents [[into]] to said sample holder.

37. (Currently Amended) The system~~device~~ of claim [[9]]17, ~~further comprising, one or more microprocessors with software for automating or controlling said time and temperature parameter of said heating or cooling apparatus~~wherein said temperature control apparatus further comprises a temperature sensor configured to provide a feedback signal to said thermal control system.

38. (Currently Amended) The ~~device~~ system of claim [[9]]33, wherein at least one of said one or more emission filters is configured to selectively filter light having a wavelength of about 510 nm, about 570 nm, about 620 nm, or about 680 nm to said one or more detectors from said volume to said one or more detectors~~further comprising, one or more microprocessors with software for automating or controlling image detection and acquisition of said optical system~~.

39. (New) The system of claim 9, wherein said one or more light sources comprises two or more light sources, wherein each light source of said two or more light sources is configured to excite fluorescence in a unique color channel.

40. (New) The system of claim 39, wherein each optically-encoded signal of said plurality of optically-encoded signals comprises at least one of said unique color channels.

41. (New) The system of claim 9, wherein at least one of the plurality of optically-encoded signals comprises two or more colors detected in series.

42. (New) The system of claim 39, wherein said two or more light sources comprise four light sources.

43. (New) The system of claim 9, wherein said optical system is configured for wide-field microscopy.

44. (New) The system of claim 9, wherein a numerical aperture of said objective is about 1.0.

45. (New) A system comprising:

(a) a stage comprising:

(i) a sample holder configured to receive a substrate comprising a sample having a volume;

(ii) a temperature control apparatus operatively coupled to said sample holder; and

(iii) an XY motion control system;

(b) a fluidics system comprising:

(i) one or more reservoirs configured to contain one or more fluid reagents; and

(ii) a fluid dispenser operationally coupled to a pump, which pump is configured to provide said one or more fluid reagents from said one or more reservoirs to said sample holder; and

(c) an optical system comprising:

(i) an objective;

(ii) two or more light sources, wherein each light source of said two or more light sources is configured to deliver light to said volume, wherein said two or more light sources are configured to emit two or more colors of light; and

(iii) one or more detectors configured to receive light signals from said volume;

(d) a Z-axis motion control system configured for Z-motion of an objective focal plane through said volume; and

(e) a computing system comprising at least one computer readable storage medium having program instructions stored thereon, which program instructions are executable by at least one processor of said computing system to cause said at least one processor to perform a method comprising:

receiving a plurality of images of at least a portion of said volume from a plurality of read-out cycles, wherein said plurality of images comprises a plurality of light signals received from said volume during said plurality of readout cycles, wherein said plurality of light signals is associated with one or more nucleic acid molecules within said volume, wherein each light signal of said plurality of light signals has a three-dimensional spatial position within said volume;

determining a plurality of optically-encoded signals from said plurality of light signals obtained from said plurality of read-out cycles, wherein each optically-encoded signal is associated with a nucleic acid molecule of said one or more nucleic acid molecules, wherein said plurality of optically-encoded signals comprises said two or more colors of light detected by said one or more detectors in series;

identifying said one or more nucleic acid molecules based on said plurality of optically-encoded signals; and

determining three-dimensional positional information of said one or more nucleic acid molecules.

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-22)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Not for submission under 37 CFR 1.99)

Application Number	17395534
Filing Date	2021-08-06
First Named Inventor	Evan R. Daugharthy
Art Unit	1637
Examiner Name	D. C. Thomas
Attorney Docket Number	010498.01437\US

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Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	7989166		2011-08-02	Koch et al.	
	2	9551032		2017-01-24	Landegren et al.	

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	1	20090280559	A1	2009-11-12	McCarthy	
	2	20170067096	A1	2017-03-09	Wassie et al.	
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	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

	1	2009-538123	JP	A	2009-11-05	Applera Corp.		☒
	2	2015-090458	JP	A	2015-05-11	Hitachi High Tech Corp		☒
	3	2007-526772	JP	A	2007-09-20			☒

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	1	BIBIKOVA et al. "Quantitative Gene Expression Profiling in Formalin-Fixed, Paraffin-Embedded Tissues Using Universal Bead Arrays" American Journal of Pathology, Vol. 165, No. 5, November 2004	
	2	CAPODIECI et al. "Gene expression profiling in single cells within tissue" Nature Methods, September 14, 2005, 2(9) pp. 663-665	
	3	CONZE et al. "Single molecule analysis of combinatorial splicing" Nucleic Acids Research, June 29, 2010, Vol. 38, No. 16; e163	
	4	FEMINO et al. "Visualization of Single RNA Transcripts in Situ" Science, April 24, 1998, Vol. 280, pp. 585-90	
	5	GAVRILOVIC et al. "Automated Classification of Multicolored Rolling Circle Products in Dual-Channel Wide-Field Fluroescence Microscopy" Cytometry Part A, July 2011, 79(7), pp. 518-27	
	6	GEISS et al. "Direct multiplexed measurement of gene expression with color-coded probe pairs" Nature Biotechnology, Vol. 26, No. 3, March 2008, pp. 317-25	

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	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
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	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

	7	GUNDERSON et al. "Decoding Randomly Ordered DNA Arrays" Genome Research, May 2004, 14(5), pp. 870-7	
	8	HAN et al. "Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules" Nature Biotechnology, July 2001, Vol. 19, No. 7, pp. 631-5	
	9	ITZKOVITZ et al. "Validating Transcripts with Probes and Imaging Technology" Nature Methods, April 2011, 8(4 Suppl): S12-S19	
	10	ITZKOVITZ et al. "Single molecule transcript counting of stem cell markers in the mouse intestine" Nat Cell Biol., November 2011, 14(1), pp. 106-114	
	11	LAGUNAVICIUS et al. "Novel application of Phi29 DNA polymerase: RNA detection and analysis in vitro and in situ by target RNA-primed RCA" RNA, May 2009, 15(5), pp. 765-771	
	12	LARSSON et al. "In situ detection and genotyping of individual mRNA molecules" Nature Methods, Vol. 7, No. 5, May 2010, pp. 395-7	
	13	LEVSKY et al. "Fluorescence in situ hybridization: past, present and future" Journal of Cell Science, July 15, 2003, 116 (Pt 14), pp. 2833-8	
	14	LEVSKY et al. "Single-Cell Gene Expression Profiling" Science, August 2, 2002, 297(5582), pp. 836-40	
	15	MAIERHOFER et al. "Multicolor Deconvolution Microscopy of Thick Biological Specimens" American Journal of Pathology, Vol. 162, No .2, February 2003, pp. 373-9	
	16	MEADE et al. "Multiplexed DNA detection using spectrally encoded porous" Analytical Chemistry, April 1, 2009, 81(7), pp. 2618-2625	
	17	RAJ et al. "Imaging individual mRNA molecules using multiple singly labeled probes" Nature Methods, October 2009, 5 (10), pp. 877-879	

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	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

	18	SUN et al. "Composite Organic-Inorganic Nanoparticles as Raman Labels for Tissue Analysis" Nano Letters, February 2007, Vol. 7, No. 2, pp. 351-356	
	19	WAHLBY et al. "Sequential Immunofluorescence Staining and Image Analysis for Detection of Large Numbers of Antigens in Individual Cell Nuclei" Cytometry, January 1, 2002, 47(1), pp. 32-41	
	20	WEIBRECHT et al. "Simultaneous Visualization of Both Signaling Cascade Activity and End-Point Gene Expression in Single Cells" PLoS One, May 2011, Vol. 6, Issue 5, e20148	
	21	WILSON et al. "Encoded Microcarriers For High-Throughput Multiplexed Detection" Angewandte Chemie International Edition, September 18, 2006, 45(37), pp. 6104-17	
	22	ZHAO et al. "Advances of multiplex and high throughput biomolecular detection technologies based on encoding microparticles" Science China Chemistry, August 2011, Vol. 54, No. 8, pp. 1185-1201	

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Examiner Signature		Date Considered	

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	Application Number	17395534
	Filing Date	2021-08-06
	First Named Inventor	Evan R. Daugharthy
	Art Unit	1637
	Examiner Name	D. C. Thomas
	Attorney Docket Number	010498.01437\US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

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☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

☒ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/John P. Iwanicki/	Date (YYYY-MM-DD)	2022-06-06
Name/Print	John P. Iwanicki	Registration Number	34,628

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Application Number:	17395534
Filing Date:	06-Aug-2021
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Filer:	John P. Iwanicki/Jennifer Hazzard
Attorney Docket Number:	010498.01437\US

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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Pages:				
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Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**260**

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EFS ID:	45890415
Application Number:	17395534
International Application Number:	
Confirmation Number:	5147
Title of Invention:	Method and Apparatus for Volumetric Imaging
First Named Inventor/Applicant Name:	Evan R. Daugharthy
Customer Number:	22907
Filer:	John P. Iwanicki/Jennifer Hazzard
Filer Authorized By:	John P. Iwanicki
Attorney Docket Number:	010498.01437\US
Receipt Date:	07-JUN-2022
Filing Date:	06-AUG-2021
Time Stamp:	09:30:45
Application Type:	Utility under 35 USC 111(a)

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7	Non Patent Literature	BIBIKOVA_et_al_American_J_of_Pathol_2004_21929845.PDF	546891 05d633c77dccb26cf355d9d42e0ccfa54c20b39b	no	9
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8	Non Patent Literature	CAPODIECI_et_al_Nature_Methods_2005_21929935.PDF	561392 9b7ecced859959c8b186c0914f5de8a483c68e9e	no	6
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22	Non Patent Literature	MEADE_et_al_Analytical_Chemistry_2009_21930154.PDF	3274269 694e7218d183f16d1af956b8b705e2c4c70ebc44	no	20
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New International Application Filed with the USPTO as a Receiving Office

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Espacenet

Bibliographic data: JP2009538123 (A) — 2009-11-05

REAGENTS, METHODS, AND LIBRARIES FOR GEL-FREE BEAD-BASED SEQUENCING

Inventor(s):

Applicant(s):

Classification: - international: ***C12M1/00; C12N15/09; C12Q1/68***
- cooperative: **C12Q1/6837 (EP); C12Q1/6869 (EP); C12Q1/6874 (EP)** more

Application number: JP20090506766 20070419
Global Dossier

Priority number(s): US20060793702P 20060419 ; WO2007US66931 20070419

Also published as: AU2007237909 (A1) CA2649725 (A1) CN101495654 (A) EP2007907 (A2) US2009062129 (A1) more

Abstract not available for JP2009538123 (A)
Abstract of corresponding document: WO2007121489 (A2)

The present invention provides methods for determining a nucleic acid sequence by performing successive cycles of duplex extension along a single stranded template. The cycles comprise steps of extension, ligation, and, preferably, cleavage. In certain embodiments the methods make use of extension probes containing phosphorothiolate linkages and employ agents appropriate to cleave such linkages. In certain embodiments the methods make use of extension probes containing an abasic residue or a damaged base and employ agents appropriate to cleave linkages between a nucleoside and an abasic residue and/or agents appropriate to remove a damaged base from a nucleic acid. The invention provides methods of

determining information about a sequence using at least two distinguishably labeled probe families. In certain embodiments the methods acquire less than 2 bits of information from each of a plurality of nucleotides in the template in each cycle. In certain embodiments the sequencing reactions are performed on templates attached to microparticles, which are immobilized in or on a semi-solid support or attached to a substrate. The invention further provides sets of labeled extension probes containing phosphorothiolate linkages or trigger residues that are suitable for use in the method. In addition, the invention includes performing multiple sequencing reactions on a single template by removing initializing oligonucleotides and extended strands and performing subsequent reactions using different initializing oligonucleotides. The invention further provides efficient methods for preparing templates, particularly for performing sequencing multiple different templates in parallel. The invention also provides methods for performing ligation and cleavage. The invention also provides new libraries of nucleic acid fragments containing paired tags, and methods of preparing microparticles having multiple different templates (e.g., containing paired tags) attached thereto and of sequencing the templates individually. The invention also provides automated sequencing systems, flow cells, image processing methods, and computer-readable media that store computer-executable instructions (e.g., to perform the image-processing methods) and/or sequence information. In certain embodiments the sequence information is stored in a database. The invention further provides blocking oligonucleotides and methods of use thereof to facilitate sequencing. Further provided are arrays comprising microparticles having templates attached thereto and attached to a substrate in the absence of a semi-solid medium and methods of sequencing the templates. The invention also provides arrays of nucleic acid colonies produced using microparticles to "imprint" templates on a semi-solid medium or substrate, and methods of sequencing thereof.

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(71)出願人　509130413
アプライド　バイオシステムズ,　エルエルシー
アメリカ合衆国　カリフォルニア　９２００８,　カールスバッド,　バン　アレン　ウェイ　５７９１
(74)代理人　100078282
弁理士　山本　秀策
(74)代理人　100062409
弁理士　安村　高明
(74)代理人　100113413
弁理士　森下　夏樹

最終頁に続く

(54)【発明の名称】ゲル非含有ビーズベースの配列決定のための試薬、方法およびライブラリー

(57)【要約】
本発明は、単鎖鋳型に沿った二本鎖伸長の連続的サイクルを行なうことにより、核酸配列を決定する方法を提供する。該サイクルは、伸長、ライゲーション、および好ましくは、切断の工程を含む。ある特定の実施形態において、該方法では、ホスホロチオレート結合を含有する伸長プローブを利用し、かかる連結を切断するのに適した試薬を用いる。ある特定の実施形態において、該方法では、無塩基残基または損傷塩基を含有する伸長プローブを利用し、ヌクレオシドと無塩基残基間の連結を切断するのに適した試薬および／または損傷塩基を核酸から除去するのに適した試薬を用いる。本発明は、少なくとも２つの識別可能に標識されたプローブファミリーを用いて配列に関する情報を決定する方法を提供する。ある特定の実施形態において、該方法により、各サイクルで複数の鋳型内のヌクレオチドの各々から２ビット未満の情報が取得される。

【特許請求の範囲】
【請求項1】
鋳型ポリヌクレオチドチド内のヌクレオチドの配列の同定方法であって、以下：
（ａ）　オリゴヌクレオチドプローブを該鋳型ポリヌクレオチドにライゲートさせ、伸長された二本鎖を形成することにより、初期オリゴヌクレオチドを該鋳型ポリヌクレオチドチドに沿って伸長させる工程、ここで、該オリゴヌクレオチドプローブは微粒子に結合し、該微粒子は基材に結合し、該微粒子は半固体支持体に固定化されていない、工程；
（ｂ）　該ポリヌクレオチドチドの１つ以上のヌクレオチドを同定する工程；ならびに
（ｃ）　該ヌクレオチドの配列が決定されるまで工程（ａ）および（ｂ）を反復する工程を含む、方法。
【請求項2】
前記オリゴヌクレオチドプローブはホスホロチオレート結合を含む、請求項１に記載の方法。
【請求項3】
前記同定する工程が、最も新しくライゲートされたオリゴヌクレオチドプローブに結合された標識を検出することを含む、請求項１に記載の方法。
【請求項4】
前記ホスホロチオレート結合を、Ａｇ、Ｈｇ、Ｃｕ、Ｍｎ、ＺｎおよびＣｄからなる群より選択される原子を含む切断剤で切断することにより、伸長可能なプローブ末端を生成させる工程をさらに含む、請求項３に記載の方法。
【請求項5】
前記切断剤が$AgNO_3$である、請求項４に記載の方法。
【請求項6】
前記鋳型ポリヌクレオチドを伸長させる前に、該鋳型ポリヌクレオチドとブロッキングオリゴヌクレオチドとを接触させる工程を包含する、請求項１に記載の方法。
【請求項7】
前記ブロッキングオリゴヌクレオチドが酵素的に伸長させることができない、請求項６に記載の方法。
【請求項8】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合している、請求項１に記載の方法。
【請求項9】
一本鎖鋳型が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記微粒子を前記基材に繋いでいる、請求項８に記載の方法。
【請求項10】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合し、該ビオチン結合タンパク質が、該基材に結合している、請求項１に記載の方法。
【請求項11】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合し、該ビオチン結合タンパク質が、該基材に結合し、前記鋳型がビオチンを含む、請求項１に記載の方法。
【請求項12】
前記基材が実質的に平面状で剛性である、請求項１に記載の方法。
【請求項13】
鋳型ポリヌクレオチドチドのヌクレオチドの配列の決定方法であって、以下：
（ａ）　鋳型ポリヌクレオチドチドにハイブリダイズされたプローブであって、伸長可能な末端を有するプローブを含むプローブ－鋳型二本鎖を提供する工程；
（ｂ）　伸長オリゴヌクレオチドプローブを該伸長可能な末端にライゲートし、伸長されたオリゴヌクレオチドプローブを含有する伸長された二本鎖を形成する工程であって、こ

こで、該オリゴヌクレオチドプローブは微粒子に結合し、該微粒子は基材に結合し、該微粒子は半固体支持体に固定化されていない、工程；
（ｃ）　該伸長された二本鎖において、（１）ライゲートしたばかりの該伸長プローブに相補的であるか、もしくは（２）該伸長されたオリゴヌクレオチドプローブのすぐ下流にある該鋳型ポリヌクレオチドチド内のヌクレオチド残基であるかのであるかのいずれかである、該鋳型ポリヌクレオチドチド内の少なくとも１つのヌクレオチドを同定する工程；
（ｄ）もし伸長可能な末端が既に存在しない場合は、生成される末端が、最後の伸長プローブがライゲートされた末端と異なるように、該伸長されたオリゴヌクレオチドプローブ上に伸長可能な末端を生成させる工程；ならびに
（ｅ）　該鋳型ポリヌクレオチドチドのヌクレオチドの配列が決定されるまで、工程（ｂ）、（ｃ）および（ｄ）を反復する工程、
を含む、方法。
【請求項14】
前記伸長プローブはホスホロチオレート結合を含む、請求項１３に記載の方法。
【請求項15】
各伸長プローブが伸長不可能な部分を一方の末端に有する、請求項１３に記載の方法。
【請求項16】
前記同定する工程が、最も新しくライゲートされた伸長プローブに結合された標識を検出することを含む、請求項１３に記載の方法。
【請求項17】
前記同定する工程が、前記伸長不可能な部分を除去すること、および前記伸長されたオリゴヌクレオチドプローブを核酸ポリメラーゼにより、１種類以上の標識された連鎖停止ヌクレオシド三リン酸の存在下で伸長させることを含む、請求項１３に記載の方法。
【請求項18】
前記ライゲーション工程において伸長プローブが前記伸長可能な末端にライゲートされなかった場合はいつでも、伸長されたオリゴヌクレオチドプローブをキャッピングする工程をさらに含む、請求項１３に記載の方法。
【請求項19】
前記生成させる工程が、前記ホスホロチオレート結合を、Ａｇ、Ｈｇ、Ｃｕ、Ｍｎ、ＺｎおよびＣｄからなる群より選択される原子を含む切断剤で切断することを含む、請求項１３に記載の方法。
【請求項20】
前記切断剤が$AgNO_3$である、請求項１９に記載の方法。
【請求項21】
（ｆ）前記ライゲートされたプローブおよび前記初期オリゴヌクレオチドを前記鋳型から除去する工程；（ｇ）前記鋳型ポリヌクレオチドチドと異なる配列に結合させた第２のオリゴヌクレオチドを用いて工程（ａ）を反復する工程；ならびに（ｈ）工程（ｂ）～（ｅ）を反復する工程をさらに含む、請求項１３に記載の方法。
【請求項22】
前記鋳型ポリヌクレオチドチドと異なる配列に結合させた初期オリゴヌクレオチドを用いて複数回反復される、請求項２１に記載の方法。
【請求項23】
前記伸長プローブが伸長不可能な部分を一方の末端に有する、請求項２２に記載の方法。
【請求項24】
各反復で、前記同定する工程が、最も新しくライゲートされた伸長プローブに結合された標識を検出することを含む、請求項２２に記載の方法。
【請求項25】
前記ライゲーション工程において、伸長プローブが前記伸長可能な末端にライゲートされなかった場合はいつでも、伸長されたオリゴヌクレオチドプローブをキャッピングする工程をさらに含む、請求項２２に記載の方法。

【請求項26】
前記生成させる工程が、前記ホスホロチオレート結合を、Ａｇ、Ｈｇ、Ｃｕ、Ｍｎ、ＺｎおよびＣｄからなる群より選択される原子を含む切断剤で切断することを含む、請求項２２に記載の方法。
【請求項27】
前記切断剤が$AgNO_3$である、請求項２６に記載の方法。
【請求項28】
前記プローブ－鋳型二本鎖を提供する前に、前記鋳型ポリヌクレオチドとブロッキングオリゴヌクレオチドとを接触させる工程を包含する、請求項１３に記載の方法。
【請求項29】
前記ブロッキングオリゴヌクレオチドが酵素的に伸長させることができない、請求項２８に記載の方法。
【請求項30】
前記プローブ－鋳型二本鎖を提供する前に、
（ａ）　前記鋳型ポリヌクレオチドとブロッキングオリゴヌクレオチドとを接触させる工程、および
（ｂ）　プローブ－鋳型二本鎖を形成する工程
を含む、請求項１３に記載の方法。
【請求項31】
鋳型ポリヌクレオチド内のヌクレオチドの配列の同定方法であって、以下：
（ａ）　半固相支持体内または該支持体上に固定化されているか、あるいは実質的に平面状で剛性の基材に結合されている微粒子に結合された鋳型ポリヌクレオチドを提供する工程、
（ｂ）該鋳型ポリヌクレオチドとブロッキングオリゴヌクレオチドとを接触させる工程
（ｃ）　オリゴヌクレオチドプローブを該鋳型ポリヌクレオチドにライゲートさせ、伸長された二本鎖を形成することにより、初期オリゴヌクレオチドを該鋳型ポリヌクレオチドに沿って伸長させる工程であって、ここで、オリゴヌクレオチドプローブは必要に応じて切れやすい連結を含む、工程；
（ｄ）　該ポリヌクレオチドの１つ以上のヌクレオチドを同定する工程；ならびに
（ｅ）　該ヌクレオチドの配列が決定されるまで工程（ｃ）および（ｄ）を反復する工程
を含む、方法。
【請求項32】
前記伸長させる工程が半固相支持体内で行なわれる、請求項３１に記載の方法。
【請求項33】
前記鋳型が、実質的に平面状で剛性の基材に結合された微粒子に結合されている、請求項３１に記載の方法。
【請求項34】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合している、請求項３３に記載の方法。
【請求項35】
一本鎖鋳型が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記微粒子を前記基材に繋いでいる、請求項３４に記載の方法。
【請求項36】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合し、該ビオチン結合タンパク質が、該基材に結合している、請求項３３に記載の方法。
【請求項37】
前記ビーズに結合した一本鎖鋳型が、前記微粒子を前記基材に繋いでいる、請求項３３に記載の方法。
【請求項38】
鋳型ポリヌクレオチドのヌクレオチドの配列の決定方法であって、以下：

（ａ）　該鋳型ポリヌクレオチドにハイブリダイズされるプローブであって、伸長可能な末端を有するプローブを含むプローブ－鋳型二本鎖を提供する工程であって、ここで、該鋳型は、それにハイブリダイズしたブロッキングオリゴヌクレオチドを有し、該プローブ鋳型二本鎖は、半固相支持体内または該支持体上に包埋されているか、あるいは基材に結合している微粒子に結合されている、工程；
（ｂ）　伸長オリゴヌクレオチドプローブを該伸長可能な末端にライゲートし、伸長されたオリゴヌクレオチドプローブを含有する伸長された二本鎖を形成する工程であって、ここで、該伸長プローブはホスホロチオレート結合を含む、工程；
（ｃ）　該伸長された二本鎖において、（１）ライゲートされたばかりの該伸長プローブに相補的であるか、もしくは（２）該伸長されたオリゴヌクレオチドプローブのすぐ下流にある該鋳型ポリヌクレオチド内のヌクレオチド残基であるかのいずれかである鋳型ポリヌクレオチド内の少なくとも１つのヌクレオチドを同定する工程；
（ｄ）もし該伸長可能な末端が既に存在しない場合は、生成される末端が、最後の伸長プローブがライゲートされた末端と異なるように、伸長されたオリゴヌクレオチドプローブ上に伸長可能な末端を生成させる工程；ならびに
（ｅ）　鋳型ポリヌクレオチドのヌクレオチドの配列が決定されるまで工程（ｂ）、（ｃ）および（ｄ）を反復する工程
を含む、方法。
【請求項39】
工程（ａ）の前に、鋳型とブロッキングオリゴヌクレオチドとを接触させる工程を含む、請求項３８に記載の方法。
【請求項40】
前記ライゲートする工程および前記生成させる工程が、半固相支持体内で行なわれる、請求項３８に記載の方法。
【請求項41】
前記鋳型が、実質的に平面状で剛性の基材に結合された微粒子に結合されている、請求項３８に記載の方法。
【請求項42】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合している、請求項４１に記載の方法。
【請求項43】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合し、該ビオチン結合タンパク質が、該基材に結合している、請求項４１に記載の方法。
【請求項44】
前記微粒子に結合した一本鎖鋳型が、該微粒子を前記基材に繋いでいる、請求項３８に記載の方法。
【請求項45】
鋳型ポリヌクレオチドのヌクレオチドの配列の決定方法であって、以下：
（ａ）　微粒子の存在下、エマルジョンの区画内で、該微粒子に結合した鋳型ポリヌクレオチドのクローン性の集団を有する微粒子が生成されるように、鋳型ポリヌクレオチド分子を増幅する工程；
（ｂ）　該微粒子を該エマルジョンから回収する工程；
（ｃ）　該微粒子を、半固相支持体内または該支持体上に包埋させるか、該微粒子を基材に結合させる工程；
（ｄ）　オリゴヌクレオチドプローブを該鋳型ポリヌクレオチドにライゲートさせ、伸長された二本鎖を形成することにより、初期オリゴヌクレオチドを該鋳型ポリヌクレオチドに沿って伸長させる工程であって、ここで、該オリゴヌクレオチドプローブは切れやすい連結を含む、工程；
（ｅ）　該ポリヌクレオチドの１つ以上のヌクレオチドを同定する工程；ならびに
（ｆ）　該ヌクレオチドの配列が決定されるまで工程（ｄ）および（ｅ）を反復する工程

を含む、方法。
【請求項46】
（ｉ）異なる配列を含む複数の鋳型ポリヌクレオチド分子がエマルジョンの個々の区画内で増幅され；（ｉｉ）各々が自身に結合され鋳型ポリヌクレオチドのクローン性の集団を有し、該クローン性の集団が異なる配列を有する複数の微粒子が、エマルジョンから回収され、前記支持体内または該支持体上に包埋され、そして（ｉｉｉ）　工程（ｄ）、（ｅ）および（ｆ）が、複数の配列が並行して決定されるように、包埋または結合された微粒子に結合されたクローン性の集団において並行して行なわれる、請求項４５に記載の方法。
【請求項47】
工程（ｃ）の前に、前記鋳型ポリヌクレオチドとブロッキングオリゴヌクレオチドとを接触させる工程を含む、請求項４５に記載の方法。
【請求項48】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合している、請求項４５に記載の方法。
【請求項49】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合し、該ビオチン結合タンパク質が、該基材に結合している、請求項４５に記載の方法。
【請求項50】
前記微粒子に結合した一本鎖鋳型が、該微粒子を前記基材に繋いでいる、請求項４５に記載の方法。
【請求項51】
少なくとも２つの識別可能に標識されたオリゴヌクレオチドプローブファミリーの第１のコレクションを用いて、鋳型ポリヌクレオチド内のヌクレオチドの配列に関する情報を決定する方法であって、
（ａ）　オリゴヌクレオチドプローブを該鋳型ポリヌクレオチドにライゲートさせ、伸長された二本鎖を形成することにより、初期オリゴヌクレオチドを該鋳型ポリヌクレオチドに沿って伸長させる工程であって、ここで、オリゴヌクレオチドプローブは、識別可能に標識されたオリゴヌクレオチドプローブファミリーのコレクションのメンバーであり、それにハイブリダイズしたブロッキングオリゴヌクレオチドを有する、工程；
（ｂ）　該オリゴヌクレオチドプローブと会合した標識を検出する工程；ならびに
（ｃ）　プローブファミリーの序列リスト名が得られるまで工程（ａ）および（ｂ）を反復する工程；ならびに
（ｄ）　該ヌクレオチドの配列に対する１つ以上の可能性を排除するためにプローブファミリーの該序列リスト名を使用する工程
を含む、方法。
【請求項52】
工程（ｄ）が、前記配列を決定するためにプローブファミリーの前記序列リスト名をデコード化することを含む、請求項５１に記載の方法。
【請求項53】
鋳型ポリヌクレオチドにハイブリダイズされた初期オリゴヌクレオチドプローブであって、伸長可能な末端を有するプローブを含むプローブ－鋳型二本鎖を提供することを含み、伸長させる工程が、該オリゴヌクレオチドプローブを該伸長可能な末端にライゲートさせて、伸長されたオリゴヌクレオチドプローブを含有する伸長された二本鎖を形成させることを含み、さらに、伸長させる工程においてオリゴヌクレオチドプローブが該伸長可能な末端にライゲートされなかった場合はいつでも、任意の残留する伸長可能な末端をキャッピングする工程を含む、請求項５１に記載の方法。
【請求項54】
各プローブファミリー内の前記オリゴヌクレオチドプローブが伸長不可能な部分を一方の末端に含む、請求項５１に記載の方法。

【請求項55】
各検出工程後に、（ｆ）もし伸長可能な末端が既に存在しない場合は、生成される末端が、前記最も新しくライゲートされたオリゴヌクレオチドプローブがライゲートされた末端と異なるように、伸長可能な末端を、該最も新しくライゲートされたオリゴヌクレオチドプローブ上に生成させることをさらに含む、請求項５１に記載の方法。
【請求項56】
前記オリゴヌクレオチドプローブがホスホロチオレート結合を含み、前記伸長可能なプローブ末端が、該ホスホロチオレート結合を、Ａｇ、Ｈｇ、Ｃｕ、Ｍｎ、ＺｎおよびＣｄからなる群より選択される原子を含む切断剤で切断させることにより生成される、請求項５５に記載の方法。
【請求項57】
前記切断剤が$AgNO_3$である、請求項５６に記載の方法。
【請求項58】
前記伸長させる工程が半固相支持体内または該支持体上で行なわれる、請求項５１に記載の方法。
【請求項59】
前記鋳型が、実質的に平面状で剛性の基材に結合された微粒子に結合されている、請求項５１に記載の方法。
【請求項60】
前記コレクションが２つの識別可能に標識されたプローブファミリーを含む、請求項５１に記載の方法。
【請求項61】
前記コレクションが３つの識別可能に標識されたプローブファミリーを含む、請求項５１に記載の方法。
【請求項62】
前記コレクションが４つの識別可能に標識されたプローブファミリーを含む、請求項５１に記載の方法。
【請求項63】
前記コレクションが、４つより多くの識別可能に標識されたプローブファミリーを含む、請求項５１に記載の方法。
【請求項64】
前記オリゴヌクレオチドプローブが、ヌクレオシドが独立しては選択されない拘束部分を含み、配列が異なる拘束部分を有するオリゴヌクレオチドプローブが、コード化に従ってプローブファミリーに割り当てられる、請求項５１に記載の方法。
【請求項65】
オリゴヌクレオチドプローブが、表１に示す２４個のコード化の１つに従って第１、第２、第３および第４のプローブファミリーに割り当てられる、請求項５１に記載の方法。
【請求項66】
鋳型内の少なくとも１つのヌクレオチドが既知の正体を有し、デコード化する工程が：
（ｉ）　正体を、どの正体が既知ヌクレオチドの正体と一致するか、およびその近位ヌクレオチドが、既知の正体のヌクレオチドに隣接するヌクレオチドに対向してライゲートされたプローブの拘束部分の可能な配列を調べることにより、既知の正体のヌクレオチドに隣接する該鋳型内の該ヌクレオチドに割り当てる工程；
（ｉｉ）　どの正体が、その近位ヌクレオチドが後続のヌクレオチドに対向してライゲートされたプローブの拘束部分の可能な配列と一致するかを調べることにより、正体を後続のヌクレオチドに割り当てる工程；ならびに
（ｉｉｉ）　配列が決定されるまで工程（ｉｉ）を反復する工程
を含む、請求項５２に記載の方法。
【請求項67】
（ａ）　ヌクレオチドが既知の正体を有するように、前記鋳型内のヌクレオチドの該正体

を決定する工程をさらに含み、ここで前記デコード化する工程は：
（ｉ）　正体を、どの正体が既知ヌクレオチドの正体と一致するか、およびその近位ヌクレオチドが、既知の正体のヌクレオチドに隣接するヌクレオチドに対向してライゲートされたプローブの拘束部分の可能な配列を調べることにより、既知の正体のヌクレオチドに隣接する該鋳型内のヌクレオチドに割り当てる工程；
（ｉｉ）　どの正体が、その近位ヌクレオチドが後続のヌクレオチドに対向してライゲートされたプローブの拘束部分の可能な配列と一致するかを調べることにより、正体を後続のヌクレオチドに割り当てる工程；ならびに
（ｉｉｉ）　配列が決定されるまで工程（ｉｉ）を反復する工程
を含む
をさらに含む、請求項５２に記載の方法。
【請求項68】
前記決定する工程が、鋳型プローブ二本鎖を標識されたヌクレオチドと、ポリメラーゼの存在下、該二本鎖に隣接する位置の該鋳型に相補的である場合は、標識されたヌクレオチドの組込みを可能にする条件下で接触させることを含む、請求項６７に記載の方法。
【請求項69】
前記デコード化する工程が、少なくとも１つの候補配列をプローブファミリーの前記序列リスト名から生成させること；および前記鋳型内のヌクレオチドの配列として候補配列を選択することを含む、請求項５２に記載の方法。
【請求項70】
前記生成させる工程が、少なくとも４つの候補配列を生成させることを含む、請求項６９に記載の方法。
【請求項71】
前記生成させる工程が、
（ｉ）　正体をヌクレオチドの配列内の第１のヌクレオチドと仮定すること；
（ｉｉ）　可能な正体を該第１のヌクレオチドに対応するプローブファミリー名に基づいて隣接するヌクレオチドと決定することにより、正体を第１のヌクレオチドに隣接するヌクレオチド割り当てること；
（ｉｉｉ）　その正体が最も新しく割り当てられたヌクレオチドに対応するプローブファミリー名に基づいて可能な正体を後続のヌクレオチドと決定することにより、正体を後続のヌクレオチドに割り当てること；
（ｉｖ）　候補配列が生成されるまで工程（ｉｉｉ）を反復すること；ならびに
（ｖ）　工程（ｉ）～（ｉｖ）を反復することであって、ここで、各反復において、所望数の候補配列が生成されるまで、異なる正体を該第１のヌクレオチドと仮定すること
を含む、請求項６９に記載の方法。
【請求項72】
前記選択工程が、少なくとも１つの候補配列を１つ以上の既知配列と比較すること、および該既知配列の１つ以上と所定の程度の同一性を示すか、またはほとんどほぼ同一である候補配列を選択することを含む、請求項６９に記載の方法。
【請求項73】
前記鋳型が、目的の生物体由来のものであり、前記比較工程が、少なくとも１つの候補配列を、該生物体から得られた配列を含むデータベース内の配列と比較することを含む、請求項７２に記載の方法。
【請求項74】
前記比較工程が、少なくとも１つの候補配列を、各々が、測定対象のポリヌクレオチドの配列の可能な択一的な配列を含む複数の比較配列を含むデータベース内の配列と比較することを含む、請求項７２に記載の方法。
【請求項75】
前記選択工程が、
（ｉ）　第２のプローブファミリーの序列リスト名を、前記鋳型から、識別可能に標識さ

れたコードされたプローブのファミリーの第２のコレクションを用いて得ることであって、ここで、該プローブファミリーの該第２のコレクション内のプローブファミリーは、プローブファミリーの前記第１のコレクション内のプローブファミリーと異なるようにコードされていること；
（ｉｉ）　少なくとも１つの比較配列を、該第２のプローブファミリーの序列リスト名から生成させること；
（ｉｉｉ）　候補配列の少なくとも１つの一部分を比較配列の少なくとも１つの一部分と比較すること；ならびに
（ｉｖ）　所定のレベルの同一性を示すか、または該鋳型内のヌクレオチドの配列として工程（ｃ）で比較される部分において、該比較配列とほとんどほぼ同一である候補配列を選択すること
を含む、請求項６９に記載の方法。
【請求項76】
前記比較される部分が単一のジヌクレオチドである、請求項７５に記載の方法。
【請求項77】
前記第２のプローブファミリーの序列リスト名が、単一の要素のみを含む、請求項７５に記載の方法。
【請求項78】
各プローブファミリー内のオリゴヌクレオチドプローブが、構造５’－（ＸＹ）（Ｎ）$_{k}$ N_B＊－３’または３’－（ＸＹ）（Ｎ）$_{k}$ N_B＊－５’（式中、Ｎは任意のヌクレオシド表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、ＸＹはプローブの拘束部分であり、ここで、ＸおよびＹは、同一または異なるが独立しては選択されないヌクレオシドを表し、ＸおよびＹは少なくとも２重に縮重しており、少なくとも１つのヌクレオシド間結合は切れやすい連結であり、ｋは１～１００（両端を含む）であり、ただし、検出可能な部分は、Ｙ上に、または（Ｎ）$_{k}$の任意のヌクレオシド上に、N_Bの代わりまたはこれに加えて存在し得るものとする）
を有する、請求項５０に記載の方法。
【請求項79】
前記切れやすい連結がホスホロチオレート連結である、請求項７８に記載の方法。
【請求項80】
前記検出可能な部分が、切断可能なリンカーに結合されているか、光退色性であるか、またはその両方である、請求項７８に記載の方法。
【請求項81】
前記切断可能なリンカーがジスルフィド結合を含む、請求項８０に記載の方法。
【請求項82】
４つの識別可能に標識されたオリゴヌクレオチドプローブファミリーが使用され、プローブの拘束部分の異なる配列を有するオリゴヌクレオチドプローブが、表１に示す２４個のコード化の１つに従って第１、第２、第３および第４のプローブファミリーに割り当てられる、請求項７８に記載の方法。
【請求項83】
前記検出工程が、平均２ビットの情報を同時に、任意の個々のヌクレオチドの２ビットの情報を取得することなく、少なくとも２つの前記鋳型内のヌクレオチドの各々から取得することを含む、請求項５１に記載の方法。
【請求項84】
前記検出工程が、２ビット未満の情報を同時に、前記鋳型内の少なくとも２つのヌクレオチドの各々から取得することを含む、請求項５１に記載の方法。
【請求項85】
少なくとも２つの識別可能に標識されたオリゴヌクレオチドプローブファミリーの第１のコレクションを用いて、鋳型ポリヌクレオチド内のヌクレオチドの配列に関する情報を決定する方法であって、

（ａ）　二本鎖部分を含むプローブ－鋳型複合体を伸長可能な末端と、および目的の配列決定対象の単鎖部分を少なくとも２つの識別可能に標識されたオリゴヌクレオチドプローブファミリーと、ハイブリダイゼーションが該鋳型の該二本鎖部分に直接隣接する部分に相補的な部分を含むオリゴヌクレオチドプローブ間で起こるように接触させる工程であって、該鋳型は、それにハイブリダイズしたブロッキングオリゴヌクレオチドを有する、工程；
（ｂ）　ハイブリダイズされた該オリゴヌクレオチドプローブを該伸長可能な末端にライゲートさせ、それにより、伸長された二本鎖を含むプローブ－鋳型複合体を生成させる工程；
（ｃ）　該ライゲートされたプローブと会合した標識を検出する工程；
（ｄ）　伸長可能なプローブ末端が既に存在しない場合は、該伸長可能なプローブ末端を該伸長された二本鎖上に生成させる工程；ならびに
（ｅ）　プローブファミリーの序列リスト名が得られるまで（ａ）～（ｄ）を反復する工程
を含む、方法。
【請求項86】
前記検出工程が、平均２ビットの情報を同時に、任意の個々のヌクレオチドの２ビットの情報を取得することなく、少なくとも２つの前記鋳型内のヌクレオチドの各々から取得することを含む、請求項８５に記載の方法。
【請求項87】
前記検出工程が、２ビット未満の情報を同時に、前記鋳型内の少なくとも２つのヌクレオチドの各々から取得することを含む、請求項８５に記載の方法。
【請求項88】
オリゴヌクレオチドプローブファミリーの第１のコレクションを用いて、鋳型ポリヌクレオチド内のヌクレオチドの配列に関する情報を決定する方法であって、
（ａ）　伸長、ライゲーション、検出および切断の連続的サイクルを行なう工程であって、ここで、該検出工程が、平均２ビットの情報を同時に、任意の個々のヌクレオチドの２ビットの情報を取得することなく、少なくとも２つの該鋳型内のヌクレオチドの各々から取得することを含み、該鋳型は、それにハイブリダイズしたブロッキングオリゴヌクレオチドを有する、工程；ならびに
（ｂ）　工程（ａ）で得られた情報を少なくとも１ビットのさらなる情報と組み合わせて該配列を決定する工程
を含む、方法。
【請求項89】
前記少なくとも１ビットのさらなる情報が、前記鋳型内のヌクレオチドの正体、候補配列を少なくとも１つの既知配列と比較することにより得られる情報；およびオリゴヌクレオチドプローブファミリーの第２のコレクションを用いて該方法を反復することにより得られる情報からなる群より選択される項目を含む、請求項８８に記載の方法。
【請求項90】
複数の鋳型ポリヌクレオチドの調製方法であって、
（ａ）　複数の微粒子と半固相支持体とを接触させる工程であって、該微粒子の少なくとも一部は、それに結合した鋳型を有し、該半固相支持体は、該半固相支持体に結合しているか、または該半固相支持体内に包埋したプライマーを有し、該鋳型が該プライマーにハイブリダイズする、工程；ならびに
（ｂ）　該プライマーを伸長して、該微粒子に結合した該鋳型に相補的な鋳型を形成する工程
を含む、方法。
【請求項91】
前記プライマーを伸長させることによって生成した前記鋳型を増幅する工程をさらに含む、請求項９０に記載の方法。

【請求項92】
増幅する工程が、ＲＣＡを実行することを含む、請求項９１に記載の方法。
【請求項93】
前記半固相支持体から前記微粒子を放出させる工程をさらに含む、請求項９０に記載の方法。
【請求項94】
前記鋳型を増幅する工程の後で、必要に応じて、前記プライマーを伸長させることによって生成した該鋳型を配列決定する工程をさらに含む、請求項９０に記載の方法。
【請求項95】
微粒子の集団を調製するための成分のコレクションであって、
（ａ）　個々の微粒子が、該微粒子に結合されたプライマーの少なくとも第１および第２の集団を有し、該第１の集団のプライマーが、該第２の集団のプライマーと異なる配列を有する、微粒子の集団；
（ｂ）　各核酸断片が目的の第１および第２の核酸セグメントを含み、該第１および第２のプライマーが、該目的の第１および第２の核酸セグメントの外部に位置する普遍配列に対応する、核酸断片のライブラリー；ならびに
（ｃ）　該核酸断片の共通領域に結合するブロッキングオリゴヌクレオチド
を含む、コレクション。
【請求項96】
前記目的の第１および第２の核酸セグメントが５’および３’タグの１対のタグである、請求項９５に記載の成分のコレクション。
【請求項97】
前記核酸断片が、核酸セグメントの各々がＰＣＲを用いて増幅され得るように、増幅プライマーのための１つ以上のプライマー結合部位を含む内部アダプターを含む、請求項９５に記載の成分のコレクション。
【請求項98】
前記内部アダプター内のプライマー結合部位に相補的なプライマーをさらに含む、請求項９７に記載の成分のコレクション。
【請求項99】
実質的に同一な鋳型分子の集団を含む鋳型であって、該鋳型分子は、少なくとも１つの共通領域および少なくとも１つの目的のセグメントを含み、該鋳型分子の少なくとも一部は、該共通領域にハイブリダイズしたブロッキングオリゴヌクレオチドを有する、鋳型。
【請求項100】
前記鋳型分子が、対合したタグライブラリーのメンバーである、請求項９９に記載の鋳型。
【請求項101】
前記鋳型分子が、ＲＣＡを使用して増幅される、請求項９９に記載の鋳型。
【請求項102】
前記鋳型分子は、少なくとも２つの共通領域および少なくとも１つの目的のセグメントを含み、該鋳型分子の少なくとも一部は、少なくとも２つの共通領域の各々にハイブリダイズしたブロッキングオリゴヌクレオチドを有する、請求項９９に記載の鋳型。
【請求項103】
請求項９９に記載の集団が結合した、支持体または基材。
【請求項104】
微粒子である、請求項１０１に記載の支持体または基材。
【請求項105】
半固相支持体である、請求項１０１に記載の支持体または基材。
【請求項106】
実質的に平面状で剛性の支持体である、請求項１０１に記載の支持体または基材。
【請求項107】

前記鋳型が、目的の種々のセグメントを含む、請求項９９に記載の鋳型のコレクション。
【請求項108】
請求項１０４に記載の微粒子の集団を含むアレイであって、該微粒子は、それに結合した目的の種々のセグメントを含む鋳型を有する、アレイ。
【請求項109】
前記微粒子は、半固相支持体内または該支持体上に包埋されているか、あるいは基材に結合している、請求項１０８に記載のアレイ。
【請求項110】
基材に結合した微粒子であり、該微粒子は、それに結合した鋳型を有する、微粒子。
【請求項111】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合している、請求項１１０に記載の微粒子。
【請求項112】
前記微粒子が、ビオチンおよびビオチン結合タンパク質を含む連結によって前記基材に結合し、該ビオチン結合タンパク質が、該基材に結合している、請求項１１０に記載の微粒子。
【請求項113】
前記微粒子が、前記基材に結合した一本鎖鋳型に結合しており、それによって前記鋳型が該微粒子を該基材に繋いでいる、請求項１１０に記載の微粒子。
【請求項114】
前記微粒子が、前記基材に結合した一本鎖鋳型に結合しており、それによって前記鋳型が該微粒子を該基材に繋ぎ、該鋳型が、ビオチンおよびビオチン結合タンパク質を含む連結によって該基材および該微粒子に結合している、請求項１１０に記載の微粒子。
【請求項115】
請求項１１０に記載の微粒子の集団であって、目的の種々のセグメントと共通配列とを含む鋳型が、異なる微粒子に結合している、微粒子の集団。
【請求項116】
アレイを調製するための方法であって、
　鋳型が結合した微粒子の集団を提供する工程であって、該鋳型がビオチンを含む、工程；ならびに
　ビオチンがビオチン結合タンパク質に結合する条件下で、該微粒子と該ビオチン結合タンパク質を含む基材とを接触させ、それによって微粒子のアレイを生成する工程；
を含む、方法。
【請求項117】
前記鋳型にブロッキングオリゴヌクレオチドをハイブリダイズさせる工程をさらに含む、請求項１１６に記載の方法。
【請求項118】
前記鋳型を配列決定する工程をさらに含む、請求項１１６に記載の方法。
【発明の詳細な説明】
【技術分野】
【0001】
　政府の支援
　本発明は、ＮＩＨによって与えられた助成金番号Ｒ０１－ＨＧ－００３５７０の下、米国政府の支援によって為された。政府は、本発明において一定の権利を有する。
【0002】
（関連出願の引用）
　本願は、２００６年４月１９日に出願された米国仮特許出願第６０／７９３，７０２号（この全内容は、参考として本明細書に援用される）の利益、およびそれに対する優先権を主張する。本願は、２００５年２月１日に出願された米国仮特許出願第６０／６４９，２９４号；２００５年２月２５日に出願された米国仮特許出願第６０／６５６，５９９号；２００５年４月２１日に出願された米国仮特許出願第６０／６７３，７４９号；２００

５年７月１５日に出願された米国仮特許出願第６０／６９９，５４１号；２００５年９月３０日に出願された米国仮特許出願第６０／７２２，５２６号；および米国特許出願第１１／３４５，９７９号に関する。これらは全て、本明細書中に参考として援用される。

【0003】

（発明の背景）

核酸配列決定手法は、基礎研究から臨床診断にわたる広範で多様な分野において非常に重要である。かかる手法から入手可能な結果としては、種々の程度の特異性の情報が挙げられ得る。例えば、有用な情報は、ある特定のポリヌクレオチドが参照ポリヌクレオチドと配列が異なるか否かの判定、試料中のある特定のポリヌクレオチド配列の存在の確認、部分配列情報、例えば、ポリヌクレオチド内の１つ以上のヌクレオチドの正体の決定、ポリヌクレオチド内のヌクレオチドの正体および順序の決定などからなるものであり得る。

【0004】

ＤＮＡ鎖は、典型的には、４種類のサブユニット、すなわち、塩基アデニン（Ａ）、シトシン（Ｃ）、グアニン（Ｇ）およびチミジン（Ｔ）を含有するデオキシリボヌクレオチドで構成されるポリマーである。これらのサブユニットは、一方のデオキシリボース基の５’炭素を次の基の３’炭素に連結するホスホジエステル共有結合によって互いに結合されている。ほとんどの天然に存在するＤＮＡは２つのかかる鎖からなり、これらは、逆平行の向きに整列され、相補的塩基間、すなわち、ＡとＴ間およびＧとＣ間で形成される水素結合によって互いに保持されている。

【0005】

ＤＮＡ配列決定は、最初、連鎖停止またはジデオキシヌクレオチド方法（非特許文献１）および化学的分解方法（非特許文献２）の開発によって大きさ規模において可能になり、このうち前者は、最も広く使用され、改良され、自動化されている。特に、蛍光標識された連鎖停止剤の使用が、自動ＤＮＡ配列決定装置の開発において主に重要であった。上記のアプローチの両方に共通するのは、大きさの異なる標識されたＤＮＡ断片の１つ以上のコレクションの作製であり、これは、次いで、該断片の３’末端のヌクレオチドの正体（連鎖停止法において）または該断片から最も新しく除去されたヌクレオチドの正体（化学的分解方法の場合）を決定するために、長さに基づいて分離されなければならない。

【0006】

現在利用可能な配列決定手法は、いくつかの完全ゲノムの配列決定などの主要な目的の達成を可能にするが、これらの手法は、いくつかの不都合点を有し、いくつかの領域において改善の相当な必要性がなお残っている。標識されたＤＮＡ断片の分離は、典型的には、ポリアクリルアミドゲル電気泳動を用いてなされている。しかしながら、この工程は、多くの状況で配列決定のスピードと精度の両方を制限する大きな障害であることが示されている。キャピラリー電気泳動（ＣＡＥ）は、ヒトゲノムプロジェクトの完成を可能にした飛躍的進歩であることが示されたが（非特許文献３）、大きな欠点がなお残っている。例えば、ＣＡＥは、依然として時間のかかる分離工程を必要とし、依然として大きさに基づく区別を伴ない、これらは、不正確となり得る。

【0007】

連鎖停止方法に対するさまざまな代替法が提案されている。多くの場合、「合成による配列決定」とよばれるアプローチの一例では、オリゴヌクレオチドプライマーを、まず標的鋳型にハイブリダイズさせる。次いで、プライマーを、種々に標識したヌクレオチドのポリメラーゼ触媒型付加の連続的サイクルによって伸長させる。成長鎖内へのその組込みを検出する。標識の正体は、鋳型内の相補的ヌクレオチドを同定する機能を果たす。あるいはまた、多数の反応が、各ヌクレオチドを用いて並行して行なわれ得、ある特定のヌクレオチドを使用する反応内への標識されたヌクレオチドの組込みにより、鋳型内の相補的ヌクレオチドが同定される（例えば、Ｍｅｌａｍｅｄｅ，特許文献１；Ｃｈｅｅｓｅｍａｎ，特許文献２；Ｔｓｉｅｎら，特許文献３；Ｒｏｓｅｎｔｈａｌら，特許文献４；非特許文献４；非特許文献５参照のこと）。

【0008】

任意の有意な長さのポリヌクレオチドを効率的に配列決定するため、ポリメラーゼは、各サイクルにおいて正確に１つのヌクレオチドを組み込むことが望ましい。したがって、一般的に、連鎖停止剤として作用するヌクレオチド使用すること、すなわち、その組込みにより、ポリメラーゼによるさらなる伸長が抑制されることが必要である。組み込まれたヌクレオチドは、次いで、ポリメラーゼが次のヌクレオチドを組み込むことが可能になるように酵素的または化学的のいずれかで修飾されなければならない。連鎖停止剤としての機能を果たし得るが、後続の工程で伸長され得るようにその組込み後に修飾され得るさまざまなヌクレオチドアナログが提案されている。かかる「可逆的停止剤」は、例えば特許文献２；特許文献５～７に記載されている。しかしながら、高効率でポリメラーゼ組み込まれ得る可逆的停止剤を同定することは、おそらく、ヌクレオチドの大きさが小さいことを考慮すると、ヌクレオチドが停止剤としての機能を果たす能力に影響する修飾はまた、成長ポリヌクレオチド鎖内へのその組込みにも影響するという事実のため、困難であることが示されている。

【0009】

他の配列決定アプローチとしては、ピロシーケンスが挙げられ、これは、ＤＮＡ重合中に放出されるピロリン酸塩（ＰＰｉ）の検出に基づくものである（例えば、特許文献８および９参照のこと）。電気泳動による分離の必要性は回避されるが、ピロシーケンスは、今のところその広範な適用可能性を制限している多数の欠点を有する（非特許文献６）。ハイブリダイゼーションによる配列決定もまた、代替法として提案されている（特許文献１０～１２；非特許文献７）が、いくつかの不都合点（例えば、高度に類似した配列間の識別におけるエラーに可能性など）を有する。エクソヌクレアーゼによる単一分子配列決定は、１つの鎖内の塩基ごとの標識、次いで、試料流中の切断された３’末端ヌクレオチドの連続的な検出を伴うものであるが、これは、理論的には、長鎖ＤＮＡ分子の配列を速やかに決定するに非常に強力な方法である（非特許文献８）。しかしながら、この潜在性を実現する前に克服すべき種々の技術的な障害がなお残っている（非特許文献８）。

【0010】

特定の配列異型に基づく診断用試験は、さまざまな種々の疾患のために既に使用されている。ヒトゲノムの配列決定は、治療（予防的治療を含む）が患者の特定のゲノム構成にあつらえられるか、または特定の対立遺伝子もしくは変異の同定に基づいて選択される個別化医療の時代の到来を告げると広く考えられている。ＨＩＶなどの感染性因子の配列バリアントの迅速かる正確な決定の必要性が増大している。したがって、近い将来、正確かつ迅速な配列決定に対する要望が大きく拡大することは明白である。したがって、あらゆる型の改善された配列決定法が必要とされている。

【特許文献1】米国特許第４，８６３，８４９号明細書
【特許文献2】米国特許第５，３０２，５０９号明細書
【特許文献3】国際公開第９１／０６６７８号パンフレット
【特許文献4】国際公開第９３／２１３４０号パンフレット
【特許文献5】米国特許第６，２５５，４７５号明細書
【特許文献6】米国特許第６，３０９，８３６号明細書
【特許文献7】米国特許第６，６１３，５１３号明細書
【特許文献8】米国特許第６，２１０，８９１号明細書
【特許文献9】米国特許第６，２５８，５６８号明細書
【特許文献10】米国特許第５，２０２，２３１号明細書
【特許文献11】国際公開第９９／６０１７０号パンフレット
【特許文献12】国際公開第００／５６９３７号パンフレット
【非特許文献1】Ｓａｎｇｅｒら．，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．７４：５４６３－５４６７，１９７７
【非特許文献2】Ｍａｘａｍ&Ｇｉｌｂｅｒｔ，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．７４：５６０－５６４，１９７７
【非特許文献3】Ｖｅｎｔｅｒら．，Ｓｃｉｅｎｃｅ，２９１：１３０４－１３５１，２

００１；Landerら．，Ｎａｔｕｒｅ，４０９：８６０－９２１，２００１
【非特許文献4】Ｃａｎａｒｄら，Ｇｅｎｅ，１４８：１－６（１９９４）
【非特許文献5】Ｍｅｔｚｋｅｒら，Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓｅａｒｃｈ，２２：４２５９－４２６７（１９９４）
【非特許文献6】Ｆｒａｎｃａら．，Ｑｕａｒｔｅｒｌｙ　Ｒｅｖｉｅｗｓ　ｏｆ　Ｂｉｏｐｈｙｓｉｃｓ，３５（２）：１６９－２００，２００２
【非特許文献7】Ｄｒｍａｎａｃら．，Ａｄｖａｎｃｅｓ　ｉｎ　Ｂｉｏｃｈｅｍｉｃａｌ　Ｅｎｇｉｎｅｅｒｉｎｇ／Ｂｉｏｔｅｃｈｎｏｌｏｇｙ，７７：７６－１０１，２００２
【非特許文献8】Ｓｔｅｐｈａｎら．，Ｊ　Ｂｉｏｔｅｃｈｎｏｌ．，８６：２５５－２６７，２００１

【発明の開示】
【課題を解決するための手段】
【0011】
（発明の要旨）
本発明は、断片分離を行なう必要性を回避する、またある特定の実施形態においては、ポリメラーゼ酵素の使用の必要性を回避する新しい改善された配列決定法を提供する。背景の項目に記載の方法の代替法は、Ｍａｃｅｖｉｃｚ（Ｍａｃｅｖｉｃｚ）の米国特許第５，７４０，３４１号および同第６，３０６，５９７号に記載されている。この方法は、単鎖鋳型に沿った二本鎖伸長の反復サイクルに基づくものである。これらの方法の好ましい実施形態において、ヌクレオチドは各サイクルで同定される。本発明は、これらの方法に対する改善を提供する。該改善により、該方法の効率的な実施が可能になり、ハイスループット配列決定に特に好適である。また、本発明は、単鎖鋳型に沿った二本鎖伸長の反復サイクルを伴うが、各サイクル中での個々のヌクレオチドの同定をなんら伴わない配列決定法を提供する。
【0012】
一態様において、本発明は、単鎖鋳型に沿った二本鎖伸長、標識された伸長プローブのライゲーションおよび標識の検出の連続的サイクルに基づく配列決定のための改善された方法を提供する。一般に、伸長は、初期化（ｉｎｉｔｉａｌｉｚｉｎｇ）オリゴヌクレオチドおよび鋳型によって形成された二本鎖から開始される。初期オリゴヌクレオチドは、オリゴヌクレオチドプローブをその末端にライゲートして伸長された二本鎖を形成させ、次いで、これを、ライゲーションの連続的サイクルによって反復的に伸長させることにより伸長される。各サイクル中、鋳型内の１つ以上のヌクレオチドの正体が、成功裏にライゲートされたオリゴヌクレオチドプローブ上またはこれと会合した標識を同定することにより決定される。新たに付加されたプローブの標識はまた、ライゲーション前、あるいはそれに加えて、ライゲーション後に検出され得る。一般的に、ライゲーション後に標識を検出することが好ましい。
【0013】
好ましい実施形態において、伸長された二本鎖のたった１回の伸長が１サイクルで起こるように、プローブは伸長不可能な部分を、末端位置に（二本鎖の成長核酸鎖にライゲートされたヌクレオチドと反対のプローブ末端に）有する。「伸長不可能な」により、該部分が、修飾のないリガーゼ基質としての機能を果たさないことが意図される。例えば、該部分は、５’リン酸基または３’ヒドロキシル基がない。該部分は、ライゲーションを抑制するブロッキング基が自身に結合されたヌクレオチドであり得る。本発明の好ましい実施形態において、伸長不可能な部分は、ライゲーション後に除去され、伸長可能な末端が生成され、その結果、二本鎖は後続のサイクルでさらに伸長され得る。
【0014】
伸長不可能な部分の除去を可能にするため、本発明のある特定の実施形態において、プローブに、ホスホジエステル結合が実質的に切断されない条件下で切断され得る少なくとも１つのヌクレオシド間結合を含有させる。かかる連結は、本明細書において「切れやす

いヌクレオシド間結合」または「切れやすい連結」という。切れやすいヌクレオシド間結合の切断により、伸長不可能な部分が除去され、伸長可能なプローブ末端が再生されるか、または修飾されて伸長可能なプローブ末端を形成する末端残基が残るかのいずれかとなる。切れやすいヌクレオシド間結合は、プローブ内の任意の２つのヌクレオシド間に位置し得る。好ましくは、切れやすい連結は、新たに形成された結合から少なくとも数個のヌクレオチド離れて（すなわち、遠位に）位置する。伸長可能な末端ライゲートされた末端ヌクレオチドと切れやすい連結間の伸長プローブ内のヌクレオチドは、鋳型に完璧にハイブリダイズする必要はない。これらのヌクレオチドは、「スペーサー」としての機能を果たし、鋳型に沿った区間に位置するヌクレオチドの同定を、該区間該の各ヌクレオチドについてサイクルを行なうことなく可能にし得る。

【0015】

切れやすいヌクレオシド間結合および標識は、好ましくは、切れやすいヌクレオシド間結合の切断により、標識された部分内の伸長プローブと成長核酸鎖の一部のままである部分とが分離され、標識された部分の核酸が可能になる（例えば、温度上昇時）ように配置させる。例えば、標識は、伸長プローブの末端ヌクレオチドのライゲートされるヌクレオチドと反対の末端に結合させ得る。あるいはまた、標識は、任意のいくつかのアプローチを用いて除去してもよい。

【0016】

本発明者らは、ホスホジエステル結合内の橋絡酸素原子の１つがイオウ原子で置き換えられたホスホロチオレート結合が、特に好都合な切れやすいヌクレオシド間結合であることを見出した。ホスホロチオレート結合内のイオウ原子は、一方のヌクレオシドの３’炭素または隣接するヌクレオシドの５’炭素のいずれかに結合され得る。

【0017】

上記の方法のある特定の実施形態では、複数の配列決定反応が行なわれる。該反応では、最初のライゲーションが起こる末端が、鋳型に対して異なる位置に位置するように、鋳型の異なる配列にハイブリダイズする初期オリゴヌクレオチドが使用される。例えば、最初のライゲーションが起こる位置は、１個のヌクレオチド増加によって、互いに対してシフトまたは「位相ずれ（ｏｕｔ　ｏｆ　ｐｈａｓｅ）」させ得る。したがって、同じ長さのオリゴヌクレオチドプローブを用いた各伸長サイクル後、同じ相対位相が、異なる鋳型上の初期オリゴヌクレオチドの両末端間に存在する。該反応は、並行して、各々が同じ鋳型コピーを含む独立した区画において、または順次（すなわち、第１の初期オリゴヌクレオチドを用いて配列情報を得た後、伸長された二本鎖を鋳型から除去し、次いで鋳型の異なる配列にハイブリダイズする初期オリゴヌクレオチドを用いてさらなる反応（１つまたは複数）を行なうことにより）行なわれ得る。

【0018】

別の態様において、本発明は、さまざまな核酸操作に有用な溶液を提供する。一実施形態において、本発明は、１．０～３．０％ＳＤＳ、１００～３００ｍＭ　ＮａＣｌおよび５～１５ｍＭ重硫酸ナトリウム（$NaHSO_4$）を水中に含有するか、またはそれらから本質的になる溶液を提供する。該溶液は、約２％ＳＤＳ、約２００ｍＭ　ＮａＣｌおよび約１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を水中に含有するものであるか、または本質的にそれらからなるものであり得る。例えば、一実施形態において、該溶液は、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を水中に含有するものである。別の実施形態において、該溶液は、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含む水から本質的になるものである。ある特定の実施形態において、該溶液は、２．０～３．０、例えば２．５のｐＨを有する。該溶液は、二本鎖核酸、例えば二本鎖ＤＮＡを個々の鎖に分離、すなわち、二本鎖核酸を変性（融解）させるのに有用である。ある特定の実施形態において、両方の鎖がＤＮＡである。他の実施形態において、両方の鎖がＲＮＡである。他の実施形態において、一方の鎖がＤＮＡであり、他方の鎖はＲＮＡである。他の実施形態において、一方または両方の鎖がＲＮＡおよびＤＮＡの両方を含有する。他の実施形態において、該鎖の一方

または両方がＡ、Ｇ、ＣまたはＴ以外の少なくとも１つのヌクレオチドを含有する。ある一部の実施形態において、該鎖の一方または両方が、非天然に存在するヌクレオチドを含有する。また他の実施形態において、該残基の１つ以上がトリガー（ｔｒｉｇｇｅｒ）残基、例えば、無塩基残基または損傷塩基である。ある一部の実施形態において、１つ以上の残基が、普遍（ｕｎｉｖｅｒｓａｌ）塩基を含有する。ある一部の実施形態において、該鎖の一方または両方が切れやすい連結を含有する。

【0019】

二本鎖核酸は、完全な二本鎖または部分二本鎖であり得る。これは、溶液中で遊離状態であり得るか、または一方もしくは両方の鎖が固相もしくは半固相支持体もしくは基材と物理的に会合（例えば、共有結合もしくは非共有結合）　された状態であり得る。特に注目すべきことには、このような溶液中でインキュベートされた二本鎖核酸は、ゲル離層を引き起こし得る（例えば、核酸が、ポリアクリルアミドゲルなどの半固相支持体内に位置するか、これに結合されている場合）か、またはストレプトアビジン（ＳＡ）－ビオチン結合（例えば、核酸が支持体もしくは基材にＳＡ－ビオチン結合によって結合されている場合）などの非共有結合を破壊し得る熱または刺激の強い変性剤の非存在下で効果的に単鎖に分離される。一実施形態において、該溶液は、核酸の一方がビーズにＳＡ－ビオチン結合によって結合されている二本鎖核酸を分離するために使用される。

【0020】

本発明はまた、二本鎖核酸と任意の前述の溶液、例えば、約１．０～３．０％ＳＤＳ、約１００～３００ｍＭ　ＮａＣｌおよび約５～１５ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含有する水溶液、例えば、１．０～３．０％ＳＤＳ、１００～３００ｍＭ　ＮａＣｌおよび５～１５ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含有する水溶液と接触させる工程を含む、二本鎖核酸の鎖を分離する方法を提供する。一実施形態において、該溶液は、約２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）、例えば、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）　を含有するものである。別の実施形態において、該溶液は、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含む水から本質的になるものである。ある特定の実施形態において、該溶液は、２．０～３．０、例えば２．５のｐＨを有する。ある一部の実施形態において、二本鎖核酸は、溶液中でインキュベートされる。他の実施形態において、二本鎖核酸（好ましくは、支持体または基材に結合されている）を該溶液で洗浄する。ある一部の実施形態において、二本鎖核酸を該溶液と、二本鎖核酸分子の少なくとも１０％が単鎖に分離されるのに充分な時間接触させる。ある一部の実施形態において、二本鎖核酸を該溶液と、二本鎖核酸少なくとも２０％、３０％、４０％、５０％、６０％、７０％、８０％、９０％、９５％、９８％、９９％またはそれ以上が単鎖に分離されるのに充分な時間接触させる。例示的な実施形態において、二本鎖核酸を該溶液と１５秒間～３時間接触させる。別の実施形態において、二本鎖核酸を該溶液と１分間～１時間接触させる。ある特定の実施形態において、二本鎖核酸を該溶液と、約１、２、３、４、５、１０、１５、２０、２５、３０、３５、４０、４５、５０、５５または６０分間接触させる。該方法は、インキュベーション期間後に、該溶液を除去または一部もしくは全部の核酸を該溶液から除去するさらなる工程を含むものであってもよい。

【0021】

該溶液には、いくつかの本明細書に記載した配列決定法の１つ以上の工程における用途が見出され、任意のこれらの方法において使用され得る。例えば、該溶液は、伸長された二本鎖を鋳型から分離するために使用され得る。該溶液は、切れやすい連結を切断して伸長プローブの伸長された二本鎖にもはや結合されていない部分を除去した後に使用され得る。該溶液はまた、三重鎖核酸の分離、または単一の核酸鎖の互いににハイブリダイズした自己相補性部分を含有する二本鎖領域の分離に有用である。

【0022】

別の態様において、本発明は、少なくとも２つの識別可能に標識されたオリゴヌクレオ

チドプローブファミリーのコレクションを用いて、配列に関する情報を得るための方法を提供する。該プローブファミリー内のプローブは、非拘束部分および拘束部分を含有する。上記の方法のように、伸長は、初期オリゴヌクレオチドおよび鋳型によって形成された二本鎖から開始される。初期オリゴヌクレオチドは、オリゴヌクレオチドプローブをその末端にライゲートして伸長された二本鎖を形成させ、次いで、これを、ライゲーションの連続的サイクルによって反復的に伸長させることにより伸長される。伸長された二本鎖のたった１回の伸長が１サイクルで起こるように、プローブは伸長不可能な部分を、末端位置に（二本鎖の成長核酸鎖にライゲートされたヌクレオチドと反対のプローブ末端に）有する。各サイクル中、成功裏にライゲートされたプローブ上の、またはこれと会合させた標識が検出され、伸長不可能な部分が除去または修飾されて、伸長可能な末端が生成する。標識は、該プローブが属するプローブファミリーに対応する。

【0023】

伸長、ライゲーションおよび検出の連続的サイクルにより、連続的な成功裏にライゲートされたプローブが属するプローブファミリーの序列リスト（ｏｒｄｅｒｅｄ　ｌｉｓｔ）がもたらされる。プローブファミリーの序列リストは、配列に関する情報を得るために使用される。しかしながら、新たにライゲートされたプローブがどのプローブファミリーに属するのかがわかることは、それ自体では、鋳型内のヌクレオチドの正体を決定するのに充分でない。それどころか、新たにライゲートされたプローブがどのプローブファミリーに属するのかがわかることは、ある特定の配列がプローブの拘束部分の配列である可能性を排除し、各位置におけるヌクレオチドの正体の少なくとも２つの可能性を残す。したがって、新たにライゲートされたプローブの拘束部分内のヌクレオチド（すなわち、プローブの拘束部分内のヌクレオチドに相補的なヌクレオチド）と反対の位置に位置する鋳型内のヌクレオチドの正体には少なくとも２つの可能性がある。

【0024】

ある特定の実施形態において、所望回数サイクルを行なった後、該一連の序列のプローブファミリー正体を用いて１組の候補配列を作製する。この組の候補配列により、目的を達成するのに充分な情報が提供され得る。本発明の好ましい実施形態において、候補配列の中から正確な配列を選択するため、１つ以上のさらなる工程を行なう。例えば、該配列が既知配列のデータベースと比較され得、データベース内の該配列の１つに最も近い候補配列を正確な配列として選択する。他の実施形態において、異なるようにコード化された組のプローブファミリー用い、鋳型を、伸長、ライゲーション、検出および切断の連続的サイクルによるさらなる回の配列決定に供し、２回目で得られた情報を用いて正確な配列を選択する。他の実施形態において、少なくとも１つの情報項目を、プローブファミリー正体の序列リストから得られた情報と組み合わせ、配列を決定する。

【0025】

本発明はまた、プローブファミリーを用いて鋳型を配列決定する場合のエラーチェックを行なう方法を提供する。該方法のある特定のものは、一塩基多型（ＳＮＰ）と配列決定エラーを識別する。

【0026】

本発明はまた、目的の少なくとも２つのセグメント（例えば、少なくとも２つのタグ）および少なくとも３つのプライマー結合領域（ＰＢＲ）を、各々が目的のセグメントに対応する少なくとも２つの異なる鋳型が各断片から増幅され得るように含有する核酸断片（例えば、ＤＮＡ断片）を提供する。「プライマー結合領域」は、オリゴヌクレオチドが、該オリゴヌクレオチドが増幅プライマー、配列決定プライマー、初期オリゴヌクレオチドなどとしての機能を果たし得るようにハイブリダイズし得る核酸の一部分である。したがって、プライマー結合領域は、適当な相補的オリゴヌクレオチドの選択を可能にするため、既知配列を有するものであるのがよい。本明細書および図面で用いる場合、本発明の方法に用いられる核酸鎖の一部分は、該方法の実施において、プライマーが実際に、該領域に結合するか、核酸鎖の相補鎖の対応する部分に結合するかに関係なく、プライマー結合領域とよばれ得る。したがって、核酸の一部分は、本発明の方法に用いられる場合、プラ

イマーが実際に、該領域に結合するのか（この場合、プライマーの配列は、該領域のものに相補的または実質的に相補的である）、または該領域の相補的部分に結合するのか（この場合、プライマーの配列は、プライマー結合領域の配列と同一または実質的に同一である）とは無関係にプライマー結合領域とよばれ得る。目的のセグメントは、その配列情報が所望される核酸の任意のセグメントである。例えば、目的の配列は、タグであり得、本発明の開示の目的のためには、目的のセグメントがタグである（本明細書および他で「末端タグ」とも称する）ことが想定される。しかしながら、本発明は、目的のセグメントがタグであることに限定されないことを理解されたい。ある特定の実施形態において、少なくとも２つのタグは１対のタグである。該核酸断片は、１対以上のタグ、例えば、１つ以上の対のタグ、例えば、２、３、４、５対以上の対のタグを含有するものであり得る。本発明は、さらに、かかる核酸断片を含有するライブラリー、ならびに該鋳型およびライブラリーの作製方法を提供する。

【0027】

本発明は、さらに、自身に結合された核酸の少なくとも２つの異なる集団を有する微粒子（例えば、ビーズ）であって、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、該集団は、単一の核酸断片からの増幅（例えば、ＰＣＲ増幅）によって生成されたものである微粒子（例えば、ビーズ）を提供する。ある一部の実施形態において、単一の核酸断片は、１対のタグである５’タグおよび３’タグを含有する。単一の核酸断片が対である５’タグおよび３’タグを含有するある一部の実施形態において、微粒子に結合された核酸の集団の１つが５’タグの少なくとも一部分を含み、微粒子に結合された核酸の集団の１つが３’タグの少なくとも一部分を含む。好ましい実施形態において、該集団の１つが完全な５’タグを含み、該集団の１つが完全な３’タグを含む。

【0028】

該核酸断片は多数のＰＢＲを含有するものであり、その少なくとも１つがタグ間に位置し、その少なくとも２つが、該核酸断片の該タグを含有する一部分と隣接し、その結果、５’タグの少なくとも一部分を含む領域が増幅され得、３’タグの少なくとも一部分を含む領域が増幅され得、２つの異なる核酸集団がもたらされる。好ましい実施形態において、５’タグ全体および３’タグ全体が増幅され得る。例えば、該核酸断片は、５’タグと隣接する第１および第２のプライマー結合部位、さらに３’タグと隣接する第３および第４のプライマー結合部位を含有するものであり得る。第１および第２のプライマー結合部位に結合するプライマーを用いるＰＣＲ増幅により、５’タグが増幅される。第３および第４のプライマー結合部位に結合するプライマーを用いるＰＣＲ増幅により、３’タグが増幅される。プライマーは、各プライマーからの伸長により、増幅されるタグを含有するＤＮＡ断片の領域に向かって進行するように選択されるのがよいことは認識されよう。あるいはまた、第１のプライマー結合部位を一方のタグの上流に配置させ得、第２のプライマー結合部位を他方のタグの下流に配置させ得、第３のプライマー結合部位を、この２つのタグ間に配置させ得る。第３のプライマー結合部位は、一方のタグを増幅させるＰＣＲ増幅のためのフォワードプライマーの結合部位としての機能を果たし、他方のタグを増幅させるＰＣＲ増幅のためのリバースプライマー結合部位としての機能を果たす。したがって、一実施形態において、本発明は、自身に結合された核酸の少なくとも２つの異なる集団を有する微粒子（例えば、ビーズ）であって、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、第１の異なる集団が５’タグを含み、第２の異なる集団が３’タグを含む微粒子（例えば、ビーズ）を提供する。

【0029】

本発明は、さらに、個々の微粒子が自身に結合された核酸の少なくとも２つの異なる集団を有し、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、該集団が単一のＤＮＡ断片からの増幅（例えば、ＰＣＲ増幅）によって生成されたものである微粒子（例えば、ビーズ）の集団を提供する。実質的に同一の集団は、例えば、５’タグと３’タグであり得る。本発明は、さらに、かかる微粒子のアレイ、および実質的に同一の核酸の集団の配列決定を伴う配列決定法を提供する。例えば、一実施形態において、個々

の微粒子に結合された２つの実質的に同一の核酸の集団の各々が異なるプライマー結合領域（ＰＢＲ）を含み、その結果、異なる配列決定プライマーを用いることにより、一方の集団は、他方の集団から障害されることなく配列決定され得る。実質的に同一の核酸の２つより多くの実質的に同一の集団が単一の微粒子に結合されている場合、該集団の各々が特有のＰＢＲ有し得、その結果、所与のＰＢＲに結合するプライマーは、微粒子に結合された核酸の他方の実質的に同一の集団内に存在するＰＢＲに結合しない。したがって、本発明の方法は、自身に結合された核酸の少なくとも２つの異なる実質的に同一の集団（例えば、５’タグを含有する多コピーの鋳型と３’タグを含有する多コピーの鋳型）を有する微粒子であって、タグが対のタグである微粒子の作製を可能にする。本発明の方法によれば、鋳型は、配列決定プライマーの結合部位を提供する異なるＰＢＲを含むものである。したがって、５’タグを含有する鋳型内のＰＢＲに相補的な配列決定プライマーを選択することにより、配列情報は５’タグから、３’タグを含有する鋳型もまた同じ微粒子上に存在していても３’タグを含有する鋳型から障害されることなく得られ得る。３’タグを含有する鋳型内のＰＢＲに相補的な配列決定プライマーを選択することにより、配列情報は３’タグから、５’タグを含有する鋳型もまた同じ微粒子上に存在していても５’タグを含有する鋳型から障害されることなく得られ得る。対のタグの両方が同じ微粒子上に存在するという事実は、先行技術において単一の鋳型内に存在した場合に起こり得るように、５’および３’の対のタグの配列が互いに会合し得ることを意味する。

【0030】

基材に付着した微粒子のアレイも提供される。一実施形態において、微粒子は、一方の末端が微粒子に付着し、他方の末端が基材に付着した一本鎖鋳型によって基材に繋がれる。一端または両端における付着手段は、共有結合的である場合もあれば、非共有結合的である場合もある。ある実施形態において、一端または両端の付着手段は、ビオチン結合部分およびビオチンを含む。

【0031】

また、微粒子に付着した鋳型を複製し、場合によりその複製した鋳型を増幅させることによって作成される核酸コロニーを含むアレイも提供される。さらにまた、ブロッキングオリゴヌクレオチドとその使用方法、ならびにブロッキングオリゴヌクレオチドを含む組成物も提供される。

【0032】

本発明はまた、例えば、実質的に平面状の支持体内または該支持体上のアレイ状の鋳型を配列決定するために使用され得る自動配列決定システムを提供する。本発明は、さらに、コンピュータ可読媒体、例えば、ハードディスク、ＣＤ、ジップディスク、フュラッシュメモリーなど上に保存され得る画像処理方法を提供する。ある特定の実施形態において、該システムは、１秒当たり４０，０００個のヌクレオチド以上の同定を達成する。ある特定の好ましい実施形態において、該システムは、１日（２４時間）あたり、８．６ギガバイト（Ｇｂ）以上の配列データをもたらす。ある特定の好ましい実施形態において、該システムは、１日あたり、４８Ｇｂ以上の配列情報（ヌクレオチド同定）をもたらす。

【0033】

また、本発明は、本発明の配列決定法を適用することによってもたらされる情報を保存するコンピュータ可読媒体を提供する。情報は、データベースに保存してもよい。

【0034】

本出願書類において、種々の特許、特許出願、学術論文および他の刊行物に言及するが、これらはすべて、引用により本明細書に組み込まれる。また、以下の標準的な参考文献の研究：Ｃｕｒｒｅｎｔ　Ｐｒｏｔｏｃｏｌｓ　ｉｎ　Ｍｏｌｅｃｕｌａｒ　Ｂｉｏｌｏｇｙ，　Ｊｏｈｎ　Ｗｉｌｅｙ　＆　Ｓｏｎｓ，　Ｎ．Ｙ．，編集２００２年７月；Ｓａｍｂｒｏｏｋ，　ＲｕｓｓｅｌｌおよびＳａｍｂｒｏｏｋ，　Ｍｏｌｅｃｕｌａｒ　Ｃｌｏｎｉｎｇ：Ａ　Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ，第3版，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ　Ｌａｂｏｒａｔｏｒｙ　Ｐｒｅｓｓ，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ，２００１は、引用により本明細書に組み込まれる。本明細書と引用により

組み込まれる任意の文献とで矛盾する場合は、本明細書が支配するものとし、本発明者らの裁量に矛盾または不一致が存在するか否かの決定は、任意の時点で行なわれ得ることを理解されたい。

【0035】

図１～３５の多くは、米国特許出願第１１／３４５，９７９号ではカラーとなっており、これらの図が参考として本明細書で援用され、本明細書に示す図の代わりに使用できることに留意されたい。

【発明を実施するための最良の形態】

【0036】

定義

本発明の記載の理解を助長するために、以下に定義を示す。一般に、特に定義のない用語は、当該技術分野で一般的に認められている意味（１つまたは複数）を示すものとすることを理解されたい。

【0037】

本明細書で用いる場合、「無塩基残基」は、窒素含有塩基の除去または窒素含有塩基の充分な部分の除去後に残留し、得られた分子が、もはや、ヌクレオシドまたはヌクレオチドに特徴的な水素結合に関与しないようなヌクレオシドまたはヌクレオチドの一部分の構造を有する残基である。無塩基残基は、窒素含有塩基をヌクレオシドまたはヌクレオチドから除去することにより生成させ得る。しかしながら、用語「無塩基」は、該残基の構造的特徴をいうために使用され、残基が生成される様式とは無関係である。用語「無塩基残基」および「無塩基部位」は、本明細書において、プリンまたはピリミジン塩基がない核酸内の残基をいうために使用される。

【0038】

「無プリン（ａｐｕｒｉｎｉｃ）／無ピリミジン（ａｐｙｒｉｍｉｄｉｎｉｃ）（ＡＰ）エンドヌクレアーゼ」は、本明細書で用いる場合、ポリヌクレオチド内の無塩基残基の５’側、３’側または５’および３’側両方いずれかの結合を切断する酵素をいう。本発明のある特定の実施形態において、ＡＰエンドヌクレアーゼは、ＡＰリアーゼである。ＡＰエンドヌクレアーゼの例としては、限定されないが、大腸菌エンドヌクレアーゼＶＩＩＩおよびその類縁体ならびに大腸菌エンドヌクレアーゼＩＩＩおよびその類縁体が挙げられる。特定の酵素、例えば、大腸菌Ｅｎｄｏ　ＶＩＩＩ、Ｅｎｄｏ　Ｖなどのエンドヌクレアーゼに対する言及は、当該技術分野において類縁体であると、および損傷塩基の除去および／または無塩基残基もしくは他のトリガー残基を含有するＤＮＡの切断に関して類似した生化学的活性を有すると認められる他の種由来の類縁体を包含することが意図されることを理解されたい。

【0039】

本明細書で用いる場合、用語「アレイ」は、支持体マトリックス全体または該マトリックス内に分布された正体のコレクションをいい、好ましくは、個々の正体は、任意のさまざまな手法によってアレイの個別の特徴の同定を可能にするのに充分な距離で互いに間隔があいている。正体は、例えば、核酸分子、核酸分子のクローン性の集団、微粒子（任意選択で、自身に結合された核酸分子のクローン性の集団を有する）などであり得る。動詞で用いる場合、用語「アレイ」およびその変形体は、アレイを形成するため、例えば、正体を支持体マトリックス全体または該マトリックス内に分布させるための任意のプロセスをいう。

【0040】

「損傷塩基」は、Ａ、Ｇ、ＣまたはＴがＤＮＡグリコシラーゼによるＤＮＡからの除去のための基質となるような様式で異なるプリンまたはピリミジン塩基である。ウラシルは、本発明の目的のために損傷塩基とみなす。本発明のある一部の実施形態において、損傷塩基はヒポキサンチンである。

【0041】

ポリヌクレオチド集団うちの１つのポリヌクレオチド内の位置に関する「縮重している

」は、該位置を占めるヌクレオシドの一部を構成する塩基の正体が、該集団の種々のメンバー間で異なることを意味する。したがって、該集団は、その配列が縮重位置において異なる個々のメンバーを含む。用語「位置」は、一般的に、５’または３’末端に関してポリヌクレオチド内の各ヌクレオシドに割り当てられた数値をいう。例えば、伸長プローブの３’末端のヌクレオシドには１位が割り当てられ得る。したがって、構造３’－ＸＸＸＮＸＸＸＸ－５’の伸長プローブのプールにおいて、Ｎは４位である。４位は、該プールの異なるメンバーにおいてＮの正体が異なり得る場合、縮重しているとみなされる。また、該伸長プローブのプールはＮ位が縮重していると言える。位置は、任意のｋ個の異なる正体を有するヌクレオシドによって占められ得る場合、ｋ重に縮重していると言える。例えば、２つの異なる塩基のいずれかを含むヌクレオシドによって占められ得る位置は、２重に縮重している。

【0042】

「配列に関する情報を調べること」は「配列決定」を包含し、また、他のレベルの情報、例えば、配列に関する１つ以上の可能性の排除なども包含する。ポリヌクレオチドにおいて配列決定を行なうことにより、典型的には、完璧に相補的（１００％相補的）なポリヌクレオチドの配列に関して等価な情報がもたらされる。したがって、完璧に相補的なポリヌクレオチドにおいて直接行なわれる配列決定と等価である。

【0043】

複数の要素、例えば、オリゴヌクレオチドプローブ分子またはその部分内のヌクレオシドに関する「独立して」は、各要素の正体が、任意の他方の要素の正体を制限しないこと、およびそれらに制限されないことを意味し、例えば、各要素の正体は、任意の他方の要素（１つまたは複数）の正体とは無関係に選択される。したがって、１つ以上の要素の正体がわかることは、任意のその他の要素の正体に関する任意の情報を提供することにはならない。例えば、配列ＮＮＮＮ内のヌクレオシドは、各Ｎの正体が任意の他のＮの正体とは無関係にＡ、Ｇ、ＣまたはＴであり得る場合、独立している。

【0044】

「ライゲーション」は、鋳型駆動型反応において、２つ以上の核酸、例えば、オリゴヌクレオチドおよび／またはポリヌクレオチドの末端間に、共有結合または連結を形成することを意味する。結合または連結の性質は広く異なり得、ライゲーションは、酵素的または化学的に行なわれ得る。

【0045】

用語「微粒子」は、本明細書において、５０ミクロン以下、好ましくは１０ミクロン以下の最小の断面寸法を有する粒子をいうために用いる。ある特定の実施形態において、最小断面寸法は、およそ３ミクロン以下、およそ１ミクロン以下、およそ０．５ミクロン以下、例えば、およそ０．１、０．２、０．３または０．４ミクロンである。微粒子は、さまざまな無機または有機材料、例えば、限定されないが、ガラス（例えば、細孔性ガラス）、シリカ、ジルコニア、架橋ポリスチレン、ポリアクリレート、ポリメチルメタクリレート、二酸化チタン、ラテックス、ポリスチレンなど作製されたものであり得る。例えば、種々の適当な材料および他の考慮事項については、米国特許第６，４０６，８４８号を参照のこと。Ｄｙｎａビーズ（Ｄｙｎａｌ（オスロ、ノルウェー）から入手可能）は、本発明に有用な市販の微粒子の一例である。磁気応答性微粒子も使用され得る。ある種の好ましい微粒子磁気応答性により、増幅後の微粒子結合鋳型の容易な回収および濃縮が可能になり、さらなる工程（例えば、洗浄、試薬除去など）が容易になる。本発明のある特定の実施形態において、異なる形状（例えば、いくらか球状および他の非球状のもの）を有する微粒子の集団
が使用される。

【0046】

用語「ミクロスフィア」または「ビーズ」は、本明細書において、５０ミクロン以下、好ましくは１０ミクロン以下の直径を有する実質的に球状の微粒子をいうために用いる。ある特定の実施形態において、直径は、およそ３ミクロン以下、およそ１ミクロン以下、

およそ０．５ミクロン以下、例えば、およそ０．１、０．２、０．３、または０．４ミクロンである。本発明のある特定の実施形態において、単分散性ミクロスフィアの集団が使用される、すなわち、ミクロスフィアは、実質的に均一な大きさのものである。例えば、微粒子の直径は、５％未満、例えば２％以下、１％以下などの変動係数を有し得る。しかしながら、他の実施形態において、微粒子集団の変動係数は、５％以上、例えば、５％、５％～１０％（両端を含む）、１０％～２５％（両端を含む）などである。ある特定の実施形態において、微粒子の混合集団が使用される。例えば、各々が５％未満の変動係数を有する２つの集団の混合物を用い、単分散性でない混合集団がもたらされ得る。一例として、１ミクロンおよび３ミクロンの直径を有するミクロスフィアの混合物が使用され得る。本発明のある特定の実施形態において、配列決定が、単分散性でない集団のミクロスフィアに結合された鋳型を用いて行なわれる場合、さらなる情報がミクロスフィアの大きさによって提供される。例えば、鋳型の異なるライブラリーを、異なる大きさのミクロスフィアに結合させ得る。また、より少数の鋳型分子をより小さい粒子に結合させ得るため、シグナル強度は異なり得、これにより多重配列決定が容易になり得る。

【0047】

用語「核酸配列」は、本明細書で用いる場合、核酸材料それ自体をいうことがあり得る。特定の核酸、例えば、ＤＮＡまたはＲＮＡ分子を生化学的にキャラクタライズする配列情報（すなわち５つの塩基の文字Ａ、Ｇ、Ｃ、ＴまたはＵから選択される文字の連続）に限定されない。本明細書に示す核酸は、特に記載のない限り、５’→３’方向で示す。

【0048】

「ヌクレオシド」は、糖分子に連結された窒素含有塩基を含む。本明細書で用いる場合、該用語は、その２’－デオキシおよび２’－ヒドロキシル形態において天然のヌクレオシド（ＫｏｒｎｂｅｒｇおよびＢａｋｅｒ，　ＤＮＡ　Ｒｅｐｌｉｃａｔｉｏｎ，　第２版．（Ｆｒｅｅｍａｎ，　Ｓａｎ　Ｆｒａｎｃｉｓｃｏ，１９９２）に記載のものなど）ならびにヌクレオシド類縁体を含む。例えば、天然ヌクレオシドとしては、アデノシン、チミジン、グアノシン、シチジン、ウリジン、デオキシアデノシン、デオキシチミジン、デオキシグアノシンおよびデオキシシチジンが挙げられる。ヌクレオシド「類縁体」は、修飾された塩基部分および／または修飾された糖部分を有する合成ヌクレオシドをいい、例えば、Ｓｃｈｅｉｔ，　ヌクレオチドＡｎａｌｏｇｓ（Ｊｏｈｎ　Ｗｉｌｅｙ，　Ｎｅｗ　Ｙｏｒｋ，　１９８０）に一般的に記載されている。かかる類縁体としては、結合特性の増強、縮重の低減、特異性の増大などのために設計された合成ヌクレオシドが挙げられる。ヌクレオシド類縁体としては、２－アミノアデノシン、２－チオチミジン、ピロロ－ピリミジン、３－メチルアデノシン、Ｃ５－プロピニルシチジン、Ｃ５－プロピニルウリジン、Ｃ５－ブロモウリジン、Ｃ５－フルオロウリジン、Ｃ５－ヨードウリジン、Ｃ５－メチルシチジン、７－デアザアデノシン、７－デアザグアノシン、８－オキソアデノシン、８－オキソグアノシン、Ｏ（６）－メチルグアニン、２－チオシチジンなどが挙げられる。ヌクレオシド類縁体は、本明細書に記載する任意の普遍塩基を含むものであり得る。

【0049】

用語「生物体」は、本明細書において、複製可能であり、配列決定の対象となる核酸を含む、任意の生体的（ｌｉｖｉｎｇ）または非生体的正体を示すために用いる。これには、プラスミド；ウイルス；原核生物、古細菌および真核生物の細胞、細胞株、真菌、原生動物、植物、動物などが含まれる。

【0050】

プローブおよび鋳型ポリヌクレオチドの突出した鎖に関する「完璧にマッチングした二本鎖」は、一方の突出した鎖が他方と二本鎖構造を、該二本鎖構造内の各ヌクレオシドが、対向する鎖上のヌクレオシドとワトソン－クリック塩基対合を行なうように形成することを意味する。該用語はまた、ヌクレオシド類縁体の対合（例えば、デオキシイノシン、ヌクレオシドと２－アミノプリン塩基となど）を包含し、これは、プローブの縮重を低減させるために使用され得る（かかる対合が水素結合の形成を伴うものであってもそうでな

くても）。
【0051】
用語「複数」は、１つより多いことを意味する。
【0052】
用語「多型」は、当該技術分野におけるその通常の意味で示され、同じ種の個体間でのゲノム配列における差をいう。「一塩基多型」（ＳＮＰ）は、単一の位置における多型をいう。
【0053】
「ポリヌクレオチド」、「核酸」または「オリゴヌクレオチド」は、ヌクレオシド間結合によって連結されたヌクレオシド（デオキシリボヌクレオシド、リボヌクレオシドまたはその類縁体を含む）の線状ポリマーをいう。典型的には、ポリヌクレオチドは少なくとも３個のヌクレオシドを含む。本発明のある特定の実施形態において、伸長プローブ内の１個以上のヌクレオシドは普遍塩基を含む。通常、オリゴヌクレオチドは、大きさが数個のモノマー単位（例えば、３～４個）から数百個のモノマー単位の範囲に及ぶ。オリゴヌクレオチドなどのポリヌクレオチドが「ＡＴＧＣＣＴＧ」などの一列の文字で表される場合はいつでも、特に記載のない限り、ヌクレオチドは、左から右に向かって５’→３’の順であること、および「Ａ」はデオキシアデノシンを表し、「Ｃ」はデオキシシチジンを表し、「Ｇ」はデオキシグアノシンを表し、「Ｔ」はチミジンを表すことは理解されよう。Ａ、Ｃ、ＧおよびＴの文字は、当該技術分野において標準的であるように、塩基自体、ヌクレオシドまたは該塩基を含むヌクレオチドをいうために使用され得る。
【0054】
天然に存在するポリヌクレオチドでは、ヌクレオシド間結合は、典型的にはホスホジエステル結合であり、サブユニットは「ヌクレオチド」とよばれる。しかしながら、ホスホロチオレート結合などの他のヌクレオシド間結合を含むオリゴヌクレオチドプローブが、本発明のある特定の実施形態において使用される。非ホスホジエステル結合を有するかかるオリゴヌクレオチドプローブを構成するサブユニットの１つ以上は、リン酸基を含むものであり得ることは認識されよう。ヌクレオチドのかかる類縁体は、本明細書で用いる用語「ヌクレオチド」の範囲に含まれるとみなし、ホスホジエステル結合でない１個以上のヌクレオシド間結合を含む核酸もやはり、「ポリヌクレオチド」、「オリゴヌクレオチド」などとよばれる。他の実施形態において、オリゴヌクレオチドプローブなどのポリヌクレオチドは、ＡＰエンドヌクレアーゼ感受性部位を含有する連結部を含む。例えば、オリゴヌクレオチドプローブは、無塩基残基、ＤＮＡグリコシラーゼによる除去のための基質である損傷塩基を含有する残基、またはＡＰエンドヌクレアーゼによる切断のための基質である別の残基または連結部を含有するものであり得る。別の実施形態において、オリゴヌクレオチドプローブは、二糖ヌクレオシドを含有するものである。
【0055】
用語「プライマー」は、短鎖ポリヌクレオチドをいい、典型的には、約１０～１００ヌクレオチド長であり、標的ポリヌクレオチド、すなわち「鋳型」に、該標的とハイブリダイズすることにより結合するものである。プライマーは、好ましくは、標的に相補的なポリヌクレオチドの鋳型指向型合成（これは、適切な酵素（１種類または複数種）、補因子、基質、例えば、ヌクレオチド、オリゴヌクレオチドなどの存在下で行なわれ得る）の開始点を提供するものである。プライマーは、典型的には、伸長が行なわれ得る起点となる末端を提供する。ＤＮＡポリメラーゼなどのポリメラーゼ酵素によって触媒される合成のためのプライマーの場合（例えば、「合成による配列決定」、ポリメラーゼ連鎖反応（ＰＣＲ）　増幅などにおいて）、プライマーは、典型的には、遊離３’ＯＨ基を有するものであるか、またはこれを有するように修飾されたものであり得る。典型的には、ＰＣＲ反応では、増幅される領域の境界を定める１対のプライマー（第１および第２の増幅プライマー）、例えば、「上流」（または「フォワード」）プライマーおよび「下流」（または「リバース」）　プライマーが使用される。伸長、ライゲーション（および任意選択で、切断）の連続的サイクルによる合成のためのプライマーの場合、プライマー典型的には、

ＤＮＡリガーゼの基質としての機能を果たす遊離５’リン酸基または３’ＯＨ基を有するものであるか、またはこれらを有するように修飾されたものであり得る。

【0056】

本明細書で用いる場合、「プローブファミリー」は、その各々が同じ標識を含む一群のプローブをいう。

【0057】

本明細書で用いる場合、ポリヌクレオチドに関する「配列の決定）」、「ヌクレオチド配列を決定すること」、「配列決定」などの用語は、ポリヌクレオチドの部分配列ならびに完全な配列の情報の決定を含む。すなわち、該用語は、配列の比較、フィンガープリンティングなどのレベルの標的ポリヌクレオチドに関する情報、ならびに同定という表示（ｅｘｐｒｅｓｓ）および目的の領域内の標的ポリヌクレオチド各ヌクレオシドの順序を含む。本発明のある特定の実施形態において、「配列の決定」は、単一のヌクレオチドを同定することを含み、一方、他の実施形態においては、１個より多くのヌクレオチドが同定される。本発明のある特定の実施形態において、それ自体では１サイクルにおいて任意のヌクレオチドを同定するのに不充分な配列情報が収集される。ヌクレオシド、ヌクレオチドおよび／または塩基の同定は、本明細書において等価とみなす。ポリヌクレオチドにおいて配列決定を行なうことにより、典型的には、完璧に相補的（１００％相補的）なポリヌクレオチドの配列に関して等価な情報がもたらされ、したがって、完璧に相補的なポリヌクレオチドにおいて直接行なわれる配列決定と等価であることに注意されたい。

【0058】

「配列決定反応」は、本明細書で用いる場合、伸長、ライゲーションおよび検出の１組のサイクルをいう。伸長された二本鎖が鋳型から除去され、第２の組のサイクルが該鋳型において行なわれる場合、各組みのサイクルは、独立した配列決定反応とみなすが、得られる配列情報を組み合わせて単一の配列を生成させ得る。

【0059】

「半固形」は、本明細書で用いる場合、固形および液状の両方の成分を有し、液状成分が固形マトリックス要素間の細孔、空間または他の隙間を占めている圧縮性のマトリックスをいう。例示的な半固形マトリックスとしては、ポリアクリルアミド、セルロース、ポリアミド（ナイロン）、および架橋アガロース、デキストランおよびポリエチレングリコールで構成されたマトリックスが挙げられる。半固相支持体は、該半固相支持体を支持する第２の支持体、例えば、実質的に平面状で剛性の支持体（基材ともいう）上に設けられたものであってもよい。

【0060】

「支持体」は、本明細書で用いる場合、その上面または内部に核酸分子、微粒子などが固定化され得るマトリックス、すなわち、これらが互いに対して自由に拡散または移動することが広くまたは全体的に妨げられるように、これらが共有結合または非共有結合され得るか、またはその内部もしくは上面にこれらが部分的または完全に包埋され得るマトリックスをいう。

【0061】

「トリガー残基」は、核酸内に存在する場合、それ以外は同一であるトリガー残基を含有しない核酸のおそらくの場合よりも、該核酸を、切断剤（例えば、酵素、硝酸銀など）または薬剤の組合せによる切断（例えば、核酸主鎖の切断）に対してより感受性にする残基、および／または該核酸をかかる切断に対してより感受性にする残基を生成させる修飾に対して感受性である残基である。したがって、核酸内のトリガー残基の存在は、該核酸内に切れやすい連結の存在をもたらし得る。例えば、無塩基残基は、核酸内の無塩基残基の存在によって該核酸がＡＰエンドヌクレアーゼなどの酵素による切断に対して感受性となるため、トリガー残基である。損傷塩基を含有するヌクレオシドは、核酸内に損傷塩基を含むヌクレオシドの存在によってもまた、例えば、ＤＮＡグリコシラーゼによる損傷塩基の除去後、該核酸がＡＰエンドヌクレアーゼなどの酵素による切断に対してより感受性となるため、トリガー残基である。切断部位は、トリガー残基と隣接残基間の結合部分で

あり得るか、またはトリガー残基から除去される１つ以上の残基である結合部分であり得る。例えば、デオキシイノシンは、核酸内のデオキシイノシンの存在によって、該核酸が大腸菌エンドヌクレアーゼＶおよびその類縁体による切断に対してより感受性となるため、トリガー残基である。かかる酵素は、デオキシイノシンに対して３’側の第２のホスホジエステル結合を切断する。本明細書に開示された任意のプローブは、１つ以上のトリガー残基を含有し得る。トリガー残基は、リボースまたはデオキシリボース部分を含むものであり得るが、そうである必要はない。好ましくは、切断剤は、トリガー残基の非存在下では実質的に核酸を切断しないが、同じ条件下（該条件は、該核酸が切断剤に対して感受性となるように修正する薬剤の存在を含み得る）で、トリガー残基を含有する核酸に対して有意な切断活性を示すものである。例えば、好ましくは、切断剤が一方がトリガー残基を含有し、他方がトリガー残基を含有しない以外は長さと組成が同一である核酸を含有する組成物内に存在する場合、トリガー残基を含有する核酸が切断される尤度は、少なくとも：１０；２５；５０；１００；２５０；５００；１０００；２５００；５０００；１０，０００；２５，０００；５０，０００；１００，０００；２５０，０００；５００，０００；１，０００，０００またはそれより大きく、トリガー残基を含有しない核酸が切断される尤度と同定度に大きく、例えば、トリガー残基を含有する核酸の切断の尤度の、トリガー残基を含有しないがそれ以外は同一である核酸の切断の尤度に対する比は１０～10^6または任意のその整数（ｉｎｔｅｇｒａｌ）部分的範囲である。この比は、トリガー残基の具体的な核酸および位置およびヌクレオチド状況に応じて異なり得ることは認識されよう。

【0062】

好ましくは、トリガー残基を含有する核酸が、該核酸を切断剤による切断に対して感受性とするために修飾される必要がある場合、かかる修飾は、適当な修飾剤（１種類または複数種）の存在下で、容易に行なわれ、例えば、該修飾は、適度な収率および適度な時間行なわれる。例えば、本発明のある特定の実施形態において、トリガー残基を含有する核酸の少なくとも５０％、少なくとも６０％、少なくとも７０％、好ましくは少なくとも８０％、少なくとも９０％またはより好ましくは少なくとも９５％が、例えば、２４時間以内に、好ましくは１２時間以内、より好ましくは１分未満～４時間以内に修飾される。

【0063】

さまざまな適当なトリガー残基および対応する切断試薬を本明細書において例示する。本明細書に記載のものと類似した活性を有する任意のトリガー残基および切断試薬が使用され得る。当業者なら、ある特定のトリガー残基および切断試薬組合せが本発明における使用に適しているか否か、例えば、切断の効率および速度、トリガー残基を含有する核酸の切断剤の選択性などが本発明における使用に適しているか否かを判定することができよう。「トリガー残基」は、単に制限酵素部位の一部を構成するヌクレオチドとは、トリガー残基が切断に対する感受性の増大を与える能力は、一般的に、トリガー残基が見出される特定の配列状況に有意に依存しないが、上記のように、該状況は修飾および／または切断に対する感受性に対し、ある程度の影響を及ぼし得るという点において区別されることに注意されたい。もちろん、その周囲のヌクレオチドに応じて、トリガー残基は、制限部位の一部を構成し得る。したがって、ほとんどの場合、切断剤は制限酵素ではないが、制限酵素であることと、非配列特異的切断可能性を有することの両方である酵素の使用は除外されない。

【0064】

「普遍塩基」は、本明細書で用いる場合、天然に存在する核酸内に典型的に見られる塩基の１つより多くと「対合し」得、したがって、二本鎖内のかかる天然に存在する塩基に代用され得る塩基である。該塩基は、天然に存在する塩基の各々と対合できるものである必要はない。例えば、ある特定の塩基は、プリンのみと、もしくはこれと選択的に、またはピリミジンのみと、もしくはこれと選択的に対合する。ある特定の好ましい普遍塩基（完全普遍塩基）は、天然に存在する核酸内に典型的に見られる任意の塩基と対合し得るものであり、したがって、二本鎖内の任意のこれらの塩基に代用され得る塩基である。該塩

基は、天然に存在する塩基の各々と等しく対合できるものである必要はない。プローブミックスが、天然に存在するヌクレオチドのすべてとは対合しない普遍塩基を（１つ以上の位置に）含むプローブを含有する場合、該普遍塩基の少なくとも１つがＡと対合するか、該普遍塩基の少なくとも１つがＧと対合するか、該普遍塩基の少なくとも１つがＣと対合するか、該普遍塩基の少なくとも１つがＴと対合するように、その特定のプローブ内の該位置に２つ以上の普遍塩基を用いることが望ましいことがあり得る。

【0065】

いくつかの普遍塩基が、当該技術分野で知られており、限定されないが、ヒポキサンチン、３－ニトロピロール、４－ニトロインドール、５－ニトロインドール、４－ニトロベンズイミダゾール、５－ニトロインダゾール、８－アザ－７－デアザアデニン、６Ｈ，８Ｈ－３，４－ジヒドロピリミド［４，５－ｃ］［１，２］オキサジン－７－オン（Ｐ．Ｋｏｎｇ　Ｔｈｏｏ　Ｌｉｎ．およびＤ．Ｍ．Ｂｒｏｗｎ，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，１９８９，　１７，１０３７３－１０３８３）、２－アミノ－６－メトキシアミノプリン（Ｄ．Ｍ．ＢｒｏｗｎおよびＰ．Ｋｏｎｇ　Ｔｈｏｏ　Ｌｉｎ，　Ｃａｒｂｏｈｙｄｒａｔｅ　Ｒｅｓｅａｒｃｈ，１９９１，２１６，１２９－１３９）などが挙げられる。ヒポキサンチンは、好ましい完全普遍塩基の一例である。ヒポキサンチンを含むヌクレオシドとしては、限定されないが、イノシン、イソイノシン、２’－デオキシイノシンおよび７－デアザ－２’－デオキシイノシン、２－アザ－２’デオキシイノシンが挙げられる。

【0066】

さらなる普遍塩基が当該技術分野で知られており、例えば、Ｌｏａｋｅｓ，　Ｄ．およびＢｒｏｗｎ，　Ｄ．Ｍ．，　Ｎｕｃｌ．Ａｃｉｄｓ　Ｒｅｓ．２２：４０３９－４０４３，　１９９４；Ｏｈｔｓｕｋａ，　Ｅ．ら、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．　２６０（５）：２６０５－２６０８，１９８５；Ｌｉｎ，　Ｐ．Ｋ．Ｔ．およびＢｒｏｗｎ，　Ｄ．Ｍ．，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．２０（１９）：５１４９－５１５２，１９９２；Ｎｉｃｈｏｌｓ，　Ｒ．ら、Ｎａｔｕｒｅ　３６９（６４８０）：４９２－４９３，１９９４；Ｒａｈｍｏｎ，　Ｍ．Ｓ．およびＨｕｍａｙｕｎ，　Ｎ．Ｚ．，　Ｍｕｔａｔｉｏｎ　Ｒｅｓｅａｒｃｈ　３７７（２）：２６３－８，１９９７；Ｂｅｒｇｅｒ，　Ｍ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓｅａｒｃｈ，　２８（１５）：２９１１－２９１４，２０００；Ａｍｏｓｏｖａ，　Ｏ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．２５（１０）：１９３０－１９３４，　１９９７；ならびにＬｏａｋｅｓ，　Ｄ．，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．２９（１２）：２４３７－４７，２００１の関連部分に記載されている。普遍塩基は、対向する位置にある塩基と水素結合を形成し得るが、そうである必要はない。普遍塩基は、ワトソン－クリックまたは非ワトソン－クリック相互作用（例えば、フーグスティーン相互作用）により水素結合を形成し得る。

【0067】

本発明のある特定の実施形態において、普遍塩基を含むオリゴヌクレオチドプローブを使用するのではなく、無塩基残基を含むオリゴヌクレオチドプローブが使用される。無塩基残基は、任意の４つの天然に存在するヌクレオチドと対向する位置を占めることができ、したがって、普遍塩基を含むヌクレオチドと同じ機能を果たし得る。本発明のある一部の実施形態において、無塩基残基に隣接する連結部は、ＡＰエンドヌクレアーゼによって切断されるが、無塩基残基はまた、他の切れやすい連結（例えば、ホスホロチオレート）が存在し、他の切断試薬が使用される実施形態においても、本明細書に記載のように有用である（すなわち、普遍塩基の機能を果たすため）。

【0068】

本発明のある特定の実施形態の詳細な説明

Ａ．伸長、ライゲーションおよび切断の連続的サイクルによる配列決定

本発明の一態様の全体的なスキームを、図１Ａに図式的に示すが、これは、一般的に、米国特許第５，７４０，３４１号および同第６，３０６，５９７号（ともにＭａｃｅｖｉ

ｃｚに対して発行）に教示された方法と類似する。便宜上の目的のため、これらの特許を本明細書において集合的に「Ｍａｃｅｖｉｃｚ」という。特に、Ｍａｃｅｖｉｃｚには、ポリヌクレオチド内のヌクレオチドの配列を同定するための方法が教示されており、該方法は、（ａ）　プローブを自身にライゲートさせ、伸長された二本鎖を形成することにより、初期オリゴヌクレオチドを、ポリヌクレオチドオリゴヌクレオチドに沿って伸長させる工程；（ｂ）　該ポリヌクレオチドの１つ以上のヌクレオチドを同定する工程；および（ｃ）　該ヌクレオチドの配列が決定されるまで工程（ａ）および（ｂ）を反復する工程を含む。

【0069】

Ｍａｃｅｖｉｃｚには、さらに、鋳型ポリヌクレオチド内のヌクレオチドの配列決定するための方法が教示されており、該方法は、（ａ）鋳型ポリヌクレオチドにハイブリダイズさせた初期オリゴヌクレオチドプローブを含むプローブ－鋳型二本鎖提供する工程（ここで、前記プローブは伸長可能なプローブ末端を有する）；（ｂ）伸長オリゴヌクレオチドプローブを前記伸長可能なプローブ末端にライゲートさせ、伸長されたオリゴヌクレオチドプローブを含む伸長された二本鎖を形成する工程；（ｃ）伸長された二本鎖において、（１）　ライゲートされたばかりの伸長プローブに相補的であるか、もしくは（２）伸長されたオリゴヌクレオチドプローブのすぐ下流にある鋳型ポリヌクレオチド内のヌクレオチド残基であるかのいずれかである鋳型ポリヌクレオチド内の少なくとも１つのヌクレオチドを同定する工程；（ｄ）もし伸長可能なプローブ末端が既に存在しない場合は、生成される末端が最後の伸長プローブがライゲートされた末端と異なるように、伸長可能なプローブ末端を伸長されたプローブ上に生成させる工程；ならびに（ｅ）標的ポリヌクレオチド内のヌクレオチドの配列が決定されるまで工程（ｂ）、（ｃ）および（ｄ）を反復する工程を含む。これらの方法のある特定の実施形態において、各伸長プローブは、連鎖停止部分を、初期オリゴヌクレオチドプローブに対して遠位の末端に有する。ある特定の実施形態において、再生させる工程は、伸長されたオリゴヌクレオチドプローブ内の化学的に切れやすいヌクレオシド間結合を切断することを含む。

【0070】

図１Ａを参照すると、未知配列のポリヌクレオチド領域５０と結合領域４０とを含むポリヌクレオチド鋳型２０は、支持体１０に結合されている。結合領域４０の遠位端のヌクレオチド４１およびポリヌクレオチド領域５０の近位端のヌクレオチド５１は、互いに隣接している。結合領域４０とハイブリダイズし、結合領域４０内のある位置に二本鎖を形成する初期オリゴヌクレオチド３０が提供される。初期オリゴヌクレオチド３０はまた、本明細書において「プライマー」ともいい、結合領域４０は、「プライマー結合領域」ということがある。二本鎖は、完璧にマッチングした二本鎖であり得るが、そうである必要はない。初期オリゴヌクレオチドは伸長可能な末端３１を有する。図１Ａにおいて、初期オリゴヌクレオチドは結合領域に、伸長可能な末端３１がヌクレオチド４１と反対の位置に位置するように結合する。しかしながら、初期オリゴヌクレオチドは、結合領域内の別の場所に結合し得、これは以下にさらに論考する。長さＮの伸長オリゴヌクレオチドプローブ６０は、初期オリゴヌクレオチドに隣接する鋳型にハイブリダイズされる。伸長オリゴヌクレオチドプローブの末端ヌクレオチド６１は、伸長可能な末端３１にライゲートされる。

【0071】

末端ヌクレオチド６１は、ポリヌクレオチド領域５０内の第１の未知ヌクレオチドに相補的である。したがって、末端ヌクレオチド６１の正体によりヌクレオチド５１の正体が特定される。好ましくは、ヌクレオチド５１は、末端ヌクレオチド６１としてＡ、Ｇ、ＣまたはＴを有することがわかっている伸長プローブと会合した標識（図示せず）検出することをにより同定される。標識は、検出後に除去される。図２は、異なる標識（例えば、異なる色のフルオロフォア）を、異なる３’末端ヌクレオチドを有する伸長プローブに割り当てるためのスキームを示す。

【0072】

ライゲーションおよび検出の後、もしプローブ６０が既にかかる末端を有しない場合は、伸長可能なプローブ末端を伸長プローブ６０上に生成させる。第２の伸長プローブ７０（好ましくは、これも長さＮである）を伸長プローブ６０に隣接する鋳型にアニーリングさせ、プローブ６０の伸長可能な末端にライゲートさせる。伸長プローブ７０の末端ヌクレオチド７１の正体により、対向する位置にあるポリヌクレオチド５０内のヌクレオチド５２の正体が特定される。したがって、末端ヌクレオチド７１は伸長プローブの「配列決定部分」を構成し、これは、プローブの該部分は、そのハイブリダイゼーション特異性が鋳型内の１つ以上のヌクレオチドの正体を決定する基準として利用されることを意味することは認識されよう。典型的には、伸長プローブ内のさらなるヌクレオチドが鋳型とハイブリダイズするが、プローブ内のその正体がある特定の標識と会合しているヌクレオチドのみが、鋳型内のヌクレオチドを同定するために利用される。

【0073】

本発明の好ましい実施形態において、伸長可能な末端の生成は、ヌクレオシド間結合の切断を伴い、これは以下にさらに記載する。好ましくは、切断により標識もまた除去される。切断により、いくつかのヌクレオチドＭが伸長プローブから除去される（図示せず）。したがって、二本鎖は、各サイクルにおいてＮ－Ｍヌクレオチドによって伸長され、鋳型内のＮ－Ｍの区間内に位置するヌクレオチドが同定される。多コピーの所与の鋳型は、典型的には単一の支持体に結合され、配列決定反応が、これらの鋳型において同時に行なわれることを理解されたい。

【0074】

Ｍａｃｅｖｉｃｚには、オリゴヌクレオチドプローブは、一般的に、初期オリゴヌクレオチドまたは伸長された二本鎖にライゲートされ、次の伸長サイクルの伸長された二本鎖を生成させ得るものであるべきであること；ライゲーションは、プローブがライゲーション前に鋳型と二本鎖を形成すべきであるという点で、鋳型駆動型べきであること；プローブは、単一の伸長サイクルにおける同じ鋳型での多重プローブライゲーションを抑制するためのブロッキング部分を有するものであるべきこと；プローブは、ライゲーション後に伸長可能な末端を再生させるために処理または修飾され得るものであるべきこと；およびプローブは、成功裏のライゲーション後に、鋳型に関する配列情報の取得を可能にするシグナル伝達部分（すなわち、検出可能な部分）　を有するものであるべきことが教示されている。

【0075】

Ｍａｃｅｖｉｃｚには、一部の特定の好適な初期オリゴヌクレオチド、伸長オリゴヌクレオチドプローブ、鋳型、結合部位の特性、およびかかる成分を剛性、設計、作製または入手するための種々の方法が教示されている。Ｍａｃｅｖｉｃｚには、さらに、一部の特定の好適なリガーゼ、ライゲーション条件およびさまざまな適当な標識が教示されている。Ｍａｃｅｖｉｃｚにはまた、標識された連鎖停止ヌクレオチドを新たにライゲートされた伸長プローブに付加させるためにポリメラーゼ伸長を用いる同定の代替方法が教示されている。付加されたヌクレオチドの正体により、反対の位置に位置する鋳型内のヌクレオチドが特定される。

【0076】

当業者には認識されるように、鋳型、初期オリゴヌクレオチド、伸長プローブ、プライマーなどに対する言及は、一般的に、単一の分子ではなく、関連する領域内で実質的に同一である核酸分子の集団またはプールを意味する。したがって、例えば、「鋳型」は、一般的に、複数の実質的に同一の鋳型分子を意味し、「プローブ」は、一般的に、複数の実質的に同一のプローブ分子を意味するなどである。１つ以上の位置で縮重しているプローブの場合、ある特定のプローブを含むプローブ分子の配列は、縮重位置において異なる、すなわち、ある特定のプローブを構成するプローブ分子の配列は、非縮重位置（１つまたは複数）においてのみ、実質的に同一であり得ることは認識されよう。記載の目的のため、単数形は、単一の分子群および実質的に同一の分子の集団を含むと理解されたい。単一の核酸分子（すなわち、１つの分子）に言及することが意図される場合、用語「鋳型分子

」、「プローブ分子」、「プライマー分子」などを用いる。ある特定の状況において、実質的に同一の核酸分子の一集団の複数の性質は、明白に表示する。

【0077】

実質的に同一の核酸分子の集団は、任意のさまざまな既知の方法、例えば、化学合成、細胞内での生物学的合成、１種類以上の出発核酸分子からの酵素的インビトロ増幅などを用いて入手および作製され得る。例えば、当該技術分野でよく知られた方法を用い、目的の核酸を、適当な発現ベクター（例えば、ＤＮＡまたはＲＮＡプラスミド）内に挿入し、次いで、細胞（例えば、細菌細胞）内に導入して複製させることによりクローニングされ得る。次いで、目的の核酸コピーを含有するプラスミドＤＮＡまたはＲＮＡを該細胞から単離する。ウイルス、細胞などから単離されたゲノムＤＮＡ、またはｍＲＮＡ）の逆転写により作製されたｃＤＮＡもまた、クローニングまたはインビトロ増幅の中間工程なしで、実質的に同一の核酸分子の集団（例えば、その配列が決定される鋳型ポリヌクレオチド）の供給源となり得るが、一般的には、かかる中間工程を行なうことが好ましい。

【0078】

集団のメンバーは、１００％同一である必要はなく、例えば、ある特定数の「エラー」が、合成過程中に起こり得ることは理解されよう。好ましくは、集団のメンバーの少なくとも５０％が、参照核酸分子（すなわち、配列比較のための基準として用いられる規定配列の分子）と少なくとも９０％、またはより好ましくは少なくとも９５％同一である。より好ましくは、集団のメンバーの少なくとも６０％、少なくとも７０％、少なくとも８０％、少なくとも９０％、少なくとも９５％、少なくとも９９％またはそれ以上が、参照核酸分子と少なくとも９０％、またはより好ましくは少なくとも９５％同一であるか、さらにより好ましくは少なくとも９９％同一である。好ましくは、参照核酸分子に対して少なくとも９５％またはより好ましくは少なくとも９９％の同一性割合の集団のメンバーは、少なくとも９８％、９９％、９９．９％またはそれ以上である。同一性割合は、２つの最適にアライメントされた配列を比較し、同一の核酸塩基（例えば、Ａ、Ｔ、Ｃ、Ｇ、ＵまたはＩ）が両方の配列に存在する位置の数を測定してマッチングした位置の数を得、マッチングした位置の数を位置の合計数で除算し、結果に１００を積算して配列の同一性割合を得ることによりコンピュータ計算され得る。ある特定の状況において、鋳型、プローブ、プライマーなどの核酸分子は、より大きな核酸分子（これはまた、鋳型、プローブまたはプライマーの機能を果たさない部分も含有する）の一部分であり得ることは認識されよう。その場合、個々の集団のメンバーは、該部分に関して実質的に同一である必要はない。

【0079】

Ｍａｃｅｖｉｃｚには、図１Ａに示すような、鋳型がビーズなどの支持体に結合され、伸長が、支持体の遠位に位置する鋳型の末端に向かって進行する方法が教示されている。したがって、結合領域は未知配列よりも支持体の近くに位置し、伸長された二本鎖は、支持体から離れる方法に成長する。しかしながら、本発明者らは、予期に反して、該方法は、結合領域が支持体の遠位に鋳型の末端に位置し、伸長が支持体に向かって内方に進行する別のアプローチを用いて好都合に行なわれ得ることを見出した。この実施形態を図１Ｂに示す。図において、種々の要素は図１Ａと同様に番号付けしている。本発明者らは、鋳型の遠位端から支持体に向かう「内方への」配列決定により、優れた結果が得られることを調べた。特に、鋳型の遠位端からビーズなどの支持体に向かう配列決定により、支持体から外方への配列決定よりも高いライゲーション効率がもたらされる。

【0080】

Ｍａｃｅｖｉｃｚによってさらに教示されているように、好ましくは、オリゴヌクレオチドプローブは鋳型に、所定の長さの可能な全ての配列のオリゴヌクレオチドを含む混合物として適用する。例えば、構造ＮＮＮＮＮＮ（これは、$(N)_k$（式中、ｋ＝６）とも表示され得る）の６ヌクレオチド長（六量体）の可能な全ての配列を含有するプローブの混合物は、4^6（４０９６）のプローブ種を含有し得る。一般的に、プローブは、構造Ｘ$(N)_k$Ｎ*（式中、Ｎは任意のヌクレオチドを表し、ｋは１～１００であり、＊は標識

を表し、Ｘは、その正体が標識に対応するヌクレオチドを表す）のものである。ある特定の実施形態において、ｋは、１～１００、１～５０、１～３０、１～２０、例えば、４～１０である。該ヌクレオチドの１つ以上は普遍塩基を含むものであり得る。一般的に、プローブは、Ｎで表される位置において４重に縮重しているか、またはＮで表される１つ以上の位置に縮重低減性ヌクレオチドを含む。所望の場合は、混合物を、プローブ（「ストリンジェンシー類型」）の部分集団に分けてもよい。それらの相補配列完璧にマッチングした二本鎖は、類似した安定性または結合自由エネルギーを有する。部分集団は、Ｍａｃｅｖｉｃｚに教示されているように、独立したハイブリダイゼーション反応に使用され得る。

【0081】

プローブ混合物の複雑度（ｃｏｍｐｌｅｘｉｔｙ）（すなわち、異なる配列の数）は、いくつかの方法によって、例えば、いわゆる縮重低減性のヌクレオチドまたはヌクレオチドアナログを用いることにより低減させ得る。例えば、８ヌクレオチドの可能な全ての配列を含有するプローブのライブラリーは、4^8のプローブを含み得る。プローブの数は、２つの位置に普遍塩基を用いることにより、例えば、長さなどの八量体ライブラリーの種々の望ましい特徴を維持したまま4^6に低減させ得る。本発明は、任意の上記または上記参考文献に記載の普遍塩基の使用を包含する。

【0082】

実施形態に応じて、伸長された二本鎖または初期オリゴヌクレオチドは、オリゴヌクレオチドプローブによって５’→３’方向または３’→５’方向のいずれかに伸長され得、これは以下にさらに記載する。一般的に、オリゴヌクレオチドプローブは、鋳型と完璧にマッチングした二本鎖を形成する必要はないが、かかる結合が好ましいことがあり得る。鋳型内の単一のヌクレオチドが各伸長サイクルで同定される実施形態では、完璧な塩基対合だけが、その特定のヌクレオチドを同定するために必要とされる。例えば、オリゴヌクレオチドプローブが伸長された二本鎖に酵素的にライゲートされる実施形態では、完璧な塩基対合、すなわち適正なワトソン－クリック塩基対合が、ライゲートされるプローブの末端ヌクレオチドと、鋳型内のその相補配列との間に必要とされる。一般的に、かかる実施形態では、プローブの残余ヌクレオチドは、次のライゲーションが鋳型に沿った所定の部位または塩基数で起こることを確実にする「スペーサー」としての機能を果たす。すなわち、その対合またはその欠如は、さらなる配列情報を提供しない。同様に、塩基同定をポリメラーゼ伸長に依存する実施形態では、プローブは、主に、スペーサーとしての機能を果たすため、鋳型への特異的ハイブリダイゼーションは不可欠ではない。

【0083】

上記の方法により、配列の部分決定、すなわち、鋳型内で互いに間隔があいている個々のヌクレオチドの同定が可能になる、本発明の好ましい実施形態において、より完全な情報を取得するため、各反応で異なる初期オリゴヌクレオチドｉが使用される複数の反応が行なわれる。初期オリゴヌクレオチドｉは、結合領域の異なる部分に結合する。好ましくは、初期オリゴヌクレオチドは、結合領域にハイブリダイズされるとき、異なる初期オリゴヌクレオチドのかかる伸長可能な末端が互いに１ヌクレオチドずれた位置に結合する。例えば、図３に示すように、配列決定反応１．．．Ｎが行なわれる。初期オリゴヌクレオチドi_i．．．i_nは同じ長さを有し、その末端ヌクレオチド３１、３２、３３などが、結合領域４０内の連続的な隣接する位置４１、４２、４３などにハイブリダイズするように結合する。したがって、伸長プローブe_1．．．e_nは、鋳型の連続的な隣接する領域に結合し、初期オリゴヌクレオチドの伸長可能な末端にライゲートされる。i_πにライゲートされたプローブe_nの末端ヌクレオチド６１は、ポリヌクレオチド領域５０のヌクレオチド５５、すなわち、鋳型内の第１の未知ポリヌクレオチドに相補的である。伸長、ライゲーションおよび検出の第２のサイクルでは、プローブe_{12}の末端ヌクレオチド７１は、ポリヌクレオチド領域５０のヌクレオチド５６、すなわち、未知配列の第２のヌクレオチドに相補的である。同様に、初期オリゴヌクレオチドi_2、i_3、i_4などにより初期化された二本鎖にライゲートされた伸長プローブの末端ヌクレオチドは、未知配列５０

の第３、第４および第５のヌクレオチドに相補的である。初期オリゴヌクレオチドは、ポリヌクレオチド領域５０に対し、進行的に近づくのではなく進行的にさらに離れる領域に結合され得ることは認識されよう。

【0084】

非伸長プローブの末端ヌクレオチドのスペーサー機能により、初期オリゴヌクレオチドが結合する位置から相当除去される鋳型内の位置の配列情報の取得が、相応する多数回のサイクルが任意の所与の鋳型において行なわれることを要することなく可能になる。例えば、長さＮのプローブのライゲーションの連続的サイクル後、単一の末端ヌクレオチドを伸長プローブから除去するための切断によって、Ｎ－１ヌクレオチド区間のヌクレオチドが連続回数で同定され得る。例えば、鋳型内の１、Ｎ、２Ｎ－１、３Ｎ－２、４Ｎ－３および５Ｎ－４位のヌクレオチドが、６サイクルで同定され得、この場合、鋳型内の１位のヌクレオチドは、鋳型への初期オリゴヌクレオチドの結合によって形成される二本鎖内の伸長可能なプローブ末端にライゲートされるヌクレオチドと反対のヌクレオチドである。同様に、切断により２つのヌクレオチドが長さＮの伸長プローブから除去される場合、互いにＮ－２ヌクレオチド離れた位置のヌクレオチドが連続回数で同定され得る。例えば、鋳型内の１、Ｎ－１、２Ｎ－３、３Ｎ－５、４Ｎ－７位のヌクレオチドが６サイクルで同定され得る。したがって、プローブが８ヌクレオチド長であり、２個のヌクレオチドが各サイクルで除去される場合、１位、７、１３、１９および２５位のヌクレオチドが同定される。したがって、鋳型内の第１のヌクレオチドからＸ離れたヌクレオチドを同定するのに必要とされるサイクル数は、およそＸではなく、およそＸ／Ｍ（式中、Ｍは、切断後に残存する伸長プローブの長さである）である。

【0085】

例えば、図３Ｂに示す概略は、鋳型を６塩基ごとに読み出されるように設計された伸長プローブを用いた伸長、ライゲーションおよび切断方法の使用の最終結果を示す。結合領域内でずれる位置に結合する初期化６ヌクレオチドを用いて鋳型を逐次ストリッピングおよび配列決定し、結果を組み合わせることにより、すべての鋳型塩基が、規定された長さにわたって解明される。例えば、１０回の逐次ライゲーションが、６回の反応の各々で行なわれる場合、得られる読出し長さは６０個の連続塩基対であるが、１５回の逐次ライゲーションが各反応で行なわれる場合、得られる読出し長さは９０個の連続塩基対である。

【0086】

なんら理論に拘束されることを望まないが、本発明者らは、このアプローチとは対照的に、合成方法によるほとんどの逐次配列決定では、長い読出し長さに対する可能性を最終的に制限するエラー蓄積に取り組んでいることを提案する。ある特定の本明細書に記載の方法の好都合な特徴は、所与のサイクル数（ｙ）後、ｎ＊ｙ－（ｎ－１）番目の塩基に達する（例えば、１５サイクル後に前述の例において７１番目の塩基、または切断部位の３’側で６塩基のプローブを用いて２０サイクル後に１１５番目の塩基）ように、第ｎ塩基ごと（プローブ内の切断可能な部分の位置による）の同定を可能にすることである。ｎ－１、ｎ－２などの位置の初期オリゴヌクレオチドを「リセット」できることは、鋳型からの伸長鎖のストリッピングおよび新たな初期オリゴヌクレオチドのハイブリダイズのプロセスにより、バックグラウンドシグナルがゼロに有効にリセットされるため、所与の読出し長さのための逐次エラー蓄積（位相の散逸または自然減（ａｔｔｒｉｔｉｏｎ）により）が大いに最小化される。例えば、合成によるポリメラーゼ系配列決定と、本明細書に記載のライゲーション系アプローチを比較すると、各伸長サイクルでの信号対雑音比が９９：１である場合、１００サイクル後の比は、ポリメラーゼ系アプローチで３７：６３であり、リガーゼ系の方法では８５：１５である。リガーゼ系の方法での最終結果は、ポリメラーゼ系の方法よりも読出し長さにおいて大きく増大する。

【0087】

鋳型内の先行する各ヌクレオチドのためのサイクルを行なう必要がある場合に必要とされ得るよりも少ないサイクルを用いてヌクレオチドを同定できることは、いくつかの理由で重要である。特に、該方法において各工程が１００％効率で行なわれることはあり得な

い。例えば、一部の鋳型は、伸長プローブに成功裏にライゲートされないことがあり得、一部の伸長プローブは切断されないことがあり得るなどである。したがって、各サイクルにおいて、異なる鋳型コピーにおいて行なわれる反応は、進行的に位相の散逸となり、有用で正確な情報が取得され得る鋳型の数が低減される。したがって、初期オリゴヌクレオチドの伸長可能な末端から数個より多く離れた位置に位置するヌクレオチドの読み出しに必要とされサイクル数を最小限にすることは特に望ましい。しかしながら、伸長プローブの長さを増大させることは、潜在的にプローブ混合物のより大きな複雑度をもたらし、これは、個々の各プローブ配列の有効濃度を低減させる。本明細書に記載のように、縮重低減性ヌクレオチドは、複雑度を低減させるために使用され得るが、ハイブリダイゼーション強度の減少および／またはライゲーション効率の減少をもたらし得る。本発明者らは、結果を最適化するために、これらの競合する要素を均衡させる必要性を認識した。したがって、本発明の好ましい実施形態において、８ヌクレオチド長の伸長プローブを使用し、選択した位置に縮重低減性ヌクレオチドを用いる。また、本発明者らは、適切な切れやすい連結ならびに切断条件ならびに切断工程の効率（すなわち、各切断工程において成功裏に切断された連結の割合）およびその適切な連結に対する特異性を最適化するタイミングを選択することの重要性を認識した。

【0088】

Ｂ．オリゴヌクレオチド伸長プローブ設計

Ｍａｃｅｖｉｃｚには、縮重低減性ヌクレオシド類縁体が、オリゴヌクレオチド伸長プローブに使用され得ることは記載されているが、かかる残基を含む残基を伸長プローブ内に含めるのに特に望ましい具体的な位置は教示されておらず、縮重低減性ヌクレオシドが組み込まれた具体的なプローブ構造（すなわち、配列）は教示されていない。本発明者らは、縮重低減性ヌクレオシド（例えば、普遍塩基を含むヌクレオシド）を、オリゴヌクレオチド伸長プローブ内の特定の位置に特定の数で利用することが特に好都合であり得ると認識した。例えば、本発明のある特定の実施形態において、６位以降の（Ｘから数えて）のヌクレオチドの大部分または全部が普遍塩基を含む。例えば、６位以降のヌクレオチド少なくとも５０％、少なくとも６０％、少なくとも７０％、少なくとも８０％、少なくとも９０％または少なくとも１００％が、普遍塩基を含有するものであり得る。ヌクレオチドは、すべてが同じ普遍塩基を含有するものである必要はない。本発明のある特定の実施形態において、ヒポキサンチンおよび／またはニトロ－インドールが普遍塩基として使用される。例えば、イノシンなどのヌクレオシドが使用され得る。

【0089】

本発明者らは、優れた結果は、６ヌクレオチド長より大きく、プローブの近位末端から（ライゲートされるヌクレオチドから数えて）伸長可能なプローブ末端までの６位以降のヌクレオチドの１つ以上が縮重低減性ヌクレオチドである、例えば、普遍塩基を含む（すなわち、最も近位ヌクレオチドを１位とみなした場合、６位以降のヌクレオチドの１つ以上が普遍塩基を含む）、例えば、八量体プローブの場合は、６位以降のヌクレオチドの１、２または３個が普遍塩基を含む伸長プローブを用いて達成され得ると認識した。例えば、３’→５’方向における配列決定では、構造３’－ＸＮＮＮＮｓＩＮＩ－５’　を有するプローブが使用され得、式中、ＸおよびＮは任意のヌクレオチドを表し、「ｓ」は、切れやすい連結を表し、切断は３’末端から数えて第５と第６の間の残基で起こるようになっており、切れやすい連結と５’末端間の残基の少なくとも１つは、好ましくは、Ｘの正体に対応する標識を有する。別の設計は、３’－ＸＮＮＮＮｓＮＩＩ－５’　である。また別のプローブ設計は、３’－ＸＮＮＮＮｓＩＩＩ－５’　である。この設計により、中程度の複雑度を有する１０２４個の異なる種プローブ混合物がもたらされ、有意なアデニル化産物の形成を抑制するのに充分長く（実施例１参照）、切断後に残留する得られる伸長産物が非修飾ＤＮＡからなるものであり得るという利点を有する。欠点の１つは、このプローブがプライマーを一度に５塩基しか伸長させないことである。読出し長さは、伸長長さ×サイクル数の関数であるため、伸長長さにおける各追加の塩基は、読出し長さが１×サイクル数分増加する可能性を有する（例えば、２０　サイクルを用いる場合は２０塩

基）。別のプローブ設計は、切断後に１つ以上イノシン（または他の普遍塩基）を伸長プローブの末端に残し、６塩基以上の長さの伸長された二本鎖を創製させるものである。例えば、プローブ３’－ＸＮＮＮＮＩｓＩＩ－５’により、二本鎖は一度に６塩基伸長され得、接合に５’イノシンが残る。これらの設計の各々において、切れやすい連結と５’末端間の残基の少なくとも１つは、好ましくは、Ｘの正体に対応する標識を有する。本発明のある特定の実施形態において、プローブの遠位末端から（ライゲートされるヌクレオチドと反対の末端から数えて）伸長可能なプローブ末端までの第３のヌクレオチドが普遍塩基を含む（すなわち、遠位末端を位置Ｋとみなした場合、位置Ｋ－２のヌクレオチドが普遍塩基を含む）。

【0090】

本発明のある特定の実施形態において、ロックド核酸（ＬＮＡ）塩基が、初期オリゴヌクレオチドプローブ、伸長プローブまたは両方内の１つ以上の位置に使用される。ロックド核酸は、例えば、米国特許第６，２６８，４９０号；Ｋｏｓｈｋｉｎ，　ＡＡら、Ｔｅｔｒａｈｅｄｒｏｎ，　５４：３６０７－３６３０，１９９８；Ｓｉｎｇｈ，　ＳＫら、Ｃｈｅｍ．Ｃｏｍｍ．，４：４５５－４５６，１９９８に記載されている。ＬＮＡは、自動ＤＮＡ合成装置によって標準的なホスホロアミダイト化学反応を用いて合成され得、天然に存在するヌクレオチドおよび／またはヌクレオチドアナログもまた含有するオリゴヌクレオチド内に組み込まれ得る。これらもまた、以下に記載のものなどの標識を用いて合成され得る。

【0091】

Ｃ．鋳型、ライブラリー、担体、ブロッカー、およびそれらの調製および使用方法

本発明は、核酸鋳型および担体を調製する種々の方法を提供する。本発明はまた、ライゲーションに基づく配列決定での使用、または他の目的のためのライブラリーも提供する。本発明はまた、ブロッカーオリゴヌクレオチド、ならびに他の目的でオリゴヌクレオチドのライゲーション、検出および開裂のサイクルを連続して行うことにより、配列決定においてそれらを使用する方法も提供する。

【0092】

Ｍａｃｅｖｉｃｚには、複数の実質的に同一の鋳型分子を含む鋳型を最初に、例えば、慣用的なポリメラーゼ連鎖反応（ＰＣＲ）法のようなチューブまたは他の容器内で増幅によって合成する方法が教示されている。Ｍａｃｅｖｉｃｚには、増幅させた鋳型分子を、好ましくは、合成後に磁気微粒子（例えば、ビーズ）などの支持体に結合させることが教示されている。

【0093】

本発明者らは、配列決定される鋳型は、望ましくは、支持体自体の上またはその内部で、例えば、１対の増幅プライマーの一方を、ＰＣＲ反応を行なう前に結合させる微粒子などの支持体または種々の半固相支持体材料ゲルマトリックスを用いることにより合成させるのがよいと認識した。このアプローチにより、合成後に、鋳型分子を支持体に結合させる別途の工程の必要性が回避される。したがって、異なる配列の複数の鋳型種が、並行して簡便に増幅され得る。例えば、以下に記載する方法によれば、微粒子上での合成により、各々が自身に結合された多コピーのある特定の鋳型分子（またはその相補体）を有する個々の微粒子の集団がもたらされ、各微粒子に結合された鋳型分子は、他の微粒子に結合された鋳型分子と配列が異なる。したがって、支持体の各々は、自身に結合された鋳型のクローン性の集団を有する、例えば、支持体Ａは、自身に結合された多コピーの鋳型Ｘを有し、支持体Ｂは、自身に結合された多コピーの鋳型Ｙを有し、支持体Ｃは、自身に結合された多コピーの鋳型Ｚを有するなどである。「鋳型のクローン性の集団」、「核酸のクローン性の集団」などにより、好ましくは、目的の単一の鋳型分子（出発鋳型）から開始される連続回数の増幅によって生成される、実質的に同一の鋳型分子の集団が意図される。実質的に同一の鋳型分子は、出発鋳型またはその相補体と実質的に同一であり得る。

【0094】

増幅は、典型的には、ＰＣＲを用いて行なわれるが、他の増幅方法もまた使用され得る

（下記参照）。クローン性の集団のメンバーは１００％同一である必要はなく、例えば、ある特定数の「エラー」が、合成過程中（例えば、増幅中）に起こり得ることは理解されよう。クローン性の集団のメンバーの好ましくは少なくとも５０％が、出発鋳型分子（またはその相補体）と少なくとも９０％、またはより好ましくは少なくとも９５％同一である。より好ましくは、集団のメンバーの少なくとも６０％、少なくとも７０％、少なくとも８０％、少なくとも９０％、少なくとも９５％、少なくとも９９％またはそれ以上が、出発鋳型分子（またはその相補体）と少なくとも９０％、またはより好ましくは少なくとも９５％同一であるか、またはさらにより好ましくは少なくとも９９％同一である。好ましくは、出発鋳型分子（またはその相補体）に対して少なくとも９５％またはより好ましくは少なくとも９９％の同一性割合の集団のメンバーは、少なくとも９８％、９９％、９９．９％またはそれ以上である。

【0095】

増幅プライマーは、任意のさまざまな手法を用いて支持体に結合させ得る。例えば、プライマー（５’末端）の一方の末端を結合ペアの一メンバー（例えば、ビオチン）で官能性付与させ、支持体を、結合ペア他方のメンバー（例えば、ストレプトアビジン）で官能性付与させ得る。任意の類似した結合ペアが使用され得る。例えば、規定配列の核酸タグを、相補的核酸タグを有する支持体およびプライマーに結合させ得、支持体に結合された核酸タグにハイブリダイズさせ得る。種々のリンカーおよび架橋剤もまた使用され得る。

【0096】

ＰＣＲを行なうための方法は、当該技術分野でよく知られており、例えば、米国特許第４，６８３，１９５号、同第４，６８３，２０２号および同第４，９６５，１８８号、ならびにＤｉｅｆｆｅｎｂａｃｈ，　Ｃ．およびＤｖｅｋｓｌｅｒ，　ＧＳ，　ＰＣＲ　Ｐｒｉｍｅｒ：Ａ　Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ，第２版，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ　Ｌａｂｏｒａｔｏｒｙ　Ｐｒｅｓｓ，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ，２００３に記載されている。微粒子上で核酸を増幅させるための方法は、当該技術分野でよく知られ、報告されており、例えば、標準的なＰＣＲは、マイクロタイタープレート（ｄｉｓｈ）のウェルまたはチューブ内で、自身に結合されたプライマーを有するビーズ（例えば、実施例１２のようにして調製されるビーズ）上で行なわれ得る。ＰＣＲは簡便な増幅方法であるが、当該技術分野で知られた任意の数多くの他の方法もまた使用され得る。例えば、多重鎖置換型増幅、ヘリカーゼ置換型増幅（ＨＤＡ）、ニックトランスレーション、Ｑβレプリカーゼ増幅、ローリングサークル型増幅、および他の等温増幅方法などが使用され得る。

【0097】

鋳型分子は、任意のさまざまな供給源から得られ得る。例えば、ＤＮＡは、被験体から入手され得るか、またはこれに由来であり得る試料から単離され得る。用語「試料」は、配列決定が行なわれる鋳型の任意の供給源を表すために広義で用いる。語句「に由来する」は、被験体から直接得られた試料および／または試料中の核酸は、鋳型分子を得るためにさらに加工処理されたものであり得ることを示すために用いる。試料の供給源は、任意のウイルス、原核生物、古細菌または真核生物種であり得る。本発明のある特定の実施形態において、供給源はヒトである。試料は、例えば、細胞を含有する血液または別の体液；精子；生検試料などであり得る。目的の任意の生物体のゲノムまたはミトコンドリアのＤＮＡが配列決定されてもよい。ｃＤＮＡが配列決定されてもよい。また、例えば、ＲＴ－ＰＣＲなどの当該技術分野で知られた方法を用いて最初に逆転写することによってｃＤＮＡを得ることによりＲＮＡが配列決定されてもよい。異なる試料および／または被験体由来のＤＮＡの混合物を合わせてもよい。試料は、任意のさまざまな様式で加工処理され得る。核酸は試料から、既知の方法を用いて単離、精製および／または増幅され得る。もちろん、生物体に由来しない完全に人工的な合成核酸、組換え核酸もまた配列決定され得る。

【0098】

鋳型は、二本鎖または単鎖形態で提供され得る。典型的には、まず、鋳型を二本鎖形態

で提供し、続いて、この二本鎖を分離する（例えば、ＤＮＡを変性させる）場合、該二本鎖の一方だけを増幅して鋳型の局在クローン性の集団分子（例えば、微粒子に結合、半固相支持体内または該支持体上にに固定化など）を生成させる。

【0099】

鋳型は、さまざまなさらなる様式で選択または加工処理され得る。例えば、メチル感受性制限酵素（例えば、Ｍｓｐ１）による処理に供されたＤＮＡから得られる鋳型が使用され得る。かかる処理は、ＤＮＡ断片をもたらすものであり、増幅前に行なわれ得る。メチル化塩基を含有する断片は増幅されない。低メチル化（ｈｙｐｏｍｅｔｈｙｌａｔｅｄ）鋳型から得られる配列情報を、同じ供給源に由来する低メチル化の選択に供しなかった鋳型から得られる配列情報と比較し得る。

【0100】

鋳型は、ライブラリー内に挿入されるか、これで提供されるか、またはこれに由来するものであり得る。例えば、低メチル化ライブラリーは当該技術分野で知られている。ライブラリー内への鋳型の挿入により、鋳型の末端へのさらなるヌクレオチド配列の簡便な連鎖（例えば、タグ、プライマーまたは初期オリゴヌクレオチド結合部位など）が可能となり得る。例えば、ある特定のストラテジーにより、複数の結合部位（例えば、増幅プライマーの結合部位、初期オリゴヌクレオチドの結合部位、捕捉剤の結合部位など）を有するタグの付加が可能になる。

【0101】

さまざまな適当なライブラリーが、当該技術分野で知られている。例えば、特定の目的のライブラリー、およびその構築方法は、ＵＳＳＮ１０／９７８，２２４、ＰＣＴ公開公報ＷＯ２００５０４２７８１およびＷＯ２００５０８２０９８ならびにＳｈｅｎｄｕｒｅ，　Ｊ．ら、Ｓｃｉｅｎｃｅ，３０９（５７４１）：１７２８－３２，２００５，　Ｓｃｉｅｎｃｅｘｐｒｅｓｓ（２００５年８月４日）（ｗｗｗ．ｓｃｉｅｎｃｅｘｐｒｅｓｓ．ｏｒｇ）に記載されている。もちろん、かかるライブラリーの他の作製方法もまた使用され得ることは理解されよう。特定の目的のある特定のライブラリーは、複数の核酸断片（典型的には、ＤＮＡ）を含み、その各々は、配列決定工程で使用される増幅および／または配列決定用プライマーに相補的な配列によって分断された目的の２つの核酸セグメントを含有する、すなわち、これらの配列は、プライマー結合領域（ＰＢＲ）としての機能を果たす。特定の目的の実施形態において、核酸セグメントは、天然に存在するＤＮＡの連続片の一部分である。例えば、セグメントは、前述の参考文献に記載のようなゲノムＤＮＡの連続片の５’および３’末端由来のものであり得る。かかる核酸セグメントを本明細書において、前述の参考文献と一致する様式で、「タグ」または「末端タグ」という。単一の連続核酸に由来する（例えば、その５’および３’末端に由来する）２つのタグを、「１対のタグ」、「対のタグ」または「ジタグ（ｄｉｔａｇ）」とよぶ。「対のタグ」は、単数形で使用されていても２つのタグを含むことは認識されよう。１対のタグであるタグが由来するＤＮＡの連続片を所定の大きさの範囲内で選択することにより、該２つのタグが離れる間隔が限定される。

【0102】

配列決定および／または増幅用プライマーに相補的な配列によって分断されることに加え、ライブラリーの核酸断片はまた、典型的には、タグと隣接する配列決定および／または増幅用プライマーに相補的な配列を含む。すなわち、第１のかかる配列は、該断片の５’末端により近いタグの５’側に位置し得、第２のかかる配列は、該断片の３’末端により近いタグの３’側に位置し得る。タグが由来する連続核酸内に存在する２つのタグの位置は、種々の実施形態において、ライブラリーのＤＮＡ断片内のタグの位置に対応するものであり得るが、そうである必要はないことに注意されたい。

【0103】

核酸断片およびタグは、異なる範囲の大きさを有するものであり得る。典型的には、核酸断片は、例えば、８０～３００ヌクレオチド長、例えば１００～２００、１００～１５０、およそ１５０ヌクレオチド長、およそ２００ヌクレオチド長などであり得る。タグは

、例えば、１５～２５ヌクレオチド長、例えばおよそ１７～１８ヌクレオチド長などであり得る。これらの長さは、例示であって、限定を意図しないことに注意されたい。より短鎖またはより長鎖の断片および／またはタグが使用され得る。

【0104】

また、対のタグを単一の連続核酸から得ることによりライブラリー構築の簡便な方法が提供されるが、対のタグの重要な点は、これらが、自身が元々由来する核酸内である間隔（「分断間隔」）だけ互いに離れており、この分断間隔が所定範囲内に含まれる間隔であることに注意されたい。両タグが所定範囲内に含まれる分断間隔だけ離れていることにより、タグの配列を参照配列（例えば、参照ゲノム配列）に対してアライメントさせることが可能になる。なんら理論に拘束されることを望まないが、このことは、ゲノム再配列決定などのある特定の適用用途に好都合であり得、この場合、参照ゲノムに対する配列の正確な配置が依然として可能なまま、より短い読出し長さの使用が可能になる。５’および３’タグの１対のタグは、より大きな核酸片（例えば、ゲノムＤＮＡ）のセグメントとなり（すなわち、その配列を有する）、該セグメントは、天然に存在するＤＮＡ片内（例えば、ゲノムＤＮＡ片内）において互いから予め規定された間隔内に位置する。例えば、本発明のある特定の実施形態において、５’および３’タグの１対のタグは、天然に存在するＤＮＡ片内で、互いに５００ヌクレオチドまで以内、互いに１ｋＢまで以内、互いに２ｋＢまで以内、互いに５ｋＢまで以内、互いに１０ｋＢまで以内、互いに２０ｋＢまで以内に位置するＤＮＡセグメントである。ある特定の実施形態において、５’および３’タグの１対のタグは、天然に存在するＤＮＡ片内で、５００ヌクレオチド～２ｋＢ離れて、例えば、７００ヌクレオチド～１．２ｋＢ離れて（およそ１ｋＢ離れてなど）位置する。１対のタグの２つのタグが離れている正確な間隔はあまり重要性ではなく、典型的には未知であることに注意されたい。また、タグは、元々は、より大きな核酸片から得られたものであるが、用語「タグ」は、タグの配列を有する任意の核酸セグメント、元々の配列状況に存在したもの、ライブラリー断片で存在したもの、ライブラリー断片からの増幅産物、配列決定対象の鋳型など、いずれも適用される。

【0105】

核酸断片（例えば、ライブラリー分子）は、下記の構造を有するものであり得る。

【0106】

リンカー１－タグ１－リンカー３－タグ１－リンカー２

タグ１およびタグ２は、は、５’および３’タグの１対のタグであり得る。タグのいずれかが５’タグまたは３’タグであり得る。リンカー１およびリンカー２は、１つ以上のプライマーのプライマー結合領域を含む。ある特定の実施形態において、リンカー１および２は、各々が増幅プライマーのＰＢＲおよび配列決定プライマーのＰＢＲを含む。各リンカー内のプライマーは、配列決定プライマーＰＢＲが増幅プライマーＰＢＲに対して内部に位置するようにネスト化（ｎｅｓｔｅｄ）されたものであり得る。リンカー３は、タグ１およびタグ２の配列決定を可能にするため、１つ以上配列決定プライマーのＰＢＲを含むものであり得る。核酸断片のライブラリーに関して使用される用語「リンカー」は、ライブラリーの多数の核酸断片（例えば、ライブラリーの実質的にすべての断片）内に存在する核酸配列をいう。リンカーは、ライブラリーの構築中に実際に連結機能を果たしたものであってもそうでなくてもよく、単に、所与のライブラリーの大部分または全部のメンバーに共通する規定配列とみなされるものであってもよい。かかる配列はまた、「普遍配列」ともいう。したがって、リンカーまたはその一部分に相補的な核酸は、ライブラリーの多数のメンバーにハイブリダイズされ得、ライブラリー内の大部分または全部の分子の増幅プライマーまたは配列決定プライマーとして使用され得る。

【0107】

本発明のある特定の実施形態において、核酸断片は下記の構造を有する。

【0108】

リンカー１－タグ１－内部アダプター－タグ２－リンカー２

タグ１およびタグ２ならびにリンカー１およびリンカー２は、上記のようなＰＢＲを含

む。内部アダプターは、ＩＡおよびＩＢ（以下にさらに論考する）ということがある２つのプライマー結合領域を含む。これらのＰＢＲは、一方の集団の核酸がタグ１を含み、他方の集団の核酸がタグ２を含む自身に結合された核酸の２つの異なる実質的に同一の集団を有する微粒子生成させるのに有用である。核酸の２つの異なる集団は少なくとも部分的に異なる配列を有し、例えば、これらはタグの配列領域が異なる。内部アダプターは、スペーサー領域を２つのプライマー結合領域間に含むものであり得る。スペーサー領域は無塩基残基を含み得、これにより、該スペーサーを介するポリメラーゼの伸長が抑制される。もちろん、スペーサーを介するポリメラーゼ伸長が抑制され得る任意の他のブロッキング基を含有するスペーサー領域が使用され得る。

【0109】

他の実施形態において、核酸断片は、１つ以上（例えば、２、４、６など）のさらなるタグおよび１つ以上のさらなる内部アダプターを含む。例えば、核酸断片は下記の構造を有する。

【0110】

リンカー１－タグ１－内部アダプター１－タグ２－リンカー２－タグ３－内部アダプター２－タグ４－リンカー３

本発明の核酸断片およびかかる断片のライブラリー、核酸の２つ以上の実質的に同一の集団を含有する微粒子、ならびにかかる微粒子のアレイは、本明細書に記載のライゲーション系配列決定法以外の広範なさまざまな配列決定法に使用され得ることに注意されたい。例えば、ＦＩＳＳＥＱ、ピロシーケンスなどの配列決定法が使用され得る。例えば、ＷＯ２００５０８２０９８を参照のこと。もちろん、ライゲーション系の方法もまた好都合に使用され得る。本明細書に記載のライゲーション系の方法の状況では、用語「配列決定プライマー」は、「初期オリゴヌクレオチド」を意味すると理解され得ることは認識されよう。

【0111】

本発明のある特定の実施形態において、配列決定対象の鋳型は、ＰＣＲによって、エマルジョンの個々の水性区画（「反応器」ともいう）内で合成される。好ましくは、該区画は、各々が適当な自身に結合された第１の増幅プライマー、第１の鋳型コピー、第２の増幅プライマーおよびＰＣＲ反応に必要な成分（例えば、ヌクレオチド、ポリメラーゼ、補因子など）を有するビーズなどの微粒状の支持体を含む。エマルジョンの調製方法は、例えば、米国特許第６，４８９，１０３号（Ｇｒｉｆｆｉｔｈｓ）；同第５，８３０，６６３号（Ｅｍｂｌｅｔｏｎ）；および米国特許公開公報第２００４０２５３７３１号（Ｇｈａｄｅｓｓｙ）に記載されている。ＰＣＲをエマルジョンの個々の区画内で行ない、微粒子に結合された鋳型のクローン性の集団を生成させる方法（「エマルジョンＰＣＲ」）は、例えば、Ｄｒｅｓｓｍａｎ，　Ｄ．ら，　Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ，１００（１５）：８８１７－８８２２，２００３およびＰＣＴ公開公報ＷＯ２００５０１０１４５に記載されている。

【0112】

前述の参考文献に記載の方法またはその変形法は、配列決定のために微粒子に結合された鋳型のクローン性の集団を生成させるために使用され得る。好ましい非限定的な実施形態において、ＰＣＲに適した短鎖（＜５００ヌクレオチド）鋳型が、普遍アダプター配列を異なる標的配列（鋳型）の集団の各末端に結合させることにより（例えば、ライゲーションによって）創製される（この状況における普遍は、同じアダプター配列が各鋳型に結合され、単一の１対のＰＣＲ増幅プライマーを用いて増幅され得る「アダプター付加」鋳型創製されることを意味する）。バルクＰＣＲ反応液は、アダプター付加鋳型、１種類の遊離増幅プライマー、自身に結合された第２の増幅プライマーを有する微粒子、および他のＰＣＲ試薬（例えば、ポリメラーゼ、補因子、ヌクレオチドなど）を用いて調製される。水性ＰＣＲ反応液を油相（低分子（ｌｉｇｈｔ）鉱油および界面活性剤を含有）と１：２の比で混合する。この混合物をボルテックスして油中水型エマルジョンを創製する。４×１０^{9}より多い水性区画（各々が潜在的ＰＣＲ反応器）をエマルジョン内に創製するの

に１ミリリットルの混合物で充分である。エマルジョン試料のアリコートを、マイクロタイタープレート（例えば、９６ウェルプレート、３８４ウェルプレートなど）のウェル内に分配し、サーマルサイクルに供し、微粒子上での固相ＰＣＲ増幅を行なう。クローン性を確保するため、微粒子および鋳型の濃度は、反応器が１つより多くのビーズまたは鋳型分子を含むことは稀であるように注意深く制御する。例えば、本発明のある特定の実施形態において、反応器の少なくとも１０％、２０％、３０％、４０％、５０％、６０％、７０％、８０％、９０％、９５％またはそれ以上が単一のビーズおよび単一の鋳型を含む。したがって、各鋳型のクローン性の集団のメンバーは、その微粒子への結合の結果、空間的に互いに近接して局在化される。一般に、鋳型の結合点は、該粒子の表面上に実質的に均一に分布され得る。増幅手順後の微粒子に付着したクローン性の集団（代表的に数千から数百万コピーの鋳型）の鋳型を有する微粒子は、鋳型増幅を受けたという。

【0113】

個々の微粒子が、自身に結合された５’タグおよび３’タグの１対のタグを含む増幅された核酸断片の異なる集団を有する微粒子の集団を生成させるためにＰＣＲエマルジョン法を用いることは、特に重要である。換言すると、個々の粒子がライブラリー由来の異なる核酸断片（例えば、増幅され、自身に結合された上記のものなど）を有する微粒子の集団生成させることは、特に重要である。

【0114】

ＤＮＡをエマルジョン中で増幅させるための当該技術分野で知られた方法（例えば、上記の参考文献に記載のもの）は、大きな核酸分子の増幅およびこれらの分子の微粒子への結合を達成するその能力に関して制限がある。例えば、より長いアンプリコンを用いると、ＰＣＲ効率は指数関数的に減少することが示されている。このＰＣＲ効率の減少により、対のタグおよびプライマー結合部位を含有する核酸断片（例えば、上記のものなど）が、ＰＣＲエマルジョンで増幅され得、かかる増幅によって微粒子に結合される効率が低下する。したがって、第１および第２のタグの１対のタグを含有する実質的に同一の核酸断片の単一の集団がＰＣＲエマルジョンで増幅され、かかる増幅によってビーズに結合される方法は、いくつかの制限があるという欠点を有する。

【0115】

本発明は、５’および３’タグの１対のタグを含有する単一の核酸断片を増幅によって微粒子に結合させた場合にもたらされる対のタグ情報は依然として保存されたまま、より小さいアンプリコンの使用を可能にするアプローチを提供する。本発明は、自身に結合された核酸の少なくとも２つの異なる集団を有する微粒子（例えば、ビーズ）を提供し、ここで、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、第１の実質的に同一の核酸の集団は、第１の核酸の目的のセグメント（例えば、５’タグ）を含み、核酸の第２の集団は、第２の核酸の目的のセグメント（例えば、３’タグ）を含む。第１および核酸の第２の集団は、該２つのタグを含み、かつ該タグと隣接し分断する適切に配置されたプライマー結合部位も含む単一の大きな核酸断片から増幅され、その結果、２つの増幅反応は、連続的または（好ましくは）同時のいずれかで、ＰＣＲエマルジョン単一の反応器内にて微粒子および増幅試薬の存在下で行なわれ得る。微粒子は自身に結合されたプライマーの２つの異なる集団を有し、該集団の一方は、該核酸断片内の一方のタグの外部のプライマー結合領域を有する配列に対応し、他方は、該核酸断片内の他方のタグの外部のプライマー結合領域を有する配列に対応する。すなわち、プライマー結合領域は該２つのタグと隣接している。

【0116】

また、２つの独立したＰＣＲ反応が行なわれ、各々が、核酸断片の一方のタグを含有する部分を増幅し得るように、該２つのタグ間に位置するプライマー結合領域に結合するプライマーが提供される。増幅された核酸セグメントは、互いに異なるさらなるプライマー結合領域を含み、これらのさらなるプライマー結合領域は、核酸断片内に存在し、増幅プライマーのＰＢＲに対して内部に位置する、すなわち、これらはネスト化されている。これらのさらなるＰＢＲは、２つの異なる配列決定プライマーの結合領域としての機能を果

たす。したがって、２つの異なる配列決定プライマーの一方または他方を自身に結合された実質的に同一の核酸セグメントの２つの集団を有する微粒子に適用することにより、２つの核酸セグメントの一方または他方のいずれかが、他方の核酸セグメントの存在による障害なく配列決定され得る。核酸セグメントの各々は、増幅された核酸断片よりも有意に短く、したがって、対のタグを含有する断片のライブラリーを用いてエマルジョン系ＰＣＲが行なわれ得る効率は改善されるが、１対のタグのタグ間の会合は依然として保持されたままである。

【0117】

上記の方法は、図３４および３５の種々のパネルを参照することによって、より良好に理解され得る。図において、同じ配列を有する核酸の部分には同じ色を割り当てている。上記の記載は、図３４および３５と一致するよう解釈されたい。図３４Ａおよび３５Ａは図３５Ａと同じ工程を示すが、さらなる詳細を提供する。図３４Ａおよび３５Ａに示すように、２つのタグ（タグ１およびタグ２）を含有する対末端ライブラリー断片は、内部アダプターカセット（ＩＡ－ＩＢ）および特有の隣接リンカー配列（Ｐ１およびＰ２）を有するように構築される（すなわち、Ｐ１およびＰ２は互いに異なる）。内部アダプターカセットおよび隣接リンカー配列はともに、ＰＣＲ増幅およびＤＮＡ配列決定の両方をもたらすヌクレオチド配列を含む。ＰＣＲプライマー領域は、ネスト化されたＤＮＡ配列決定プライマーの使用が可能になるように設計される。ＤＮＡ捕捉微粒子（ビーズ）は、同一である２つのオリゴヌクレオチド配列を特有の隣接リンカー配列に結合させることにより生成させる。ＰＣＲ増幅のため、Ｐ１およびＰ２　配列を有するオリゴヌクレオチドに結合させたＤＮＡ捕捉微粒子を、単一のジタグライブラリー断片（すなわち、５’タグおよび３’タグの１対のタグを含有するライブラリー断片）および溶液系ＰＣＲプライマーを含有する反応液にシード（ｓｅｅｄ）する。

【0118】

溶液系隣接リンカープライマー（Ｐ１およびＰ２）は、内部アダプタープライマー（ＩＡおよびＩＢ）と比べて限定的な量で添加され、ＰＣＲ生成タグ産物の効率的なビーズ駆動型（ｄｒｉｖｅ－ｔｏ－ｂｅａｄ）増幅を促進する機能を果たす（すなわち、［Ｐ１＜＜ＩＢ］，　［Ｐ２＜＜ＩＡ］）。所望の場合は、プライマーの量を適切に制御することによってもまた、核酸の集団が実質的に同じ数の核酸を含むことが確保され得る（例えば、個々の微粒子上のおよそ半数の核酸が第１の集団に属し、個々の微粒子上のおよそ半数の核酸が第２の集団に属する）。したがって、所望により、種々の集団の比を制御するため、非対称形態のＰＣＲが使用され得る。

【0119】

増幅中、図３４Ｂおよび３５Ｂに示すように（この場合も、図３５Ｂは、図３４Ｂに関するさらなる詳細を提供する）、単一の対末端ライブラリー断片が、４種類のオリゴヌクレオチドプライマー（Ｐ１、Ｐ２、ＩＡおよびＩＢ）の存在下で、２種類の特有のＰＣＲ産物を生成させる。一方の集団は、Ｐ１とＩＡに隣接したタグ１を含み、第２の集団は、Ｐ２とＩＢに隣接したタグ２を含む。

【0120】

増幅微後、粒子には、最初のライブラリー断片から生成されたタグ１およびタグ２に対応する２つの特有のＰＣＲ集団が負荷される。したがって、各タグは、図３４Ｃ、３５Ｃおよび３５Ｄに示すような、各タグの逐次配列決定を可能にする特有の組のプライマー（ｐｒｉｍｉｎｇ）領域を含む。図３５Ｃおよび３５Ｄは、異なる配列決定プライマーを用いたタグ１および２の連続的配列決定を示す。任意のさまざまな配列決定法が使用され得る。

【0121】

上記の方法は、（例えば、４、６、８、１２、１６、２０の集団であって、例えば、該集団は、２、３、４、６、８、１０の対のタグを含む）自身に結合された２つより多くの異なる核酸配列の集団を有する微粒子を生成させるために使用され得る。各集団は、２つのタグの場合で記載のようにして、特有のプライマー結合領域を各配列内に提供すること

により個々に配列決定され得る。

【0122】

本発明は、図３４および３５に示す上記の構造を有する核酸断片、かかる断片のライブラリー、自身に結合されたかかる断片由来の核酸セグメントを有する微粒子、かかる微粒子の集団（個々の微粒子は、他の微粒子のものと異なる核酸の集団が自身に結合されている）、微粒子のアレイ、核酸断片からの核酸セグメント（タグ）の増幅のための増幅プライマー、微粒子に結合された核酸セグメントの配列決定のための配列決定プライマー、該断片、ライブラリーおよび微粒子の作製方法、ならびに微粒子に結合された核酸の配列決定法を包含する。本発明は、前述の成分の任意の組合せを含み、任意選択で１種類以上の酵素、バッファーまたは増幅、配列決定などに有用な他の試薬もまた含むキットを包含する。

【0123】

所望の場合は、さまざまな方法が、自身に結合された鋳型を有する微粒子の富化のために使用され得る。例えば、増幅産物（鋳型）の微粒子に結合された部分に相補的なオリゴヌクレオチド（捕捉剤）を、別の（好ましくは、より大きな）微粒子、マイクロタイターウェルまたは他の表面などの捕捉実体に結合させるハイブリダイゼーション系の方法が使用され得る。増幅産物の該部分は標的領域とよばれ得る。標的領域は、増幅中、鋳型内に、例えば、鋳型の未知配列を有する部分の末端に組み込まれ得る。例えば、標的領域は、微粒子に結合されない増幅プライマー内に存在し得、その結果、相補的部分が増幅された鋳型内に存在する。したがって、多数の異なる鋳型が、同じ標的領域を含み得るため、単一の捕捉剤が、多数の異なる鋳型にハイブリダイズし、１つだけのオリゴヌクレオチド配列を捕捉剤として用い、多数の微粒子の捕捉が可能になる。増幅に供された微粒子は、ハイブリダイゼーションが起こり得る条件下で捕捉剤に曝露され、その結果、自身に結合された増幅された鋳型を有する微粒子が、捕捉剤により捕捉実体に結合される。次いで、未結合微粒子を除去し、保持された微粒子を放出させる（例えば、昇温により）。微粒状の捕捉実体が使用されるある特定の実施形態において、自身に結合された微粒子を有する捕捉実体からなる凝集体を、ハイブリダイゼーション後に、結合された微粒子のない微粒状の捕捉実体から、および捕捉実体に結合されていない微粒子から、例えば、粘性溶液（例えば、グリセロール）中での遠心分離によって分離する。大きさ、密度などに基づく他の分離方法もまた使用され得る。しかし、ハイブリダイゼーションは、富化に使用され得るいくつかの方法のうちの１つである。例えば、鋳型内に組み込まれ得る（例えば、合成中）任意のいくつかの異なるリガンドに対して親和性を有する捕捉剤が使用され得る。多数回の富化が使用され得る。

【0124】

図１４Ａは、ＰＣＲ反応が、自身に結合された第１の増幅プライマーを有するビーズ上で、蛍光標識された第２の増幅プライマーおよび過剰の鋳型を用いて行なわれた油中水型エマルジョンの区画の画像を示す。水性反応器は、拡散した遊離プライマーから弱い蛍光を発するが、ビーズは、固相増幅の結果としてビーズ上に蓄積されるプライマーから強い蛍光を発する（すなわち、蛍光プライマーが、ビーズに結合された増幅された鋳型内に第１の増幅プライマーによって組み込まれている）。ビーズシグナルは、種々の大きさの反応器において不均一である。

【0125】

増幅後、微粒子を回収し（例えば、磁気粒子の場合は磁石の使用によって）、本明細書に記載の伸長、ライゲーションおよび切断の反復サイクルによる配列決定に使用する。本発明のある特定の実施形態において、微粒子は、配列決定前に半固相支持体内または該支持体上にアレイ状にする（これは、後述する）。実施例１２、１３、１４および１５は、（ｉ）　微粒子上での鋳型の合成のための自身に結合された増幅プライマーを有する微粒子の調製（実施例１２）；（ｉｉ）ＰＣＲを行なうための複数の反応器を含むエマルジョンの調製（実施例１３）；（ｉｉｉ）エマルジョンの区画内でのＰＣＲ増幅（実施例１３）；（ｉｖ）エマルジョンの破壊および微粒子の回収（実施例１３）；（ｖ）　自身に結

合されたクローン鋳型集団を有する微粒子の富化（実施例１４）；（ｖｉ）　半固形ポリアクリルアミド支持体の基材として供するガラススライドの調製（実施例１５）；ならびに（ｖｉｉ）　微粒子と非重合アクリルアミドとの混合による自身に結合された鋳型を有する微粒子のアレイの形成、基材上のアクリルアミド内への包埋（実施例１５）ために使用され得る代表的で非限定的な方法のさらなる詳細を提供する。また、実施例１５には、ポリメラーゼトラッピング（ｔｒａｐｐｉｎｇ）のプロトコルを記載しており、これは、半固相支持体内でＰＣＲ行なう場合のある特定の方法で使用される。当業者には、これらの方法に対する数多くの変形法が使用され得ることが認識されよう。

【0126】

本発明の他の実施形態において、鋳型は、内部に固定化された適当な増幅プライマーを有するゲルなどの半固相支持体内でのＰＣＲによって増幅される。鋳型、さらなる増幅プライマー、およびＰＣＲ反応に必要な試薬を半固相支持体内に存在させる。１対の増幅プライマーの一方または両方を半固相支持体に、適当な連結部分、例えば、アクリダイト基によって結合させる。結合は、重合中に行なわれ得る。さらなる試薬（例えば、鋳型、第２の増幅プライマー、ポリメラーゼ、ヌクレオチド、補因子など）を、半固相支持体の形成の前に存在させてもよく（例えば、ゲル形成前に液体中）、または試薬の１種類以上をその形成後に半固相支持体中に拡散させてもよい。半固相支持体の細孔径は、かかる拡散を可能にするように選択される。当該技術分野でよく知られているように、ポリアクリルアミドゲルの場合は、細孔径は、主にアクリルアミドモノマー濃度によって、および程度は低いが架橋剤によって決定される。同様の考慮事項が、他の半固相支持体材料の場合に適用される。所望の細孔径を得るために適切な架橋剤および濃度が選択され得る。本発明のある特定の実施形態において、カチオン脂質、ポリアミン、ポリカチオンなどの添加剤を、重合前に該溶液中に含め、ゲル内ミセルを形成させるか、または微粒子周囲に凝集させる。米国特許第５，７０５，６２８号、同第５，８９８，０７１号、および同第６，５３４，２６２号に記載の方法もまた使用され得る。例えば、種々の「クラウディング（ｃｒｏｗｄｉｎｇ）試薬」が、クローンＰＣＲ用のビーズ付近にＤＮＡをクラウディングさせるために使用され得るＳＰＲＩ（登録商標）磁気ビーズ手法および／または条件もまた使用され得る。例えば、１０％ポリエチレングリコール（ＰＥＧ）の存在下での有効なＰＣＲ増幅を示した米国特許第５，６６５，５７２号を参照のこと。本発明の方法のある特定の実施形態において、増幅（例えば、ＰＣＲ）、ライゲーションまたは両方が、ベタイン、ポリエチレングリコール、ＰＶＰ－４０などの存在下で行なわれる。これらの試薬は、溶液に添加しても、エマルジョン中に存在させても、および／または半固相支持体中に拡散させてもよい。

【0127】

半固相支持体は、実質的に平面状の剛性の基材に配置するか、またはその上面に配設する。ある特定の好ましい実施形態において、該基材は、典型的な標識（例えば、蛍光標識、量子ドット、プラスモン共鳴粒子、ナノクラスター）の励起および検出に使用される励起および発光波長（例えば、およそ４００～９００ｎｍ）の放射線に対して透過性である。ガラス、プラスチック、石英などの材料が適当である。半固相支持体を基材に接着してもよく、任意選択で、任意のさまざまな方法を用いて基材に固定させ得る。

基質は、接着または結合を増強する物質（例えば、シラン、ポリリシンなど）でコーティングされたものであってもそうでなくてもよい。米国特許第６，５１１，８０３号には、半固相支持体内でのＰＣＲを用いて鋳型のクローン性の集団合成するための方法、実質的に平面状の基材上に半固相支持体を作製するための方法などが記載されている。同様の方法が本発明に使用され得る。基材は、半固形基材の形成前の液体を入れるためのウェルまたは凹部を有するものであってもよい。あるいはまた、高くした障壁またはマスクがこの目的のために使用され得る。

【0128】

上記のアプローチは、空間的に局在化されるクローン鋳型の集団を生成させるためのエマルジョン中の反応器（ｒｅａｃｔｏｒ）の使用の代替法を提供する。クローン性の集団

は半固相支持体内の別個の位置に、新たにライゲートされた伸長プローブを検出する（例えば、画像化によって）目的のために、シグナルが、配列決定中に各集団から取得され得るように存在する。本発明のある一部の実施形態において、２つ以上の異なるクローン性の集団が単一の核酸断片から増幅され、半固相支持体内の別個の位置に混合物として存在する。混合物中のクローン性の集団の各々は、例えば、別個の位置に５’タグを含有する断片および３’タグを含有する断片が含まれるようにタグを含み得る。５’タグおよび３’タグを含むクローン鋳型は異なる配列決定プライマーを含み、その結果、これらは、互いに独立して配列決定され得る。このアプローチは、多数の実質的に同一の核酸の集団を微粒子上に生成させ、単一の微粒子から１対のタグの両方のメンバーの配列決定情報得るための上記のアプローチ同一である。

【0129】

一般に、任意の本発明の方法における使用のための半固相支持体は、約１００ミクロン以下の厚さ、例えば約５０ミクロン厚以下、例えば約２０～４０ミクロン厚（両端を含む）の層を形成したものである。カバースリップまたは実質的に平面状の表面を有する他の類似した物体を、半固相支持体材料の上に、好ましくは重合前に配設し得、均一なゲル層の作製、例えば、実質的に平面状および／または実質的に均一な厚さのゲル層の作製が補助される。

【0130】

また本発明の他の実施形態において、上記の方法に対し、鋳型が、自身に結合された適当な増幅プライマーを有する微粒子上でのＰＣＲによって合成され、該微粒子が鋳型合成前に半固相支持体内または該支持体上に固定化される、すなわち、これらは、完全または部分的に半固相支持体内に包埋される変形法が使用される。一般的に、微粒子は半固相支持体材料に完全に囲まれるが、基材上に載っていてもその下方にあってもよい。したがって、微粒子は、半固相支持体が破壊されな限り、互いに対して実質的に固定された位置に維持される。このアプローチは、空間的に局在化されるクローン鋳型の集団を生成させるためのエマルジョンの使用別の代替法を提供する。微粒子は、半固相支持体の形成前に液体と混合してもよい。あるいはまた、微粒子を、実質的に平面状の基材上でアレイ状にし、重合、架橋などの前に液体を微粒子アレイに添加してもよい。微粒子は、自身に結合された第１の増幅プライマーを有する。第２の増幅プライマーは、半固相支持体に結合されたものであり得るが、そうである必要はない。さらなる試薬（例えば、鋳型、第２の増幅プライマー、ポリメラーゼ、ヌクレオチド、補因子など）を、半固相支持体の形成前に存在させてもよく（例えば、ゲル形成前に液体中）、またはこれらの試薬の１種類以上をゲル形成後に半固相支持体中に拡散させてもよい。半固形基材は、一般的に、上記のようにして例えばガラススライド上に形成される。

【0131】

本発明のある特定の実施形態において、ゲルは、鋳型合成後に、クローン鋳型集団が結合された微粒子が簡便に回収され得るように（例えば、磁気粒子の場合は磁石の使用によって）、可溶化（例えば、消化または解重合または溶解）させ得る。可溶化、消化、解重合、溶解させなどさせ得るゲルを、本明細書において「可逆的」という。慣用的なポリアクリルアミド重合は、Ｎ－Ｎ’メチレンビスアクリルアミド（ＢＩＳ）を架橋剤と、重合を開始させる適当な触媒（例えば、Ｎ，Ｎ，Ｎ’，Ｎ’－テトラメチルエチレンジアミン（ＴＥＭＥＤ））と一緒に使用する。可逆的ゲルを作製するため、代替的な架橋剤、例えば、Ｎ－Ｎ’ジアリル酒石酸ジアミド（ＤＡＴＤ）などが使用され得る。この化合物は、ＢＩＳに構造的に類似しているが、過ヨウ素酸によって（例えば、過ヨウ素酸ナトリウムを含有する溶液中）切断され得るシス－ジオール基を有する（Ａｎｋｅｒ，　Ｈ．Ｓ．：Ｆ．Ｅ．Ｂ．Ｓ．Ｌｅｔｔ．，　７：２９３，１９７０）。したがって、ＤＡＴＤゲルは、容易に可溶化され得る。架橋剤としてＤＡＴＤを用いて作製されたゲルは、高度に透明であり、ガラスに良好に結合する。可逆的ゲルを形成するＤＡＴＤ様特性を有する別の架橋剤は、エチレンジアクリレートである（Ｃｈｏｕｌｅｓ，　Ｇ．Ｌ．およびＺｉｍｍ，Ｂ．Ｓ．：Ａｎａｌ．Ｂｉｏｃｈｅｍ．，１３：３３６－３３９，１９６５）。Ｎ，Ｎ

’－ビスアクリリルシスタミン（ＢＡＣ）は、可逆的ポリアクリルアミドゲルを形成するために使用され得る別の架橋剤である。過ヨウ素酸塩に溶解されるゲルを形成するために使用され得る別の架橋剤はＮ，Ｎ’－（１，２－ジヒドロキシエチレン）ビス－アクリルアミド（ＤＨＥＢＡ）である。可逆的半固相支持体を形成する任意のさまざまな他の材料もまた使用され得る。例えば、熱可逆的ポリマー、例えば、Ｐｌｕｒｏｎｉｃｓ（ＢＡＳＦから入手可能）　などが使用され得る。Ｐｌｕｒｏｎｉｃｓは、ポリ（エチレンオキシド）－ポリ（プロピレンオキシド）－ポリ（エチレンオキシド）（ＰＥＯ－ＰＰＯ－ＰＥＯ）トリブロックコポリマーのファミリーの１つである（Ｎａｃｅ，　Ｖ．Ｍ．ら、Ｎｏｎｉｏｎｉｃ　Ｓｕｒｆａｃｔａｎｔｓ，　Ｍａｒｃｅｌ－Ｄｅｋｋｅｒ，　ＮＹ，１９９６）。これらの材料は、高温（例えば、室温より高い温度）で半固形（ゲル）になり、冷却すると液化する。種々の方法が、Ｐｌｕｒｏｎｉｃｓを化学的に誘導体化し、例えば、プライマーとの結合を助長するために使用され得る（例えば、Ｎｅｆｆ，　Ｊ．Ａ．ら、Ｊ．Ｂｉｏｍｅｄ．Ｍａｔｅｒ．Ｒｅｓ．，４０：５１１，１９９８；Ｐｒｕｄ’ｈｏｍｍｅ，　ＲＫら、Ｌａｎｇｍｕｉｒ，１２：４６５１，１９９６を参照）。

【0132】

可溶化後、微粒子を回収し、伸長、ライゲーションおよび切断の反復サイクルを用いた配列決定に供し得る。配列決定前に、微粒子を第２の半固相支持体内または該支持体上に、例えば、第１の半固相支持体内または該支持体上に存在したときより高い密度でアレイ状にしてもよい。半固相支持体は、典型的には、それ自体が、実質的に平面状で剛性の基材、例えばガラススライドによって支持されている。

【0133】

したがって、２つの一般的なアプローチは、半固相支持体内または該支持体上に包埋されたクローン鋳型集団を担持する微粒子のアレイを有する半固相支持体を作製するために使用され得る。第１のアプローチは、半固相支持体内に存在しない微粒子上で増幅を行ない（例えば、エマルジョン系ＰＣＲによって）、次いで、微粒子を半固相支持体内または該支持体上に固定化することを伴う。第２の一般的なアプローチは、微粒子を半固相支持体内または該支持体上に固定化し、次いで、増幅を行なうことを伴う。いずれの場合も、微粒子の凝集を低減させるため、および／または微粒子を実質的に単一の焦点面に整列させるための手順を用いることが望ましいことがあり得る。例えば、粒子をポリアクリルアミドゲル内に固定化させる場合、モノマーおよび架橋剤の濃度は、重合が完了する前に粒子が溶液の底面に沈降し、その結果、下方の平面状の基材上に沈殿し、したがって、単一の平面状に整列するように選択される。本発明のある特定の実施形態において、カバースリップなどの実質的に平面状の表面を有する物体を、微粒子を含有する液状アクリルアミド（または半固相支持体を形成できる他の材料）の上面に配置し、アクリルアミドが２つの層間に捕捉され「サンドイッチ」構造となるようにする。次いで、該サンドイッチを裏返し、重力の作用によって、微粒子が下方に沈降し、カバースリップ（実質的に平面状の表面を有する他の物体）上に堆積するようにする。重合後、カバースリップを取り除く。したがって、微粒子は、実質的に単一の平面状に半固相支持体の表面に近接して（例えば、表面に接して（ｔａｎｇｅｎｔ））包埋される。

【0134】

上記のように微粒子などの支持体を半固形マトリックス内に固定化するのではなく、本発明のある特定の実施形態では、微粒子を、固定化のための半固相支持体を使用することなく、共有結合または非共有結合のいずれかで実質的に平面状で剛性の基材に結合させ、それによって「ゲル非含有」または「ゲル無し」微粒子アレイをもたらす。微粒子を基材、例えば、ガラス、プラスチック、石英、シリコンなどなどに結合させるためのさまざまな方法が、当該技術分野で知られている。基材は、コーティングされたものであってもそうでなくてもよく（例えば、スピンコーティング）、または結合を助長する材料（例えば、任意のさまざまなポリマー）もしくは薬剤により官能性付与させたものであってもよい。コーティングは、薄膜、自己組織型単層などであり得る。微粒子、微粒子に付着した部分、または微粒子に付着したオリゴヌクレオチド（例えば、鋳型）のいずれかを基材に付

着させることができる。本発明のある実施形態において、基材はシラン化剤で処理されず、あるいはそのように処理されたとしても、その処理は有効なシラン化をもたらさず、すなわち例えば、本明細書に記載のライゲーションに基づく配列決定を複数回繰り返す時に行われるような、その後の操作および／または流体との接触に対して安定した形で、平坦なガラス表面上のポリアクリルアミド層により固定された微粒子のアレイを形成するように、シラン化が有効に作用しない。この文脈において「安定」とは、一般的に、操作および／または流体との接触時にゲルが基材に固着したまま維持され、著しい屈曲や、脱離、離層を生じないことを意味する。本発明者等は、微粒子アレイの作成にゲルなどの半固形培地の使用を避けることにより、多くの利点が得られることを認識した。例えば、（ｉ）半固形培地の非存在下では、試薬の拡散がより早くなり、かつライゲーションされていないプローブや酵素などの望ましくない種の除去もより早くなり、（ｉｉ）有効なシラン化が行われなければ、アクリルアミドなどのゲルが基材に安定して固着される状態が維持されず、（ｉｉｉ）重合は酸素などの環境特性に敏感であることから、重合手順を排除することで、アレイ生成プロセスにおける不均一性の潜在的な原因が除去され、（ｉｖ）半固形培地を使用しないことで、一つの焦点面により多くの微粒子が収まるようになり、（ｖ）微粒子は、基材に付着した場合の方が、半固形培地（特に重合が損なわれるもの）に埋め込まれた場合よりも、適切な位置により安定して固着される。

【0135】

一般的に、当該技術分野で既知の多様な方法のいずれを使用しても、このような核酸が微粒子あるいはその他の担体または基材に付着しやすくなるように、オリゴヌクレオチドプライマー、プローブ、鋳型などの核酸を修飾することができる。さらに、当該技術分野で既知の多様な方法のいずれを使用しても、核酸が付着しやすくなるように微粒子またはその他の担体を修飾して、担体や基材などに微粒子が付着しやすくなるようにすることができる。所望の官能基が付着しやすくなるような界面化学物質を有するミクロスフェアが使用可能である。これらの界面化学物質のいくつかの例には、アミノ基（例えば、脂肪族および芳香族アミン）、カルボン酸、アルデヒド、アミド、クロロメチル基、ヒドラジド、ヒドロキシル基、スルホン酸塩、および硫酸塩が含まれるが、これらに限定されない。これらの基は核酸中に存在する基と反応する場合があり、あるいは核酸が反応基の付着によって修飾される場合もある。さらに、ホモ二官能性およびヘテロ二官能性リンカーを含めた多くの安定した二官能基も当該技術分野で周知である。例えば、ＵＲＬ：ｗｗｗ．ｐｉｅｒｃｅｎｅｔ．ｃｏｍのウェブサイトで閲覧可能なＰｉｅｒｃｅ　Ｃｈｅｍｉｃａｌ　Ｔｅｃｈｎｉｃａｌ　Ｌｉｂｒａｒｙ（１９９４－９５　Ｐｉｅｒｃｅ　Ｃａｔａｌｏｇに最初に公開）、およびＧ．Ｔ．Ｈｅｒｍａｎｓｏｎ，Ｂｉｏｃｏｎｊｕｇａｔｅ　Ｔｅｃｈｎｉｑｕｅｓ，Ａｃａｄｅｍｉｃ　Ｐｒｅｓｓ，Ｉｎｃ．，１９９６を参照されたい。また、米国特許第６，６３２，６５５号も参照されたい。

【0136】

一般に、結合ペアを形成するように互いに対して親和性を示す任意の対の分子が、微粒子または鋳型を基材に結合させるために使用され得る。結合ペアの第１のメンバーを共有結合または非共有結合により基材に結合させ、結合ペアの第２のメンバーを共有結合または非共有結合により微粒子または鋳型に結合させる。説明上、結合対の第１のメンバー、すなわち基材に付着した結合パートナーは、本明細書でＢＰ１と呼び、結合対の第２のメンバー、すなわち微粒子または鋳型に付着した結合パートナーは、（ＢＰ２）と呼ぶ。第１の結合パートナー（ＢＰ１）をリンカーによって基材に結合させてもよい。第２の結合パートナー（ＢＰ２）をリンカーによって微粒子または鋳型に結合させてもよい。例えば、アプローチの一例によれば、スライドまたは他の適当な基質をアミン反応性基（例えば、アミン反応性基を含有するＰＥＧリンカーを使用）で修飾する。アミン反応性基は、水性条件で（例えば、ｐＨ８．０で）任意のタンパク質（例えば、ストレプトアビジン）中のアミン（例えば、リシン）と反応する。したがって、アミンを担持する部分により官能性付与させた微粒子は、基材上に固定化された状態になる。アミン担持する部分は、タンパク質または適当に官能性付与された核酸、例えば、ＤＮＡ鋳型であり得る。多数の部分

がビーズに結合され得る。例えば、ビーズは、ＮＨＳエステルと反応してビーズを基材に結合させる自身に結合されたタンパク質を有し得、また、ＤＮＡ自身に結合された鋳型を有し得、これは、ビーズが基材に結合された後、配列決定され得る。アミン反応性ＮＨＳ部分を一方の末端に有するポリマーテザー（ｔｅｔｈｅｒ）　を担持する適当にコーティングされスライドは、例えば、Ｓｃｈｏｔｔ　Ｎｅｘｔｅｒｉｏｎ，　Ｓｃｈｏｔｔ　Ｎｏｒｔｈ　Ａｍｅｒｉｃａ，　Ｉｎｃ．，　Ｅｌｍｓｆｏｒｄ，　ＮＹ　１０５２３から市販されている。あるいはまた、コーティングされたスライド（例えば、ビオチン－コートスライド）が、Ａｃｃｅｌｒ８　Ｔｅｃｈｎｏｌｏｇｙ　Ｃｏｒｐｏｒａｔｉｏｎ，　Ｄｅｎｖｅｒ，　ＣＯから市販されている。そのＯｐｔｉＣｈｅｍ^{TM}技術は、基材への微粒子の結合方法の代表であるが一例である。例えば、米国特許第６，８４４，０２８号を参照のこと。あるいはまた、微粒子は基材に、ビーズ上のポリヌクレオチドをビオチンにより官能性付与し（例えば、末端トランスフェラーゼとビオチン－ジデオキシＡＴＰおよび／またはビオチン－デオキシＡＴＰの使用によって）、次いで、これをストレプトアビジン－コーティングされたスライドのような基材（例えば、Ａｃｃｅｌｒ８　Ｔｅｃｈｎｏｌｏｇｙ　Ｃｏｒｐｏｒａｔｉｏｎ，　Ｄｅｎｖｅｒ，　ＣＯから入手可能）（米国特許第６，８４４，０２８号を参照のこと）と、ビオチン－ストレプトアビジン結合の形成を促進する条件下で接触させることにより結合させ得る。一実施形態において、ストレプトアビジンは、ＰＥＧリンカーを使用して基材に付着する。一実施形態において、微粒子結合ポリヌクレオチドは、合成後にビオチンで官能基化される。別の実施形態において、ビオチンは、例えばエマルジョンＰＣＲを実施する時などの増幅時に、ビオチン化プライマーを使用することにより、合成時にポリヌクレオチドに組み込まれる。例えば、第１のプライマーＰ１は、微粒子に共有結合的にまたは非共有結合的に付着する。微粒子に結合していない第２のプライマーＰ２はビオチン部分を含み、したがって、得られるＰＣＲ産物はビオチンを含む。

【0137】

そのため、本発明は、核酸鋳型が付着した微粒子を捕捉し、基材（例えば、ガラススライドなどの実質的に平面状で剛性の基材）の表面に、それらの微粒子を繋ぐ方法を提供する。特に興味深い実施形態においては、鋳型の種々のクローン群が付着した微粒子の群が作成され（例えば、エマルジョンＰＣＲを使用して）、鋳型がビオチン部分を含む。ビオチンは、増幅後に標準的な方法を使用して鋳型に付着する場合がある。次に、微粒子は、ビオチン結合部分（例えば、ストレプトアビジンなどのビオチン結合性タンパク質）が付着したガラススライドなど、実質的に平面状で剛性の基材と接触する。鋳型分子上のビオチンは、ビオチン結合部分に結合し、それによってビオチンおよびビオチン結合性タンパク質を含む連結を介して、微粒子を基材に付着させる。したがって、微粒子の基材への付着は、鋳型がテザーの役割を果たす間接的なものである場合がある。一実施形態において、鋳型分子の一端は、ビーズに付着したビオチン結合部分に付着し、鋳型分子の他端は、基材に付着したビオチン結合部分に付着する。

【0138】

ある実施形態において、一本鎖鋳型の一方の末端は微粒子に付着し、一本鎖鋳型の他方の末端は基材に付着する。したがって、一実施形態においては、一本鎖鋳型の３’および５’の両末端が、微粒子を基材に付着させる役割を果たす連結に関与し、第１の連結は微粒子と鋳型の間にあり、第２の連結は鋳型と基材との間にある。得られる構造は、ハイブリダイズした核酸が解離しやすくなる傾向があるとされる熱や他の条件に対しても安定している。

【0139】

実施例１６に記載の通り、ストレプトアビジンでコーティングされた微粒子に付着した鋳型は、合成後のエマルジョンＰＣＲ時にビオチン化することができ、得られるビオチン化した鋳型は、ストレプトアビジンでコーティングされた基材に効率的かつ強固に結合することが発見されている。一実施形態において、ビオチンとストレプトアビジンの連結は、本方法において以下の２つの段階で使用される：（ｉ）鋳型の増幅前に（例えば、エマ

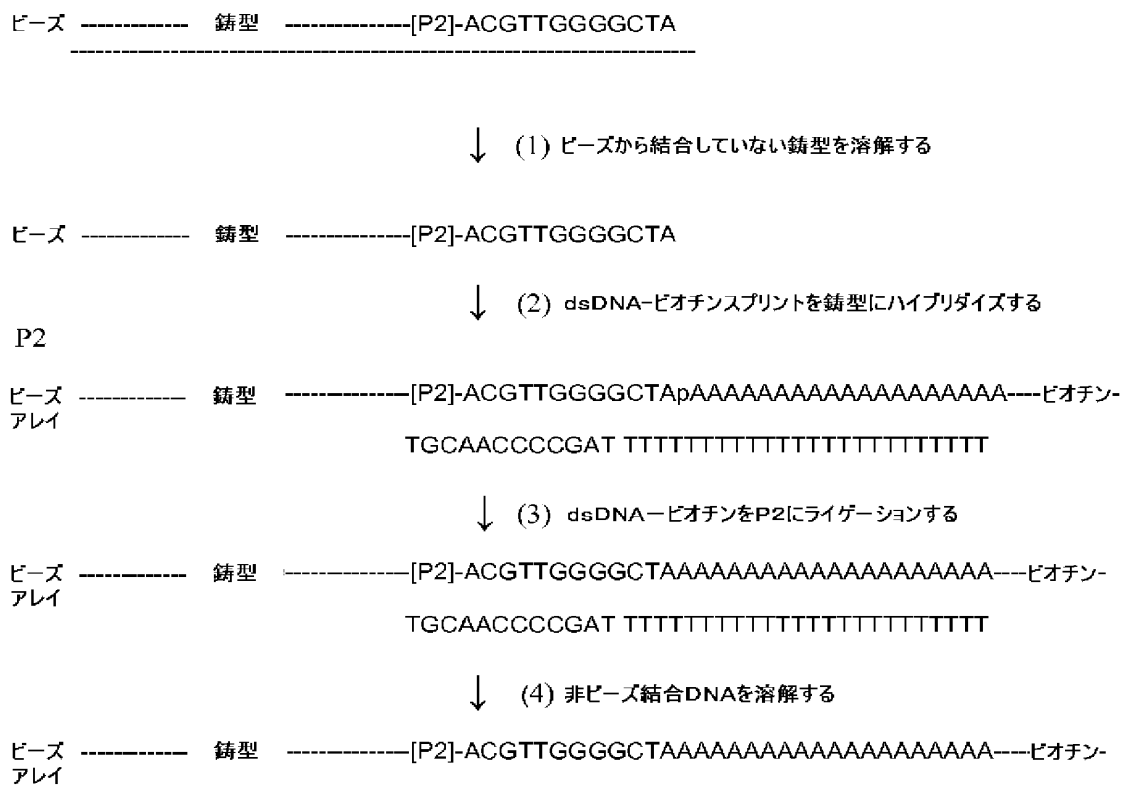
ルジョンＰＣＲ前に）、ビオチン化プライマーをストレプトアビジンでコーティングされた微粒子に付着させる；（ｉｉ）増幅後、自由端（微粒子に付着していない端部）がビオチン化された微粒子結合鋳型をストレプトアビジンでコーティングされた基材に付着させ、それによって微粒子も基材に固定する。場合により、手順（ｉ）の後、エマルジョンＰＣＲ（またはその他の増幅方法）を行った微粒子群において、増幅された微粒子を濃縮することもできる。手順（ｉｉ）の前、そして場合により濃縮の後には、ストレプトアビジンを露出した微粒子表面の部分を被覆するために、微粒子をビオチン化オリゴヌクレオチドとともにインキュベートすることもできる。これらの方法により、半固形培地を必要とすることなく、基材の表面に安定して付着した微粒子のアレイをもたらされる。特に興味深い実施形態において、基材は、ガラススライドなどの実質的に平面状で剛性の基材である。本明細書ではビオチンとストレプトアビジンの相互作用が例示されているが、ストレプトアビジンは、ビオチンに結合するいくつかのタンパク質の内の１つにすぎず、これらのタンパク質はいずれも本発明で使用できることが理解される。例えば、アビジンは卵白タンパク質であり、細菌性ストレプトアビジンのように、高い親和性と選択性を持ってビオチンに結合する。ＮｅｕｔｒＡｖｉｄｉｎは、炭水化物を除去する処理を行ったアビジンの誘導体である。ＣａｐｔＡｖｉｄｉｎは、ｐＨ９を超えるビオチン化分子に対する親和性を減少させたアビジンの誘導体である。したがって、ビオチン化分子は、中性のｐＨで結合させることができ、約ｐＨ１０で解放することができる。ＮｅｕｔｒＡｖｉｄｉｎおよびＣａｐｔＡｖｉｄｉｎについては、オンライン版のＴｈｅ　Ｈａｎｄｂｏｏｋ　ｏｆ　Ｆｌｕｏｒｅｓｃｅｎｔ　Ｐｒｏｂｅｓ　ａｎｄ　Ｒｅｓｅａｒｃｈ　Ｐｒｏｄｕｃｔｓ（ｈｔｔｐ：／／ｐｒｏｂｅｓ．ｉｎｖｉｔｒｏｇｅｎ．ｃｏｍ／ｈａｎｄｂｏｏｋ／ｓｅｃｔｉｏｎｓ／０７０６．ｈｔｍｌ；２００６年４月１７日閲覧）に記載されており、Ｉｎｖｉｔｒｏｇｅｎ（米国カリフォルニア州カールズバッド）から入手することができる。さらに、本発明は、特異的かつ高親和性の相互作用を示すいずれかの分子対の使用を包含する。例えば、特異的結合対のメンバーは、抗体と抗原、受容体と受容体リガンド（例えば、小分子またはペプチド）、金属と金属結合剤（例えば、Ｎｉ＋と６ＸＨｉｓタグ）などであると考えられる。本発明は、上述のいずれかの方法を使用して基材に付着した微粒子を提供し、さらに、種々の鋳型が付着している、基材に付着した微粒子を含むアレイも提供する。

【0140】

本発明のある実施形態において、ゲル非含有微粒子アレイの形成は、鋳型の複数の複製が付着した（例えば、鋳型の少なくとも数千、典型的には数百万の複製が付着した）微粒子を、鋳型の複数の複製が付着していない微粒子から分離する役割を果たす。一実施形態において、基材には第１の結合パートナー（ＢＰ１）が付着し、微粒子に付着した鋳型分子は第２の結合パートナー（ＢＰ２）を含み、ＢＰ１とＢＰ２は互いに特異的に結合し、すなわち、これらは特異的結合対のメンバーである。上述のようにゲル非含有微粒子アレイが形成されると、ＢＰ２が付着した鋳型を有する微粒子のみが基材に付着することになる。別の実施形態において、基材には第１の反応部分（Ｒ１）が付着しており、微粒子に付着した鋳型分子は第２の反応部分（Ｒ２）を含み、Ｒ１とＲ２は互いに反応して、共有結合を形成する。上述のようにゲル非含有微粒子アレイが形成されると、ＢＰ２またはＲ２が付着した鋳型を有する微粒子のみが基材に付着することになる。結合または反応させた後、付着していない微粒子は、例えば軽い撹拌および／または洗浄によって除去することができる。本方法は、典型的には、鋳型の種々のクローン群が付着した微粒子を含み、また鋳型の複数の複製が付着していない微粒子も含む、微粒子群に適用される。例えば、本方法は、鋳型の増幅が行われた（例えば、エマルジョンＰＣＲ時に）微粒子を、実質的な鋳型増幅が行われていない微粒子から分離させるために使用される場合がある。一実施形態において、本方法は以下の手順を含む：（ｉ）特異的結合対の第１のメンバーまたは反応部分が付着した基材を提供する（ｉｉ）（結合対のメンバー間、あるいは反応部分間で）結合を行うのに好適な条件下で、特異的結合対の第２のメンバーまたは反応部分が付着した鋳型の複数の複製を少なくともいくつか有する微粒子群と、基材を接触させる；（

ｉｉｉ）非結合微粒子を除去する。強い非共有結合連結を形成する特異的結合パートナー（例えば、ストレプトアビジンとビオチン）は、濃縮を達成するのに特に興味深い。別の実施形態において、相補性オリゴヌクレオチド間のハイブリダイゼーションが使用される。例えば、一実施形態においては、エマルジョンＰＣＲ時に鋳型に組み込まれる遊離ＰＣＲプライマー（遊離ＰＣＲプライマーとは、微粒子に付着していないプライマーである）の一部と相補的であるように選択されるオリゴヌクレオチドが、基材に付着する。遊離ＰＣＲプライマーは、増幅が成功した場合にのみ微粒子上に存在するため、鋳型増幅に成功した微粒子のみが基材に付着する。リガーゼを使用して、ハイブリダイゼーション事象の品質を確認し、ビオチン化スプリントまたはプライマーをビーズ上の鋳型の３’端に共有結合的に結合させる場合もある。例えば、以下の一連の手順を実施することができるが、これらの手順において、「ビーズ」とは微粒子を表し、Ｐ２とは増幅プライマー配列の少なくとも一部を表し、「ｄｓ」とは「二本鎖」を意味し、「アレイ」とは、増幅に成功した微粒子がビオチンを介して付着することができる基材を指す。二本鎖鋳型が付着した微粒子が提供される。第１の手順では、例えば温度を上昇させることによって、非結合鋳型が除去される。第２の手順では、一本鎖伸長物を有する二本鎖核酸が、鋳型にハイブリダイズされる。二本鎖核酸は、ビオチンを鋳型に安定して結合させることができるブリッジまたはスプリントの役割を果たす。一本鎖伸長物を有さない二本鎖核酸の鎖は、一本鎖伸長物とは反対の末端にビオチン部分が付着している。第３の手順では、リガーゼが存在する。ビオチンを含む二本鎖核酸は、ハイブリダイゼーションに成功した場合に鋳型にライゲーションされ、それによってビオチンを鋳型に安定して結合させる。第４の手順では、鋳型にライゲーションされなかったスプリントの鎖が、例えば温度を上昇させることによって解放される。ビオチンと、基材または担体に結合したストレプトアビジンとの相互作用によって、微粒子のアレイが作成される。

【数1】



本方法は、複数の鋳型が付着した微粒子を、複数の鋳型が付着していない、あるいは実質的により少ない鋳型が付着した微粒子から分離させるために使用することができ、これらの鋳型は増幅または合成後に微粒子に付着する。分離させる微粒子は、微粒子結合鋳型

の増幅または合成を行うか、あるいは増幅された鋳型の複数の複製が微粒子に付着することができるいずれかの種類の条件にさらされている場合がある。増幅方法は、ＰＣＲ増幅、ローリングサークル増幅、またはその他いずれかの種類の核酸増幅である場合がある。本方法は、本発明の他の方法および組成物のいずれかと組み合わせる、および／または併用することができる。接触手順は、典型的には液体培地内で行われる。本発明のある実施形態において、接触手順では、微粒子を含有する液体を、特異的結合対または反応部分が付着した基材に流す。基材は、例えば、流体入口と流体出口とを有するフローセルなどのチャンバー内に配置される場合がある。微粒子は、基材に付着した微粒子の所望の密度または数に達するまで、基材を流す場合がある。一定期間、密度または数の変化を監視する場合もある（例えば、画像化による）。特に興味深い実施形態において、本方法は、エマルジョンＰＣＲ時に増幅が行われた微粒子を、エマルジョンＰＣＲ時に実質的な鋳型の増幅が行われなかった微粒子から分離させるために使用される。本方法では、鋳型の増幅が行われた微粒子を濃縮する。基材に結合した微粒子に付着した鋳型には、種々のさらなる反応または操作を行うことができる。これらは、例えば、本明細書に記載のようなライゲーションに基づく配列決定、またはＦＩＳＳＥＱやパイロシーケンシングなどの他の配列決定法などの配列決定であってよい。例えば、半固形培地を使用せずに、および／または半固形培地の非存在下で、基材に付着した微粒子に付着した鋳型に対して、本明細書に記載の本発明の配列法のいずれも実施することができる。

【0141】

微粒子が基材または半固形培地に付着する本発明の実施形態のいずれにおいても、微粒子はその後に解放し、場合により除去することができる（例えば、洗浄による）。微粒子を解放するのに適切な方法は、微粒子が基材または半固形培地に付着する上で介する特定の共有結合または非共有結合連結によって異なる。ＤＮＡ鋳型を実質的に損傷しない限り、あるいは基材または半固形培地からＤＮＡ鋳型を解放しない限り、いずれの好適な方法も使用することができる。例えば、一実施形態において、微粒子は、例えばジスルフィドまたはエステル連結を含有するリンカーなどの開裂可能なリンカーによって基材または半固形培地に付着する。

【0142】

本発明のある実施形態において、微粒子は、半固形培地に安定して付着した鋳型のクローン群のアレイを作成するために使用される。本方法では、１つ以上の鋳型分子が付着した微粒子が、例えば実質的に平面状で剛性の基材上に位置するポリアクリルアミドゲルなど、基材上に位置する半固形培地の存在下でインキュベートされ、半固形培地に固定したおよび／または付着したプライマーに鋳型がハイブリダイズされる。次にプライマーが伸長され（例えば、ＤＮＡポリメラーゼを使用）、半固形培地に付着したまたは固定した相補性鋳型の合成を生じる。微粒子は、例えばインキュベーションのストリンジェンシーを上昇させることによって（例えば、温度を上昇させることによって）解放され、それによって２本の相補性鋳型鎖が分離する。例えば、微粒子に付着した鋳型を開裂させることによって、あるいは微粒子を鋳型から脱離させることによって、微粒子を解放する代替の方法を使用することもできると考えられる。

【0143】

本プロセスは、微粒子結合鋳型の複製または「インプリント」を半固形培地に転写する。本プロセスの効率は、微粒子から半固形培地に複製した鋳型分子の数を、微粒子に付着した鋳型分子の数で除算したものとして定義される場合がある。幾何学的および物理的考察に基づくと、決して本発明を限定するものではないが、２００ｂｐのサイズの鋳型分子約１５０，０００個が付着した直径１μｍの微粒子は、図４０に示す通り、直径約５００ｎｍの接触パッチを有する場合がある。接触パッチは、半固形培地または基材の中または上に位置するプライマーを伸長することによって、微粒子に付着したものと相補的な鋳型を合成できるように、培地の表面上に位置するかあるいは培地に部分的に埋め込んだ微粒子に十分に近接している、半固形培地または基材の領域を指す。具体的には、直径１ミクロンのビーズは、３．１×１０^{6} ｎｍ^{2} の面積を有し、したがってビーズ上の１５０，０

００個のＤＮＡ分子は、平均面積２０．９ｎｍ^{2}または平均距離４．５７ｎｍとなる。Ｂ－ＤＮＡの直径は約１．９ｎｍであり、２００ｂｐのＢ－ＤＮＡの長さは６８ｎｍである。したがって、６８ｎｍの分離点までの１ミクロンビーズの接触パッチは、半径２５２ｎｍまたは面積１９９，０００ｎｍ^{2}である。ＤＮＡ分子１個当たり２０．９ｎｍ^{2}で、パッチは９，５００個もの分子、またはビーズの下半分上の分子の数の約１３％を含有すると予想される。

【0144】

場合により、半固形培地と連結したままの鋳型の増幅が１回以上行われる。一実施形態において、増幅はローリングサークル増幅（ＲＣＡ；米国特許第５，８５４，０３３号；第６，１４３，４９５号）である。ＲＣＡを行う前に、（ｉ）鋳型の２つの非近接領域への環状化可能プローブ（「パドロックプローブ」）のハイブリダイゼーション、（ｉｉ）ポリメラーゼを使用した、発生したギャップの充填、（ｉｉｉ）末端のライゲーション、を含む手順が行われる場合がある。ＲＣＡで使用する鋳型分子は、配列決定する部分だけでなく、環状化可能なプローブと相補的な領域も含まなければならないことが理解される。

【0145】

プライマー伸長と場合による増幅によって、半固形培地に付着または固定した、「スポット」のアレイまたは核酸「コロニー」がもたらされる。コロニーは、微粒子を沈着した位置に対応する場所に位置する。コロニーの多くまたはほとんどは、鋳型の単一のクローン群、あるいは本発明のある実施形態においては、鋳型の２つまたは数個までのクローン群（微粒子に複数種類の鋳型が付着していた場合）から構成される。類似の手法を使用して、プライマーを、基材上に位置する半固形培地ではなく、基材自体に付着させることによって、半固形培地を使用することなく、ガラススライドなどの基材上に核酸コロニーのアレイを直接作成することもできると考えられる。

【0146】

いかなる理論にも拘束されるものではないが、上述のように微粒子を使用して核酸コロニーのアレイを形成することで、多くの利点が得られる。微粒子は、アレイを形成するために使用する前に、鋳型の増幅を行うことができ、場合により濃縮も行うことができ、それによって各核酸スポットは、単一の鋳型の増幅によってではなく、単一の微粒子に由来する鋳型の複数の複製の増幅によって生じることになる。さらに、半固形培地の表面上に互いに対して近接して配置できる微粒子を使用することで、半固形培地の表面の効果的な使用が可能になるとともに、検出時に互いに容易に区別できる離散したスポットももたらされる。スポットは、典型的には微粒子よりもサイズが小さく、互いにより明確に区別できるようになる。例えば、粒子と平坦な表面との接点の２５０ｎｍ以内に位置する直径１ミクロンの粒子上のＤＮＡが、平坦な表面に付着し、複製される場合には、粒子が解放された後に、直径５００ｎｍのＤＮＡパッチが表面にできることになる。１ミクロンのビーズ２つが接している場合には、あとにできるＤＮＡパッチの中心が１ミクロン離れており、各パッチの最も近い端部の間に５００ｎｍの空間ができる。ガラススライドなどの小さな基材の表面上に数百万の微粒子を充填する能力により、本プロセスは、近隣のコロニーに干渉されずに容易に画像化でき、また複数の配列決定サイクルにわたって簡単かつ信頼性の高い検出が可能となる十分な数の鋳型分子を含有する、鋳型コロニーの高密度アレイを実現する効率的な手段を提供する。

【0147】

基材に結合した微粒子に付着した鋳型には、種々のさらなる反応または操作を行うことができる。これは、例えば、本明細書に記載のようなライゲーションに基づく配列決定、またはＦＩＳＳＥＱやパイロシーケンシングなどの他の配列決定方法などの配列決定であってよい。例えば、上述のように微粒子を使用して形成される、半固形培地内の核酸コロニーに存在する鋳型に対して、本明細書に記載の本発明の配列法のいずれも実施することができる。

【0148】

本明細書に記載の方法に従って形成される微粒子または核酸コロニーのアレイは、一般的にランダムであり得る。本明細書で用いる場合、用語「ランダムパターンの」または「ランダム」は、不規則な非Ｃａｒｔｅｓｉａｎ分布（換言すると、格子のｘ軸およびｙ軸に沿った所定の点もしくは位置、または放射状パターンの中心からの角度もしくは半径の規定の「時計位置」で整列していない）支持体上での正体（特徴）であって、意図的な設計（もしくはかかる設計が達成され得るプログラム）または個々の正体の配置によって得られないものをいう。かかる「ランダムパターンの」または「ランダム」アレイの正体は、正体のプールを含む溶液、エマルジョン、エーロゾル、気体状のまたは乾燥した調製物を、支持体上または支持体内に滴下、噴霧、めっき、塗布、分布などさせ、支持体内または支持体上の特定の部位に指向させるなんらかの様式の介在なく支持体上または支持体内に沈降させることにより得られ得る。例えば、正体は、半固相支持体の前駆物質（例えば、アクリルアミドモノマー）を含有する溶液中に懸濁させ得る。次いで、この溶液を、第２の支持体上に分布させ、半固相支持体を第２の支持体上に形成させる。正体は、半固相支持体内または該支持体上に包埋される。もちろん、非ランダムアレイもまた使用され得る。微粒子の密接な充填は、規則正しいグリッド様のアレイの微粒子またはそれから合成される核酸コロニーをもたらし得る。一般的に、本明細書において使用されるアレイを形成するための方法は、例えば、ポリヌクレオチドの合成が、基質上の予め規定された位置の個々のヌクレオチドサブユニットの逐次適用によって行なわれる方法と異なる。

【0149】

図１４Ｂ（上）は、その上面にポリアクリルアミドゲルを有するスライド（１インチ×３インチ）の蛍光画像を示す。ビーズに結合させた鋳型にハイブリダイズされた蛍光標識されたオリゴヌクレオチドを有するビーズ（１ミクロン直径）は、ゲル内に固定化されている。この画像は、およそ２８００００００００ビーズ／スライドの画像化に充分なビーズ表面密度（すなわち、ビーズが位置する領域内の基材の単位面積あたりのビーズの数）を示す。表面密度および画像化可能な領域は、単一のスライド上で少なくとも５００００００００ビーズの画像化に充分である。例えば、図１４Ｂ（下）は、ビーズが半固相支持体層内に包埋されるポリアクリルアミドゲルなどの透明な領域を覆うテフロン（登録商標）マスクを有するスライドの概略図を示す。このマスクの面積は８６４ｍｍ^{2}である。５００００００００個のビーズで、表面密度は５７８，０００ビーズ／ｍｍ^{2}である。１ミクロンビーズの最密六方アレイでは１，１５５，０００ビーズ／ｍｍ^{2}となるため、この実施形態は、理論最大密度の５２％を有するアレイをもたらす。この特定の実施形態より少数およびより多数のビーズ、およびより大きいかより小さいビーズ表面密度が使用され得ることは認識されよう。

【0150】

微粒子は、実質的に平面状の半固相支持体内もしくは該支持体上で、または別の支持体もしくは基材上で、いくつかの様式で規定され得るさまざまな密度でアレイ状であり得る。例えば、密度は、実質的に平面状のアレイの単位面積あたりの微粒子の数（例えば、球状微粒子）に関して表示され得る。本発明のある特定の実施形態において、実質的に平面状のアレイの単位面積あたりの微粒子の数は、六方アレイ内の微粒子の数の少なくとも８０％である。（「六方アレイ」により、米国特許第６，４０６，８４８号に記載のようなアレイ内のどの微粒子も少なくとも６個の他の隣接微粒子と等しい面積で接触している微粒子の実質的に平面状のアレイが意図される）。しかしながら、本発明の他の実施形態において、微粒子密度は低く、例えば、実質的に平面状のアレイの単位面積あたりの微粒子の数は、六方アレイ内の微粒子の数の８０％未満、７０％未満、６０％未満または５０％未満である。なんら理論に拘束されることを望まないが、例えば、酵素、プライマー、補因子などの試薬の充分な拡散を可能にするため、およびある種の試薬が微粒子に対して異なる親和性を有するか、その内部の捕捉された場合に起こり得る試薬分配効果を回避するため、これらなどより小さい密度を用いることが好ましいこと場合があり得る。かかる効果は、アレイ上の異なる位置で異なる反応条件をもたらし得、これらの試薬がアレイ上のある特定の位置に接近するのを妨げることすらあり得る。このような問題は、反応がフロ

ーセル内で行なわれる場合、試薬がフローセル内を方向的に移動するため、悪化し得る。本発明のある特定の実施形態において、混合装置、例えば、機械的または音波的手段によって液体混合を行なう装置をフローセルのチャンバ内に含める。いくつかの適当な混合装置が、当該技術分野で知られている。

【0151】

本発明の配列決定法は、あらゆる型（例えば、ランダムおよび非ランダム両方のアレイ）のアレイ形式に整列させた鋳型を用いて行なわれ、アレイは、微粒子のアレイまたは鋳型自体のアレイであり得る。例えば、その上面にアレイ状の鋳型を有する支持体は、米国特許第５，６４１，６５８号およびＰＣＴ公開公報ＷＯ００１８９５７に記載されている。アレイは、広範なさまざまな基材、例えば、フィルター、膜（例えば、ナイロン）、金属表面などの上に配置させ得る。その上面で伸長、ライゲーションおよび切断の反復サイクルによる配列決定が行なわれ得るアレイ形式のさらなる例は、ウェル内で光ファイバーの束の個々の光ファイバーの末端または遠位端に配置したビーズのアレイである。例えば、ＵＳ公開公報および特許、例えば、６，０２３，５４０；６，４２９，０２７、２００４０１８５４８３、２００２１８７５１５、ＰＣＴ出願ＵＳ９８／０５０２５およびＰＣＴ　ＵＳ９８／０９１６３ならびにＰＣＴ公開公報ＷＯ００３９５８７に記載されたビーズアレイおよび「アレイのアレイ」を参照のこと。自身に結合された鋳型を有するビーズは、本明細書に記載のようなアレイ状であり得る。増幅は、好ましくは、アレイの形成前に行なわれる。かかる基材上に形成されたアレイは、必ずしも実質的に平面状である必要はない。

【0152】

他の実施形態において、ＰＣＲは、基材または支持体に結合されたオリゴヌクレオチドを含むアレイ上で行なわれる（例えば、米国特許第５，７４４，３０５号；同第５，８００，９９２号；同第６，６４６，２４３号および関連特許（Ａｆｆｙｍｅｔｒｉｘ）；ＰＣＴ公開公報ＷＯ２００４０２９５８６；ＷＯ０３０６５０３８；ＷＯ０３０４０４１０（Ｎｉｍｂｌｅｇｅｎ）　を参照）。一般に、かかるオリゴヌクレオチドは、遊離３’または５’末端を有する。所望の場合は、該末端は、例えば、リン酸基またはＯＨ基を３’末端に（既に存在しない場合）付加することにより修飾され得る。支持体または基材に結合されたオリゴヌクレオチドに相補的な領域を含む鋳型分子は、該オリゴヌクレオチドにハイブリダイズされ、ＰＣＲがアレイ上でインサイチュで行なわれ、クローン鋳型集団がアレイ上の各位置にもたらされる。アレイに結合されたオリゴヌクレオチドは、増幅プライマーの１つとしての機能を果たし得る。次いで、鋳型を本明細書に記載のライゲーション系の方法を用いて配列決定する。配列決定はまた、Ｕ．Ｓ．公開公報２００３００６８６２９に記載のように、アレイ内の鋳型上で行なわれ得る。

【0153】

表面上へのＤＮＡアレイの作製のためのさらに他の方法が使用され得る。例えば、末端アルデヒド基で修飾されたアルカンチオールが、自己組織型単層（ＳＡＭ）を金表面上に作製するために使用され得る。該単層のアルデヒド基は、アミン修飾オリゴヌクレオチドまたは他のアミン担持生体分子と反応し、シッフ塩基を形成し得、次いで、これは、シアノ水素化ホウ素ナトリウムでの処理によって安定な第２級アミンに還元され得る（Ｐｅｅｌｅｎ　＆　Ｓｍｉｔｈ，　Ｌａｎｇｍｕｉｒ，　２１（１）：２６６－７１，　２００５）。次いで、鋳型のＰＣＲ増幅が行なわれ得る。あるいはまた、自身に結合された鋳型のクローン性の集団を有する微粒子を、微粒子または鋳型上のアミン基または粒子に結合されたオリゴヌクレオチドをかかる表面と反応させることによりにより表面に結合させ得る。

【0154】

自身に結合されたクローン鋳型集団を有する微粒子を得るさらに別の方法は、米国特許第５，６０４，０９７号に記載された「固相クローニング」アプローチであり、これは、同じ配列のポリヌクレオチドだけが任意の特定の微粒子に結合されるような、微粒子上のポリヌクレオチドを分取するためのオリゴヌクレオチドタグを利用する。

【0155】
本発明のある特定の実施形態において、伸長、ライゲーションおよび切断の反復サイクルによる配列決定は、配列決定試薬（例えば、伸長プローブ、リガーゼ、ホスファターゼなど）を支持体内または支持体上に固定化された鋳型のクローン性の集団を有する半固相支持体（例えば、ゲル）中に、各クローン性の集団が支持体の空間的に異なる領域に局在化されるように拡散させることによって行なわれる。ある特定の実施形態において、鋳型は、上記のように、半固相支持体に直接結合させる。しかしながら、他の実施形態では、代わりに半固相支持体内または該支持体上に固定化させた微粒子などの第２の支持体に、鋳型を固定化させる（これも上記のようのとおり）。
【0156】
実施例１に記載のように、本発明者らは、ロバスト（ｒｏｂｕｓｔ）なライゲーションおよび切断が、ポリアクリルアミドゲル内に固定化した鋳型結合ビーズ上で行なわれ得ることを示した。したがって、本発明は、（ａ）半固相支持体内または該支持体上に固定化された第１のポリヌクレオチドを提供する工程；（ｂ）第１のポリヌクレオチドを第２のポリヌクレオチドおよびリガーゼと接触させる工程；および（ｃ）第１および第２のポリヌクレオチドを、リガーゼの存在下ライゲーションに適した条件下で維持する工程を含む、第１のポリヌクレオチドを第２のポリヌクレオチドにライゲートさせる方法を提供する。好適な条件としては、使用される具体的なリガーゼに適切なバッファー、補因子、温度、時間などの提供が挙げられる。好ましい実施形態において、半固相支持体は、アクリルアミドゲルなどのゲルである。さらに好ましい実施形態において、第１のポリヌクレオチドは、それ自体が、例えば、部分的または完全に包埋させることにより支持体マトリックス内に半固相支持体内または該支持体上に固定化されたビーズなどの支持体への結合の結果、半固相支持体内または該支持体上に固定化されている。あるいはまた、第１のポリヌクレオチドは、アクリダイト部分などの連結部によって半固相支持体に直接結合させ得る。連結は共有結合または非共有結合であり得る（例えば、ビオチン－アビジン相互作用による）。米国特許第６，５１１，８０３号には、核酸分子を本発明の好ましい支持体、すなわち、ポリアクリルアミドゲルに（ａ）結合させるために使用され得るさまざまな方法が記載されている。
【0157】
本発明は、さらに、（ａ）半固相支持体内または該支持体上に固定化され、切れやすい連結を含むポリヌクレオチドを提供する工程；（ｂ）該ポリヌクレオチドを切断剤と接触させる工程；および（ｃ）該ポリヌクレオチドを該切断剤の存在下、切断に適した条件下で維持する工程を含む、ポリヌクレオチドを切断する方法を提供する。好適な条件としては、特定の切断剤に適切なバッファー、温度、時間などの提供が挙げられる。好ましい実施形態において、半固相支持体は、アクリルアミドゲルなどのゲルである。さらに好ましい実施形態において、ポリヌクレオチドは、それ自体が半固相支持体内に固定化されたビーズなどの支持体への結合の結果、半固相支持体に固定化されている。あるいはまた、ポリヌクレオチドは、アクリダイト部分などの連結部によって半固相支持体に直接結合させ得る。連結は共有結合または非共有結合であり得る（例えば、ビオチン－アビジン相互作用による）。当然ながら、本明細書に記載の多くの方法にしたがって調製されるＤＮＡ鋳型は、典型的には配列決定する領域を含有し、また３’端および５’端のいずれかまたは両方に保存プライミング領域も含有する（ＰＢＲ）。「保存」または「共通」領域とは、配列決定する種々の領域を含有する複数の鋳型に共通する配列を指し、すなわち、鋳型は、配列の一部は異なるものの、同一の部分も含有する。鋳型は、１つ以上の保存内部アダプター配列を含有する場合もある。さらに、ＤＮＡ鋳型のローリングサークル増幅（ＲＣＡ）は、これらの保存配列のさらなる複製を作成するだけでなく、ＲＣＡプローブからさらに別の領域の保存配列の複製も導入する。そのため、配列決定するライブラリー分子の部分（「標的領域」、「対象部分」などとも呼ばれる）は、実際の鋳型核酸の半分未満に相当する場合がある。本発明は、これらの既知の／共通する非標的領域が、一本鎖の場合に、配列決定プローブを封鎖することができ、また配列決定プライマー（例えば、開始オ

リゴヌクレオチド）のミスプライムの可能性がある部位であるという認識を包含する。本発明は、ポリヌクレオチド鋳型中に存在する非標的配列と相補的なブロッキングオリゴヌクレオチドを提供する。本明細書で使用される「ブロッキングオリゴヌクレオチド」とは、鋳型の非標的配列に安定してハイブリダイズするオリゴヌクレオチドであり、この非標的配列は、配列決定に好適な条件下で種々の標的領域を含む複数の鋳型に共通する。非標的領域は、開始オリゴヌクレオチドが結合するとされる領域とは異なる。本発明はさらに、１つ以上のブロッキングオリゴヌクレオチドがハイブリダイズするポリヌクレオチド鋳型も提供する。

【0158】

本発明のある実施形態において、鋳型はエマルジョンＰＣＲを使用して合成される。

【0159】

特に興味深い実施形態において、ＤＮＡ鋳型は、断片ライブラリーのメンバーであり、図３６Ｂに示す通り前方および後方アダプターを含有する。第１のブロッキングオリゴヌクレオチドは前方アダプターと相補的であり、第２のブロッキングオリゴヌクレオチドは後方アダプターと相補的である。他の実施形態において、ＤＮＡ鋳型は、対合末端ライブラリーのメンバーであり、図３６Ａに示す通り、前方および後方アダプターと、さらに内部アダプターも含有する。第１のブロッキングオリゴヌクレオチドは前方アダプターと相補的であり、第２のブロッキングオリゴヌクレオチドは後方アダプターと相補的であり、第３のブロッキングオリゴヌクレオチドは内部アダプターと相補的である。他の実施形態において、鋳型は、ＲＣＡを使用して増幅され、図３６Ｃおよび図３７に示す通りアダプター領域とパドロック領域とを含有する。ブロッキングオリゴヌクレオチドは、鋳型に存在するアダプター領域およびパドロック領域と相補的である。ＲＣＡでは、パドロックプローブがポリメラーゼによって複製され、その相補体が生成されることが理解される。したがって、鋳型に存在するＲＣＡ相補体をブロックするために、パドロックプローブと同じ配列が、ブロッキングオリゴヌクレオチドとして使用される。図３６および図３７に示す特異的なオリゴヌクレオチドとそれらの相補体は、本発明の態様であるが、ブロッキングオリゴヌクレオチドの配列は、鋳型中に存在し、様々に異なることがある特定の保存配列と相補的になるように選択されることが認識される。さらに、本発明には、図３６または図３７に示すものと１個、２個、３個、４個または５個のヌクレオチドのみ配列が異なるオリゴヌクレオチドが含まれる。

【0160】

決して本発明を限定するものではないが、本発明者等は、例えば、鋳型の複雑性を低減させるツールとして作用する、ミスプライムの可能性がある部位を排除する、および／または鋳型の標的領域への伸長オリゴヌクレオチドのアクセスを容易にすることにより、前述の問題、あるいはこれらの共通配列の多くの複製が存在するために生じる場合があるその他の問題に対抗するために、ブロッキングオリゴヌクレオチドが使用される場合があることを認識した。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、例えばシグナル／ノイズ比の向上など、配列決定効率の向上をもたらす。

【0161】

ブロッキングオリゴヌクレオチドは、典型的には、配列決定プライマーのアニーリング前に一本鎖鋳型ＤＮＡにハイブリダイズされ、これらの領域がその後に配列決定プライマー（例えば、ライゲーションに基づく配列決定における開始オリゴヌクレオチド）またはプローブ（例えば、ライゲーションに基づく配列決定における伸長プローブ）のいずれかとハイブリダイズするのが防止される。これらのオリゴヌクレオチドは、典型的にはライゲーション、検出、（および、伸長オリゴヌクレオチドが開裂される本発明の実施形態においては、開裂）の連続サイクルを通して存在し続けるとされる。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、ポリメラーゼまたはリガーゼの基材ではなく、例えば、これらのオリゴヌクレオチドは、典型的なポリメラーゼまたはリガーゼ酵素によって酵素的に伸長可能ではない。一実施形態において、ブロッキングオリゴヌクレオチドは、３’ヒドロキシル基および５’リン酸塩が欠損している。これらの基は、存在

しない場合もあれば、合成後に除去される場合もあり、あるいはオリゴヌクレオチドの３’端および／または５’端が、伸長またはライゲーションの基材ではない部分でキャップまたはブロックされる場合もある。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、３’末端ジデオキシヌクレオシドを含む。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、３’末端ジデオキシシトシン（３‘ｄｄＣ）を含む。本発明のある実施形態において、対合タグライブラリーとともに使用するパドロックプローブは、単一タグそれぞれ（タグ１のみ、タグ２のみ）の、あるいは両タグの間（タグ１‐内部‐タグ２）のＲＣＡが可能となるように設計される（図３７）。

【0162】

ブロッキングオリゴヌクレオチドは、保存領域よりも短くてよく、すなわち、これらのオリゴヌクレオチドは、保存領域の一部のみと相補的であってよい。ブロッキングオリゴヌクレオチドは、保存領域と完全に相補的である必要はないが、そうであるのが好ましい場合がある。典型的には、ブロッキングオリゴヌクレオチドは、保存領域のすべてまたは一部と少なくとも８０％、好ましくは少なくとも９０％相補的となる。ブロッキングオリゴヌクレオチドのサイズは、ブロックする共通配列の長さによって変動する場合がある。典型的な長さは、１０～５０ヌクレオチドである。それぞれがブロックする保存領域の一部と相補的である複数のブロッキングオリゴヌクレオチドが、単一のより長いオリゴヌクレオチドの代わりに使用される場合もある。

【0163】

ブロッキングオリゴヌクレオチドは、本明細書に記載のようなライゲーションに基づく配列決定において特定の用途を見出す場合がある。したがって、本明細書に記載のいずれの方法も、鋳型を開始オリゴヌクレオチドと接触させる前に、プローブと鋳型の二本鎖を形成または提供する前に、および／または伸長した二本鎖を形成する前に、鋳型ポリヌクレオチドを１つ以上のブロッキングオリゴヌクレオチドと接触させる手順を含む場合がある。但し、ブロッキングオリゴヌクレオチドはまた、ＦＩＳＳＥＱやパイロシーケンシングなどの他の配列決定法を実施する場合に使用される場合もある。

【0164】

Ｄ．種々の初期オリゴヌクレオチドを用いた再度初期化を伴う配列決定

本発明の好ましい実施形態において、第１の初期オリゴヌクレオチドを伸長させることによって生成された伸長鎖は、充分なサイクル数の後、鋳型から除去され、第２の初期オリゴヌクレオチドが結合領域にアニーリングされた後、伸長、ライゲーションおよび検出のサイクルが行なわれる。該プロセスは、任意の数の異なる初期オリゴヌクレオチドを用いて反復される。伸長プローブが切断される実施形態では、好ましくは、使用される異なる初期オリゴヌクレオチドの数（したがって、反応の数）は、プローブの遠位部分の放出後に鋳型にハイブリダイズされたままの伸長プローブの部分の長さに等しい。したがって、この実施形態によれば、配列情報（例えば、各ヌクレオチドの順番および正体）は、単一の支持体に結合された鋳型から得られ得るが、連続したヌクレオチドが各サイクルで同定される場合に必要とされ得るよりも実質的に少ないサイクルを用いて、さらに深く読み出しが行なわれる。

【0165】

初期オリゴヌクレオチドが同じ鋳型に連続的に結合される実施形態は、Ｍａｃｅｖｉｃｚにより教示された方法などの、鋳型を多数のアリコートに分割することを要するアプローチと比べてある種の利点を有する。例えば、初期オリゴヌクレオチドを同じ鋳型に適用することにより、多数のアリコートから取得されたデータを追跡し、後で組み合わせる必要性が回避される。個々の支持体の位置が所定でないような支持体がランダム様式のアレイ状である実施形態では、各々が自身に結合された同じ配列の鋳型を有する多数の支持体からの部分配列情報を信頼できるように組み合わせることは困難または不可能であり得る。

【0166】

Ｅ．単一の鋳型における各サイクルでの多数のヌクレオチドの同定

Ｍａｃｅｖｉｃｚには、伸長、ライゲーションおよび検出の各サイクルにおける鋳型内の単一のヌクレオチドの同定が教示されている。しかしながら、本発明者らは、該方法は、各サイクルで鋳型内の多数のヌクレオチドの同定が可能となるように変形し得ると認識した。この場合、伸長プローブは、伸長された二本鎖に接合された２つ以上の、好ましくは連続したヌクレオチドの正体が標識から決定され得るように標識される。換言すると、伸長プローブの配列決定部分は、１つより多くのヌクレオチドであり、典型的には、近位ヌクレオチド、直接隣接ヌクレオチド、およびおそらく１つ以上のさらなる、好ましくは連続したヌクレオチドを含み、これらはすべて、鋳型に特異的にハイブリダイズする。例えば、塩基Ａ、Ｇ、ＣおよびＴを特定する４つの標識を用いるのではなく、１６個の識別可能に標識されたプローブまたはプローブの組合せが、１６個の可能なジヌクレオチドＡＡ、ＡＧ、ＡＣ、ＡＴ、ＧＡ、ＧＧ、ＧＣ、ＧＴ、ＣＡ、ＣＧ、ＣＣ、ＣＴ、ＴＡ、ＴＧ、ＴＣおよびＴＴを特定するために使用される。各識別可能に標識された伸長プローブの配列決定部分は、これらのジヌクレオチドの１つに相補的である。より多くの標識を用いる同様の方法により、各サイクルで、より長鎖のヌクレオチド配列の同定が可能になる。

【0167】

Ｆ．標識

用語「標識」は、本明細書において、プローブと結合または会合した任意の検出可能な部分または複数の検出可能な部分であって、それにより異なる種のプローブ（例えば、異なる末端ヌクレオチドを有するプローブ）が互いに識別され得る部分を表すために広義で用いる。したがって、標識と特異的検出可能な部分との間に１対１の対応がある必要はない。例えば、多数の検出可能な部分が単一のプローブに結合され得、プローブが、自身に結合された異なる検出可能な部分または検出可能な部分の組を有するプローブと識別されるのを可能にする複合シグナルがもたらされ得る。例えば、検出可能な部分の組合せが、米国特許第６，６３２，６０９号およびＳｐｅｉｃｈｅｒら、Ｎａｔｕｒｅ　Ｇｅｎｅｔｉｃｓ，１２：３６８－３７５，１９９６に記載された「コンビナトリアルマルチカラーコーディング（Ｃｏｍｂｉｎａｔｏｒｉａｌ　Ｍｕｌｔｉｃｏｌｏｒ　Ｃｏｄｉｎｇ）」とよばれる標識スキームに従って使用され得る。

【0168】

本発明のプローブは、さまざまな様式、例えば、基質と接触させると検出可能なシグナルを生成する蛍光性または化学発光性部分、比色性部分、酵素性部分などの直接または間接的結合によって標識され得る。Ｍａｃｅｖｉｃｚには、プローブを蛍光染料、例えば、Ｍｅｎｃｈｅｎら，米国特許第５，１８８，９３４号；Ｂｅｇｏｔら　ＰＣＴ出願ＰＣＴ／ＵＳ９０１０５５６５に開示されているもので標識させ得ることが教示されている。用語「蛍光染料」および「フルオロフォア」は、本明細書で用いる場合、規定の励起波長の光エネルギーを吸収し、異なる波長の光エネルギーを放出する部分をいう。好ましくは、所与のプローブ混合物での使用のために選択される標識は、スペクトルにより分離可能なものである。本明細書で用いる場合、「スペクトルにより分離可能な」は、標識が、作業条件下で、そのスペクトル特性、特に蛍光発光波長に基づいて識別され得ることを意味する。例えば、１つ以上の末端ヌクレオチドの正体は、最大光発光強度の異なる波長、またはおそらく異なる波長での強度比と相関し得る。標識を検出および同定するために用いられる標識のスペクトル特性（１つまたは複数）を、本明細書では「色」という。標識が、特異的スペクトル特性、例えば、単一の検出可能な部分からなる標識の場合は最大発光強度の周波数、または、多数の検出可能な部分からなる標識の場合は発光ピークの周波数に基づいて高頻度で同定されることは認識されよう。

【0169】

好ましくは、４種類のスペクトルにより分離可能な蛍光染料の各々と、４つの可能なプローブ末端ヌクレオチドとの１対１対応を可能にする４種類のプローブが提供される。スペクトルにより分離可能な色素の組は、米国特許第４，８５５，２２５号および同第５，１８８，９３４号；国際出願ＰＣＴ７ＵＳ９０／０５５６５；ならびにＬｅｅら，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓｅａｒｃｈｓ，２０：２４７１－２４８３（１９９２）

に開示されている。ある特定の実施形態において、ＦＩＴＣ、ＨＥＸ™、テキサスレッドおよびＣｙ５からなる組が好ましい。数多くの好適な蛍光染料が、例えば、Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，　Ｉｎｃ．，　Ｅｕｇｅｎｅ　ＯＲから市販されている。蛍光染料の具体例としては、限定されないが、Ａｌｅｘａ　Ｆｌｕｏｒ色素（Ａｌｅｘａ　Ｆｌｕｏｒ　３５０、Ａｌｅｘａ　Ｆｌｕｏｒ　４８８、Ａｌｅｘａ　Ｆｌｕｏｒ　５３２、Ａｌｅｘａ　Ｆｌｕｏｒ　５４６、Ａｌｅｘａ　Ｆｌｕｏｒ　５６８、Ａｌｅｘａ　Ｆｌｕｏｒ　５９４、Ａｌｅｘａ　Ｆｌｕｏｒ　６３３、Ａｌｅｘａ　Ｆｌｕｏｒ　６６０　ａｎｄ　Ａｌｅｘａ　Ｆｌｕｏｒ　６８０）、ＡＭＣＡ、ＡＭＣＡ－Ｓ、ＢＯＤＩＰＹ色素（ＢＯＤＩＰＹ　ＦＬ、ＢＯＤＩＰＹ　Ｒ６Ｇ、ＢＯＤＩＰＹ　ＴＭＲ、ＢＯＤＩＰＹ　ＴＲ、ＢＯＤＩＰＹ　５３０／５５０、ＢＯＤＩＰＹ　５５８／５６８、ＢＯＤＩＰＹ　５６４／５７０、ＢＯＤＩＰＹ　５７６／５８９、ＢＯＤＩＰＹ　５８１／５９１、ＢＯＤＩＰＹ　６３０／６５０、ＢＯＤＩＰＹ　６５０／６６５）、ＣＡＬ色素、カルボキシローダミン６Ｇ、カルボキシ－Ｘ－ローダミン（ＲＯＸ）、Ｃａｓｃａｄｅ　Ｂｌｕｅ、Ｃａｓｃａｄｅ　Ｙｅｌｌｏｗ、シアニン色素（Ｃｙ３、Ｃｙ５、Ｃｙ３．５、Ｃｙ５．５）、Ｄａｎｓｙｌ、Ｄａｐｏｘｙｌ、ジアルキルアミノクマリン、４’，５’－ジクロロ－２’，７’－ジメトキシ－フルオレセイン、ＤＭ－ＮＥＲＦ、エオシン、エリスロシン、フロレセイン、ＦＡＭ、ヒドロキシクマリン、ＩＲＤｙｅｓ（ＩＲＤ４０、ＩＲＤ　７００、ＩＲＤ　８００）、ＪＯＥ、リサミンローダミンＢ、マリンブルー、メトキシクマリン、ナフトフルオレセイン、オレゴングリーン４８８、オレゴングリーン５００、オレゴングリーン５１４、Ｏｙｓｔｅｒ色素、Ｐａｃｉｆｉｃ　Ｂｌｕｅ、ＰｙＭＰＯ、ピレン、ローダミン６Ｇ、ローダミングリーン、ローダミンレッド、Ｒｈｏｄｏｌ　Ｇｒｅｅｎ、２’，４’，５’，７’－テトラ－ブロモスルホン－フルオレセイン、テトラメチル－ローダミン（ＴＭＲ）、カルボキシテトラメチルローダミン（ＴＡＭＲＡ）、テキサスレッド、テキサスレッド－Ｘが挙げられる。Ｔｈｅ　Ｈａｎｄｂｏｏｋ　ｏｆ　Ｆｌｕｏｒｅｓｃｅｎｔ　Ｐｒｏｂｅｓ　ａｎｄ　Ｒｅｓｅａｒｃｈ　Ｐｒｏｄｕｃｔｓ，第９版，　Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，　Ｉｎｃ．，　ｆｏｒ　ｆｕｒｔｈｅｒ　ｄｅｓｃｒｉｐｔｉｏｎを参照のこと。

【0170】

それ自身が直接検出可能であるのではなく、一部の蛍光基は、非放射性蛍光共鳴エネルギー転移（ＦＲＥＴ）のプロセスにてエネルギーを別の基に転移させ、第２の基が、検出されるシグナルを生成する。すなわち、消光剤の使用もまた本発明の範囲内である。用語「消光剤」は、可視光を発光せずに励起された蛍光標識のエネルギーを、これに近接して配置された場合に吸収でき、消散できる部分をいう。消光剤の例としては、限定されないが、ＤＡＢＣＹＬ（４－（４’－ジメチルアミノフェニルアゾ）安息香酸）スクシンイミジルエステル、ジアリールローダミンカルボン酸、スクシンイミジルエステル（ＱＳＹ－７）および４’，５’－ジニトロフルオレセインカルボン酸、スクシンイミジルエステル（ＱＳＹ－３３）（すべて、Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ製）、消光剤１（Ｑ１；Ｅｐｏｃｈ製）または「ブラックホール（Ｂｌａｃｋ　ｈｏｌｅ）消光剤」ＢＨＱ－１、ＢＨＱ－２およびＢＨＱ－３（ＢｉｏＳｅａｒｃｈ，　Ｉｎｃ製）が挙げられる。

【0171】

上記の種々の検出可能な部分に加え、本発明はまた、スペクトルにより分離可能な量子ドット、金属ナノ粒子またはナノクラスターなどの使用の使用を包含し、これらは、オリゴヌクレオチドプローブに直接結合されたものであり得るか、または高分子マトリックス内に包埋もしくはこれと会合させ、次いでプローブに結合させたものであり得るかのいずれかである。上記のように、検出可能な部分は、それ自体が直接検出可能である必要はない。例えば、これらは、検出される基質に対して作用するものであってもよく、検出可能となるために修飾を必要とするものであってもよい。

【0172】

上記のように、本発明のある特定の実施形態において、標識は、複数の検出可能な部分からなる。これらの検出可能な部分からの複合シグナルにより色がもたらされ、これを、

プローブの同定のために使用する。例えば、ある特定の配列の「紫色」のプローブは、「青色」および「赤色」の検出可能な部分を結合することにより構築され得る。あるいはまた、同じ配列を有するが異なる検出可能な部分で標識された２種類のプローブを組み合わせて混合プローブを作製することにより異なる色が生成され得る。したがって、ある特定の配列の「紫色」のプローブは、該配列を有する２種類のプローブを構築することにより作製され得る。「赤色」の検出可能な部分を第１の種に結合させ、「青色」の検出可能な部分を第２の種に結合させる。これらの２種類のアリコートを混合する。種々の濃度（ｓｈａｄｅ）の紫色が、アリコートを種々の比率で混合することにより生成され得る。このアプローチはいくつかの利点をもたらす。第１に、より少数の検出可能な部分を用いて多数の識別可能なプローブの生成を可能にする。第２に、混合プローブの使用により、特定の検出可能な部分と特定のヌクレオチド間の相互作用に起因し得る偏重の低減を補助し得る冗長（ｒｅｄｕｎｄａｎｃｙ）度が提供され得る。

【0173】

本発明のある特定の実施形態において、検出可能な部分は、ライゲーションおよび検出後に検出可能な部分の除去を可能にする切断可能な連結によってオリゴヌクレオチド伸長プローブ内のヌクレオチドに結合させる。任意のさまざまな異なる切断可能な連結が使用され得る。本明細書で用いる場合、オリゴヌクレオチドプローブにおける検出可能な部分およびヌクレオチドの文脈において、用語「切断可能な連結」は、検出可能な部分をヌクレオチドに接合させ、所望の場合は、結合されたヌクレオチドまたは核酸分子を本質的に改変することなく、切断されて検出可能な部分をヌクレオチドから除去し得る化学的部分をいう。切断は、連結の性質に応じて、例えば、酸もしくは塩基処理、または連結の酸化もしくは還元、または光処理（光切断）によってなされ得る。切断可能な連結および切断剤の例は、Ｓｈｉｒｎｋｕｓら．，１９８５，　Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ　８２：２５９３－２５９７；Ｓｏｕｋｕｐら．，１９９５，　Ｂｉｏｃｏｎｊｕｇ．Ｃｈｅｍ．６：１３５－１３８；Ｓｈｉｍｉｋｕｓら．，１９８６，　ＤＮＡ　５：２４７－２５５；ならびにＨｅｒｍａｎおよびＦｅｎｎ，１９９０，　Ｍｅｔｈ．Ｅｎｚｙｍｏｌ．１８４：５８４－５８８に記載されている。より一般的には、「開裂可能な連結」とは、２つの分子または正体を連結するために使用できる部分であって、容易に開裂できることによって、例えば分子または正体の安定性と両立する条件下で、構造を実質的に変更することなく、分子または正体の分離を可能にする部分を指す。

【0174】

例えば、米国特許第６，５１１，８０３号に記載のように、ジスルフィド結合が、ジチオトレイトール（ＤＴＴ）などのチオール化合物還元剤を用いて還元され、それにより切断され得る。ヌクレオチドと反応性アリールアミノ基（例えば、ｄＣＴＰ）のように、フルオロフォアが、コンジュゲーションに利用され得るスルフヒドリル（ＳＨ）基とともに利用可能である（例えば、シアニン５またはシアニン３フルオロフォアとＳＨ基；Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｎｕｃｌｅａｒ－ＤｕＰｏｎｔ）。反応性ピリジルジチオールは、スルフヒドリル基と反応してスルフヒドリル結合をもたらし、これは、ジチオトレイトールなどの還元剤により切断可能である。ＮＨＳ－エステルヘテロ二官能性架橋剤（Ｐｉｅｒｃｅ）は、反応性アリールアミノ基を含むデオキシヌクレオチドをピリジルジチオール基に連結させるために使用され得、これは、次いでフルオロフォア上のＳＨと反応性となり、本発明の方法に有用なジスルフィド結合された切断可能なヌクレオチドフルオロフォア複合体をもたらす。あるいはまた、ヌクレオチドとフルオロフォア間のシス－グリコール連結は過ヨウ素酸塩によって切断され得る。さまざまな切断可能な連結が、米国特許第６，６６４，０７９号および同第６，６３２，６５５号、ＵＳ出願公開公報２００３０１０４４３７、ＷＯ　０４／１８４９７およびＷＯ　０３／４８３８７に記載されている。

【0175】

本発明の他の実施形態において、光などの電磁エネルギーへの曝露によって非検出可能となり得る検出可能な部分（光退色性）が使用される。

【0176】

切断可能な連結によってプローブに結合された標識を有するか、または光退色性となり得る標識を有する伸長プローブを用いる本発明の実施形態において、配列決定法は、典型的には、ライゲーションおよび標識検出が行なわれた後、１回以上のサイクルにおいて切断または光退色工程を含む。上記のように、オリゴヌクレオチド伸長プローブ内に存在する切れやすい連結の切断は、完結するまで進行し得ない（すなわち、新たにライゲートされたプローブの１００％未満が、ライゲートされたサイクルにおいて切断され得る）。かかるプローブは一般的に、伸長不可能な末端を含むか、またはキャップされているため、連続的サイクルに寄与しない。しかしながら、プローブが切断されないことは、標識が、プローブライゲートされる鋳型分子と会合したままであることを意味し、これは、後続のサイクルにおけるノイズを増大させ得るバックグラウンドシグナル（すなわち、バックグラウンド蛍光）に寄与する。標識を除去するため、または非検出可能とするための切断または光退色の工程を組み込むことは、このバックグラウンドを低減させ、信号対雑音比が改善される。切断または光退色は、各サイクルごとにできるだけ多く行なってもよく、サイクル１回おき、３回に１回、５回以上に１回などの低頻度で行なってもよい。本発明のある特定の実施形態において、切断可能なリンカーの切断を達成するための任意のさらなる工程を実際に加える必要はない。例えば、ＤＴＴなどの切断剤を、非ライゲート伸長プローブを除去するために使用され得る洗浄バッファー中に既に存在させてもよい。

【0177】

Ｇ．好ましい切れやすい連結

本発明者は、少なくとも１つのホスホロチオレート結合を有する伸長プローブが、伸長、ライゲーション、検出および切断の連続的サイクルによる配列決定のための方法の実施において特に有用であることを見出した。かかる連結では、ホスホジエステル結合の橋絡酸素原子の１つがイオウ原子で置き換えられている。ホスホロチオレート結合は、図４Ａに示すような５’－Ｓ－ホスホロチオレート結合（３’－Ｏ－Ｐ－Ｓ－５’）または図４Ｂに示すような３’－Ｓ－ホスホロチオレート結合（３’－Ｓ－Ｐ－Ｏ－５’）のいずれかであり得る。図４Ａおよび４Ｂに示すように、３’－Ｏ－Ｐ－Ｓ－５’または３’－Ｓ－Ｐ－Ｏ－５’で表される連結内のリン原子は、２つの非橋絡酸素原子に結合され得ることを理解されたい（典型的なホスホジエステル結合の場合と同様）。あるいはまた、リン原子は、任意のさまざまな他の原子または基、例えば、Ｓ、CH_3、BH_3などに結合され得る。したがって、本発明の一態様は、ホスホロチオレート結合を含む標識されたオリゴヌクレオチドプローブである。プローブには、本明細書に記載した配列決定法において具体的な用途が見られるが、さまざまな他の目的にもまた使用され得る。特に、本発明は、（ｉ）形態５’－Ｏ－Ｐ－Ｏ－Ｘ－Ｏ－Ｐ－Ｓ－（Ｎ）$_k$ N_B * －３’のオリゴヌクレオチド；および（ｉｉ）形態５’－N_B *（Ｎ）$_k$ －Ｓ－Ｐ－Ｏ－Ｘ－３’のオリゴヌクレオチドを提供する。これらのプローブの各々において、Ｎは任意のヌクレオチドを表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、Ｘはヌクレオチドを表し、ｋは１～１００である。ある特定の実施形態において、ｋは、１～５０、１～３０、１～２０、例えば４～１０である、ただし、検出可能な部分は、（Ｎ）$_k$の任意のヌクレオシド上にN_Bの代わりまたはこれに加えて存在するものとする。任意のこれらのプローブ内の末端ヌクレオチドは、リン酸基またはヒドロキシル基を含むものであってもそうでなくてもよい。さらにまた、該リン原子は、好ましい実施形態において、一般的に、２つのさらなる（非橋絡）酸素原子に結合されていることは認識されよう。

【0178】

５’－Ｓ－ホスホロチオレートまたは３’－Ｓ－ホスホロチオレート結合を含有するオリゴヌクレオチドを合成するための方法は、当該技術分野で知られており、これらの方法のある特定のものは、自動固相オリゴヌクレオチド合成に適している。合成手順は、例えば、Ｃｏｏｋ，　ＡＦ，　Ｊ．Ａｍ．Ｃｈｅｍ．Ｓｏｃ，９２：１９０－１９５，１９７０；Ｃｈｌａｄｅｋ，　Ｓ．ら、Ｊ．Ａｍ．Ｃｈｅｍ．Ｓｏｃ，９４：２０７９－２０８４，１９７２；Ｒｙｂａｋｏｖ，　ＶＮら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，９：１８９－２０１，１９８１；Ｃｏｓｓｔｉｃｋ，　Ｒ．およびＶｙｌｅ，　ＪＳ，　Ｊ

．Ｃｈｅｍ．Ｓｏｃ．ＣＨｅｍ．Ｃｏｍｍｕｎ．，９９２－９９２，１９８８；Ｍａｇ，Ｍ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，１９（７）；１４３７－１４４１，１９９１；Ｘｕ，Ｙ，およびＫｏｏｌ，ＥＴ，Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，２６（１３）：３１５９－３１６４，１９９８；Ｃｏｓｓｔｉｃｋ，Ｒ．およびＶｙｌｅ，ＪＳ，Ｔｅｔｒａｈｅｄｒｏｎ　Ｌｅｔｔ．，３０：４６９３－４６９６，１９８９；Ｃｏｓｓｔｉｃｋ，Ｒ．およびＶｙｌｅ，ＪＳ，Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，１８：８２９－８３５，１９９０；Ｓｕｎ，ＳＧおよびＰｉｃｃｉｒｉｌｌｉ，ＪＡ，Ｎｕｃｌ．Ｎｕｃｌ，１６：１５４３－１５４５，１９９７；Ｓｕｎ　ＳＧら、ＲＮＡ，３：１３５２－１３６３，１９９７；Ｖｙｌｅ，ＪＳら、Ｔｅｔｒａｈｅｄｒｏｎ　Ｌｅｔｔ．，３３：３０１７－３０２０，１９９２；Ｌｉ，Ｘ．ら、Ｊ．Ｃｈｅｍ．Ｓｏｃ．Ｐｅｒｋｉｎ　Ｔｒａｎｓ．，１：２１２３－２２１２９，１９９４；Ｌｉｕ，ＸＨ　ａｎｄ　Ｒｅｅｓｅ，ＣＢ，Ｔｅｔｒａｈｅｄｒｏｎ　Ｌｅｔｔ．，３７：９２５－９２８，１９９６；Ｗｅｉｎｓｔｅｉｎ，ＬＢら、Ｊ．Ａｍ．Ｃｈｅｍ．Ｓｏｃ，１１８：１０３４１－１０３５０，１９９６；ならびにＳａｂｂａｇｈ，Ｇ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，３２（２）：４９５－５０１，２００４に記載されている。また、本発明者らは、新たな合成方法を開発した。例えば、図7は、ｄＡの３’－ホスホロアミデートの合成スキームを示す。同様のスキームが、ｄＧの３’－ホスホロアミデートの合成に使用され得る。これらのホスホロアミデートは、プリンヌクレオシドと会合した３’－Ｓ－ホスホロチオレート結合を含有するオリゴヌクレオチドを、例えば、自動ＤＮＡ合成装置を用いて合成するために使用され得る。

【0179】

ホスホロチオレート連結は、さまざまな金属含有薬剤を用いて切断され得る。金属は、例えば、Ａｇ、Ｈｇ、Ｃｕ、Ｍｎ、ＺｎまたはＣｄであり得る。好ましくは、該薬剤は、Ag^+、Hg^{++}、Cu^{++}、Mn^{++}、Zn^+またはCd^+アニオンを提供する水溶性の塩である（他の酸化状態のイオンを提供する塩もまた使用され得る）。I_2もまた使用され得る。硝酸銀（$AgNO_3$）などの銀含有塩またはAg^+イオンを提供する他の塩，は特に好ましい。好適な条件としては、例えば、５０ｍＭ　$AgNO_3$、約２２～３７℃で１０分以上（例えば、３０分間）が挙げられる。好ましくは、ｐＨは、４．０～１０．０、より好ましくは５．０～９．０、例えば約６．０～８．０、例えば約７．０である。例えば、Ｍａｇ，Ｍ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，１９（７）：１４３７－１４４１，１９９１を参照のこと。例示的なプロトコルを実施例１に示す。

【0180】

５’→３’方向の配列決定は、３’－Ｏ－Ｐ－Ｓ－５’連結を含有する伸長プローブを用いて行なわれ得る。図５Ａは、形態５’－Ｏ－Ｐ－Ｏ－Ｘ－Ｏ－Ｐ－Ｓ－$NNNNN_B$*－３’（式中、Ｎは任意のヌクレオチドを表し、N_Bはリガーゼによって伸長可能でない部分を表し（例えば、N_Bは、３’ヒドロキシル基がないヌクレオチドであるか、またはブロッキング部分が結合されている）、＊は検出可能な部分を表し、Ｘは、その正体が検出可能な部分に対応するヌクレオチドを表す）の伸長プローブを用いたハイブリダイゼーション、ライゲーションおよび切断１サイクルを示す。あるいはまた、多重ライゲーションを抑制するため、任意の多数のブロッキング部分を３’末端ヌクレオチドに結合させ得る。例えば、バルキー基をヌクレオチドの糖鎖部分、例えば、２’位または３’位に結合させることにより、ライゲーションが抑制される。蛍光標識は、適切なバルキー基としての機能を果たし得る。

【0181】

結合領域４０および未知配列のポリヌクレオチド領域５０を含有する鋳型を支持体、例えば、ビーズに結合させる。好ましい実施形態において、図５Ａに示すように、結合領域は、支持体への結合点と反対の鋳型の末端に位置する。伸長可能な末端（この場合は、遊離３’ＯＨ基）を有する初期オリゴヌクレオチド３０を、結合領域４０にアニーリングさせる。伸長プローブ６０をポリヌクレオチド領域５０内の鋳型にハイブリダイズさせる。

ヌクレオチドＸは、鋳型内の未知ヌクレオチドＹと相補塩基対を形成する。伸長プローブ６０を初期オリゴヌクレオチドにライゲートさせる（例えば、Ｔ４リガーゼを用いて）。ライゲーション後、伸長プローブ６０に結合された標識を検出する（図示せず）。標識は、ヌクレオチドＸの正体に対応する。したがって、ヌクレオチドＹが、ヌクレオチドＸに相補的なヌクレオチドとして同定される。次いで、伸長プローブ６０をホスホロチオレート結合で切断し（例えば、$AgNO_3$ またはＡｇ$^+$ イオンを提供する別の塩を用いて）、伸長された二本鎖が得られる。切断によりリン酸基が伸長された二本鎖の３’末端に生じる。ホスファターゼ処理が、伸長可能なプローブ末端を伸長された二本鎖に生成させるために使用される。該プロセスを所望回数サイクル反復する。

【0182】

好ましい実施形態では、配列決定は、３’→５’方向に３’－Ｓ－Ｐ－Ｏ－５’連結を含有する伸長プローブを用いて行なわれる。図５Ｂは、形態５’－N_B＊－ＮＮＮＮ－Ｓ－Ｐ－Ｏ－Ｘ－３’（式中、Ｎは任意のヌクレオチドを表し、N_B はリガーゼによって伸長可能でない部分を表し（例えば、ＮＢは、５’リン酸基がないヌクレオチドであるか、またはブロッキング部分が結合されている）、＊は検出可能な部分を表し、Ｘは、その正体が検出可能な部分に対応するヌクレオチドを表す）の伸長プローブを用いたハイブリダイゼーション、ライゲーションおよび切断の１サイクルを示す。

【0183】

結合領域４０および未知配列のポリヌクレオチド領域５０を含有する鋳型を支持体、例えば、ビーズに結合させる。好ましい実施形態において、図５Ｂに示すように、結合領域は、支持体への結合点と反対の鋳型の末端に位置する。伸長可能な末端（この場合は、遊離５’リン酸基）を有する初期オリゴヌクレオチド３０を、結合領域４０にアニーリングさせる。伸長プローブ６０をポリヌクレオチド領域５０内の鋳型にハイブリダイズさせる。ヌクレオチドＸは、鋳型内の未知ヌクレオチドＹと相補塩基対を形成する。伸長プローブ６０を初期オリゴヌクレオチドにライゲートさせる（例えば、Ｔ４リガーゼを用いて）。ライゲーション後、伸長プローブ６０に結合された標識を検出する（図示せず）。標識は、ヌクレオチドＸの正体に対応する。したがって、ヌクレオチドＹが、ヌクレオチドＸに相補的なヌクレオチドとして同定される。次いで、伸長プローブ６０をホスホロチオレート結合で切断し（例えば、$AgNO_3$ またはＡｇ$^+$ イオンを提供する別の塩を用いて）、伸長された二本鎖が得られる。切断により伸長可能な一リン酸基が伸長された二本鎖の５’末端に生じ、したがって、伸長可能な末端を生成させるためのさらなる工程を行なうことは不必要である。該プロセスを所望回数サイクル反復する。

【0184】

このスキームのいくつかの変形例が使用され得ることは認識されよう。例えば、プローブは６ヌクレオチドよりも短くても長くてもよく、標識は３’末端ヌクレオチド上である必要はなく、Ｐ－Ｓ連結は任意の２つの隣接するヌクレオチド間に位置し得るなどである。上記の実施形態では、伸長、ライゲーション、検出および切断の連続的サイクルにより、隣接して位置するヌクレオチドの同定がもたらされる。しかしながら、Ｐ－Ｓ連結を伸長プローブの遠位端（すなわち、ライゲーションが起こる末端と反対の末端）に対してより近くに配置することによって、上記ならびに図１および６に示すように、連続的に同定されるヌクレオチドは鋳型に沿って間隔があく。

【0185】

図６Ａ～６Ｆは、単一の鋳型において連続的に行なわれるいくつかの配列決定反応のより詳細な概略図である。配列決定は、３’→５’方向に３’－Ｓ－Ｐ－Ｏ－５’連結を含有する伸長プローブを用いて行なわれる。各配列決定反応は、伸長、ライゲーション、検出および切断の多数のサイクルを含む。該反応では、鋳型の異なる部分に結合する初期オリゴヌクレオチドを用いる。伸長プローブは８ヌクレオチド長であり、プローブの３’末端から数えて６番目と７番目のヌクレオチド間に位置するホスホロチオレート結合を含有する。ヌクレオチド２～６は、各反応で、鋳型に沿って間隔があいている複数のヌクレオチドの同定が可能となるようにスペーサーとしての機能を果たす。多数の反応を順次行な

い、各反応で得られた部分配列情報を適切に組み合わせることにより、鋳型の一部分完全な配列が決定される。

【0186】

図６Ａは、伸長可能な二本鎖を得るための、鋳型内のアダプター配列（図６Ａ～６Ｆでは結合領域という）にハイブリダイズさせた第１の初期オリゴヌクレオチド（図６Ａ～６Ｆではプライマーという）を用いた初期化を示す。図６Ｂ～６Ｄは、ヌクレオチド同定のいくつかのサイクルを示し、この場合、鋳型は６塩基ごとに読み出しされる。図６Ｂでは、第１の未知鋳型内のヌクレオチド配列に相補的な３’末端ヌクレオチドを有する第１の伸長プローブが鋳型に結合し、プライマーの伸長可能な末端にライゲートされる。伸長プローブに結合された標識により、プローブは３’末端ヌクレオチドとしてＡを有すると同定され、したがって、鋳型配列内の第１の未知ヌクレオチドはＡであると同定される。図６Ｃは、ホスホロチオレート結合での$AgNO_3$による伸長オリゴヌクレオチドの切断および伸長プローブの標識が結合された部分の放出を示す。図６Ｄは、伸長、ライゲーションおよび切断のさらなるサイクルを示す。プローブは５ヌクレオチド長のスペーサーを含むため、配列決定反応により、鋳型は６ヌクレオチドごとに同定される。

【0187】

所望回数サイクル後、第１の初期化ヌクレオチドを含む伸長鎖が除去され、第１の初期オリゴヌクレオチドが結合した部分と異なる結合領域の部分に結合する第２の初期オリゴヌクレオチドが、鋳型にハイブリダイズされる。図６Ｅは、第２の配列決定反応を示し、この場合、初期化は、第２の初期オリゴヌクレオチドにより行なわれ、その後、数回サイクルのヌクレオチド同定が行なわれる。図６Ｆは、第３の初期オリゴヌクレオチドを用いた初期化後、数回サイクルのヌクレオチド同定を示す。第２の初期オリゴヌクレオチドからの伸長により、第１の配列決定反応同定されたヌクレオチドと異なる「フレーム」内で６塩基ごとの同定が可能になる。

【0188】

ホスホロチオレート結合を含む伸長プローブは、本発明のある特定の実施形態において好ましいが、さまざまな他の切れやすい連結が好都合に使用され得る。例えば、天然に存在する核酸に見られるＯ－Ｐ－Ｏ連結に対する多数のバリエーションが知られている（例えば、Ｍｉｃｋｌｅｆｉｅｌｄ，　Ｊ．Ｃｕｒｒ．Ｍｅｄ．Ｃｈｅｍ．，８：１１５７－１１７９，２００１を参照）。本明細書に記載されたＰ－Ｏ結合を含む任意の構造は、切れやすいＰ－Ｓ結合を含むように修飾され得る。例えば、ＮＨ－Ｐ－Ｏ結合をＮＨ－Ｐ－Ｓ結合に変更してもよい。

【0189】

本発明のある一部の実施形態において、伸長プローブは、核酸を、切断剤またはその組合せによる切断に対して感受性とする（任意選択で、修飾剤によるトリガー残基の修飾後）トリガー残基を含む。特に、本発明者らは、ＤＮＡ修復に関与する酵素は、伸長、ライゲーション、検出および切断の連続的サイクルによる配列決定のための方法の実施における使用のための好都合な切断試薬であることを見出した。一般に、伸長プローブ内における損傷塩基または無塩基残基などのトリガー残基の存在により、プローブは、１種類以上のＤＮＡ修復酵素による切断に対して感受性となり得る（任意選択で、ＤＮＡグリコシラーゼによる修飾後）。したがって、ＡＰエンドヌクレアーゼなどのＤＮＡ修復に関与する酵素による切断の基質である連結を含む伸長プローブは、本発明において有用である。修飾によりプローブがＡＰエンドヌクレアーゼによる切断に対して感受性となるＤＮＡグリコシラーゼなどのＤＮＡ修復に関与する酵素による修飾の基質である残基を含有する伸長プローブもまた、本発明に特に有用である。ある一部の実施形態において、伸長プローブは、無塩基残基を含む、すなわちプリンまたはピリミジン塩基がない。無塩基残基と隣接ヌクレオシド間の連結は、ＡＰエンドヌクレアーゼによって切断に対して感受性であり、したがって、切れやすい連結である。本発明のある特定の実施形態において、無塩基残基は２’デオキシリボースを含む。ある一部の実施形態において、伸長プローブは損傷塩基を含む。損傷塩基は、ＤＮＡグリコシラーゼなどの損傷塩基を除去する酵素の基質である

。損傷塩基の除去後、得られる無塩基残基と隣接ヌクレオシド間の連結は、ＡＰエンドヌクレアーゼによる切断に対して感受性であり、したがって、本発明に従う切れやすい連結とみなされる。

【0190】

多くの異なるＡＰエンドヌクレアーゼが本発明における切断試薬として有用である。ＡＰエンドヌクレアーゼの２つの主な類型が、無塩基残基に隣接する連結を切断する機構に基づいて識別されている。類型ＩのＡＰエンドヌクレアーゼ、例えば、大腸菌のエンドヌクレアーゼＩＩＩ（Ｅｎｄｏ　ＩＩＩ）およびエンドヌクレアーゼＶＩＩＩ（Ｅｎｄｏ　ＶＩＩＩ）ならびにヒト類縁体ｈＮＴＨ１、ＮＥＩＬ１、ＮＥＩＬ２およびＮＥＩＬ３などは、ＡＰ残基の３’側のＤＮＡを切断し、３’末端リン酸基を有する５’部分および５’末端リン酸基を担持する３’部分をもたらすＡＰリアーゼである。類型ＩＩのＡＰエンドヌクレアーゼ、例えば、例えば、大腸菌のエンドヌクレアーゼＩＶ（Ｅｎｄｏ　ＩＶ）およびエクソヌクレアーゼＩＩＩ（Ｅｘｏ　ＩＩＩ）などは、ＡＰ部位の５’のＤＮＡを切断し、３’ＯＨおよび５’デオキシリボースリン酸基部分を生じる断片の末端に生成させる。種々の類型Ｉおよび類型ＩＩのＡＰエンドヌクレアーゼならびに損傷塩基のＤＮＡからのを除去および／または無塩基残基含有ＤＮＡの切断の条件のさらなる論考については、例えば、Ｄｏｕｂｌｉｅ，　Ｓ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ　１０１（２８），１０２８４－１０２８９，２００４；Ｈａｌｔｉｗａｎｇｅｒ，　Ｂ．Ｍ．ら，　Ｂｉｏｃｈｅｍ　Ｊ．，３４５，８５－８９，２０００；Ｌｅｖｉｎ，　Ｊ．ａｎｄ　Ｄｅｍｐｌｅ，　Ｂ．，　Ｎｕｃｌ．Ａｃｉｄｓ．Ｒｅｓ，１８（１７），１９９０、および前記文献中の参考文献を参照のこと。当業者には、これらの酵素のさまざまな類縁体が、他の生物体（例えば、酵母）に存在し、本発明において有用であることが認識されよう。

【0191】

ある種の酵素は、グリコシラーゼ活性を有し、損傷塩基を除去してＡＰ残基を生成させ、また、グリコシラーゼ活性によって生成されたＡＰ部位の３’側でホスホジエステル主鎖を切断するリアーゼ活性を示すという両方の点で二官能性である。したがって、このような二重活性酵素は、ＡＰエンドヌクレアーゼとＤＮＡグリコシラーゼの両方である。例えば、Ｅｎｄｏ　ＶＩＩＩは、Ｎ－グリコシラーゼとＡＰ－リアーゼの両方として作用する。Ｎ－グリコシラーゼ活性により損傷ピリミジンが二本鎖ＤＮＡから放出され、無プリン（ＡＰ部位）をもたらされる。ＡＰ－リアーゼ活性により、ＡＰ部位の３’および５’側が切断され、５’リン酸基および３’リン酸基が生じる。エンドヌクレアーゼＶＩＩＩによって認識および除去される損傷塩基としては、尿素、５，６－ジヒドロキシチミン、チミングリコール、５－ヒドロキシ－５－メチルヒダントン、ウラシルグリコール、６－ヒドロキシ－５、６－ジヒドロチミンおよびメチルタートロニル（ｔａｒｔｒｏｎｙｌ）尿素が挙げられる。例えば、Ｄｉｚｄａｒｏｇｌｕ，　Ｍ．ら、Ｂｉｏｃｈｅｍｉｓｔｒｙ，３２，１２１０５－１２１１１，１９９３およびＨａｔａｈｅｔ，　Ｚ．ら、Ｊ　Ｂｉｏｌ．Ｃｈｅｍ．，２６９，１８８１４－１８８２０，１９９４；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．，　２７２（５１），３２２２０－３２２２９，１９９７；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂａｃｔ，１７９（１１），　３７７３－３７８２，　１９９７を参照のこと。

【0192】

Ｆｐｇ（ホルムアミドピリミジン［ｆａｐｙ］－ＤＮＡグリコシラーゼ）（８－オキソグアニンＤＮＡグリコシラーゼとしても知られる）もまた、Ｎ－グリコシラーゼとＡＰ－リアーゼの両方として作用する。Ｎ－グリコシラーゼ活性により損傷プリンが二本鎖ＤＮＡから放出され、無プリン（ＡＰ部位）がもたらされる。ＡＰ－リアーゼ活性により、ＡＰ部位の３’および５’側両方が切断され、それによりＡＰ部位が除去され、１塩基ギャップが生じる。Ｆｐｇ認識および除去される損傷塩基の一例としては、７，８－ジヒドロ－８－オキソグアニン（８－オキソグアニン）、８－オキソアデニン、ｆａｐｙ－グアニン、メチル－ｆａｐｙ－グアニン、ｆａｐｙ－アデニン、アフラトキシンＢ１－ｆａｐｙ

－グアニン、５－ヒドロキシ－シトシンおよび５－ヒドロキシ－ウラシルが挙げられる。例えば、Ｔｃｈｏｕ，　Ｊ．ら．Ｊ．Ｂｉｏｌ　Ｃｈｅｍ．，２６９，１５３１８－１５３２４，１９９４；Ｈａｔａｈｅｔ，　Ｚ．ら．Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．，２６９，１８８１４－１８８２０，　１９９４；Ｂｏｉｔｅｕｘ，　Ｓ．ら，　ＥＭＢＯ　Ｊ．，５，３１７７－３１８３，１９８７；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．，２７２（５１），３２２２０－３２２２９，１９９７；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂａｃｔ，　１７９（１１），３７７３－３７８２，１９９７を参照のこと。

【0193】

いくつかのＤＮＡグリコシラーゼおよびＡＰエンドヌクレアーゼは、例えば、Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ，　Ｉｐｓｗｉｃｈ，　ＭＡから市販されている。

【0194】

本発明のある一部の実施形態において、ＡＰエンドヌクレアーゼによる切断のための基質である部位を含む伸長プローブが、ホスホロチオレート結合を含有する伸長プローブの上記のような配列決定法、または配列決定法ＡＢ（下記参照）に使用される。任意のこれらの方法では、伸長プローブのライゲーションにより核酸鎖を成長させた後、伸長プローブをＡＰエンドヌクレアーゼを用いて切断し、プローブの標識を含む部分を除去する。

【0195】

具体的なＡＰエンドヌクレアーゼに応じて、および配列決定が３’→５’または５’→３’方向のどちらで行なわれるかに応じて、切断後、伸長された二本鎖をポリヌクレオチドキナーゼまたはホスファターゼで処理し、伸長可能なプローブ末端を伸長された二本鎖上に生成させることが必要または望ましいことがある（伸長可能なプローブ末端を示す図５Ａおよび５Ｂ参照）。したがって、本発明のある特定の方法では、伸長可能な末端を、ポリヌクレオチドキナーゼまたはホスファターゼでの処理によって生成させる。当業者には、種々の酵素に適切なバッファーが使用され、酵素を除去し、該方法の後続の工程に適切な条件を提供するための洗浄するさらなる工程を含め得ることが認識されよう。

【0196】

他の実施形態において、伸長プローブは、ＤＮＡグリコシラーゼによる除去のための基質である損傷塩基を含む。広範な細胞傷害性および変異原性ＤＮＡ塩基が、ＤＮＡの損傷後に塩基切除修復経路を起始させる種々のＤＮＡグリコシラーゼによって除去される（Ｋｒｏｋａｎ，　Ｈ．Ｅ．ら，　Ｂｉｏｃｈｅｍ　Ｊ，３２５（Ｐｔ　１）：１－１６，１９９７）。ＤＮＡグリコシラーゼは、損傷塩基とデオキシリボース間のＮ－グリコシド結合を切断し、したがって、遊離塩基を放出させ無プリン／無ピリミジン（ＡＰ）部位をもたらす。ある一部の実施形態において、伸長プローブは、ウラシル－ＤＮＡグリコシラーゼ（ＵＤＧ）によって除去されるウラシル残基を含む。ＵＤＧは、これまでに研究されたあらゆる生きた生物体に見られ、これらの酵素の多くが当該技術分野で知られており、本発明に有用である（Ｆｒｅｄｅｒｉｃａら，　Ｂｉｏｃｈｅｍｉｓｔｒｙ，２９，　２３５３－２５３７，１９９０；Ｋｒｏｋａｎ、前掲）。例えば、哺乳動物は、少なくとも４種類のＵＤＧ：ミトコンドリアＵＮＧ１および核ＵＮＧ２、ＳＭＵＧ１、ＴＤＧおよびＭＢＤ４を含む（Ｋｒｏｋａｎら、Ｏｎｃｏｇｅｎｅ，２１，８９３５－８９４８，２００２）。ＵＮＧ１およびＵＮＧ２は、大腸菌Ｕｎｇに代表される高度に保存されたファミリーに属する。伸長プローブが損傷塩基を含む実施形態では、伸長可能なプローブ末端への伸長プローブのライゲーション後、伸長された二本鎖を、損傷塩基除去するグリコシラーゼと接触させ、それにより無塩基残基を生成させる。グリコシラーゼによる除去に供された損傷塩基を含む伸長プローブは、「切れやすい連結を含むように容易に修飾可能である」とみなされる。次いで、伸長された二本鎖を、上記のような無塩基残基と隣接ヌクレオシド間の連結を切断するＡＰエンドヌクレアーゼと接触させる。本発明のある特定の実施形態において、ＤＮＡグリコシラーゼとＡＰエンドヌクレアーゼの両方である二重活性酵素は、これらの反応の両方を行うために使用される。ある一部の実施形態では、損傷塩基を含有する伸長された二本鎖を、ＤＮＡグリコシラーゼおよびＡＰエンドヌクレアーゼと接触させる。これらの酵素は、本発明の種々の実施形態において、組合せて、または連続

的に（すなわち、グリコシラーゼの後エンドヌクレアーゼ）使用され得る。

【0197】

本発明のある一部の実施形態において、伸長プローブは、デオキシイノシンであるトリガー残基を含む。上記のように、大腸菌エンドヌクレアーゼＶ（Ｅｎｄｏ　Ｖ）（デオキシイノシン３’エンドヌクレアーゼともいわれる）およびその類縁体は、デオキシイノシン含有する核酸を、デオキシイノシン残基の３’側の第２のホスホジエステル結合で切断し、３’ＯＨおよび５’リン酸基末端をもたらす。したがって、この結合は、伸長プローブ内の切れやすい連結としての機能を果たす。Ｅｎｄｏ　Ｖおよびその切断特性は、当該技術分野で知られている（Ｙａｏ，　Ｍ．およびＫｏｗ　Ｙ．Ｗ．，　Ｊ　Ｂｉｏｌ．Ｃｈｅｍ．，２７１，３０６７２－３０６７３（１９９６）；Ｙａｏ，　Ｍ．およびＫｏｗ　Ｙ．Ｗ．，　Ｊ　Ｂｉｏｌ．Ｃｈｅｍ．，２７０，２８６０９－２８６１６（１９９５）；Ｈｅ，　Ｂら、Ｍｕｔａｔ　Ｒｅｓ．，　４５９，　１０９－１１４（２０００）。デオキシイノシンに加え、Ｅｎｄｏ　Ｖはまた、デオキシウリジン、デオキシキサントシンおよびデオキシオキサノシンを認識する（Ｈｉｔｃｈｃｏｃｋ，　Ｔ．ら、Ｎｕｃ．Ａｃｉｄｓ　Ｒｅｓ．，３２（１３），３２（１３）（２００４）。ｍＥｎｄｏ　Ｖなどの哺乳動物の類縁体もまた切断活性を示す（Ｍｏｅ，　Ａ．ら、Ｎｕｃ．Ａｃｉｄｓ　Ｒｅｓ．，３１（１４），３８９３－３９００（２００４）。Ｅｎｄｏ　Ｖは、デオキシイノシンを含むプローブの好ましい切断剤であるが、他の切断試薬もまた、デオキシイノシンを含むプローブを切断するために使用され得る。例えば、損傷塩基としてヒポキサンチンが、適切なＤＮＡグリコシラーゼによる除去に供され得、生じた無塩基残基含有伸長プローブが、次いで、エンドヌクレアーゼによる切断に供される。

【0198】

デオキシイノシンがトリガー残基として使用される場合、プローブ内のどこか、特に、伸長可能なプローブ末端にライゲートされる末端とトリガー残基間の位置でのデオキシイノシンの使用を回避することが望ましいことがあり得ることは認識されよう。したがって、プローブが１つ以上の普遍塩基を含む場合、デオキシイノシン以外のヌクレオシドが使用され得る。また、トリガー残基を含有する核酸を、ある特定の切断剤による切断に感受性にするトリガー残基が伸長プローブ内に使用される場合、同じ切断剤による切断を誘発し得る他の残基をプローブ内に（または該伸長プローブとともに配列決定反応に使用され得る他のプローブ内に）を含めることを回避することが望ましいことがあり得ることも認識されよう。

【0199】

本発明は、トリガー残基を含む核酸を切断する任意の酵素の使用を包含する。さらなる酵素は、Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ（登録商標），　Ｉｎｃ．などの酵素供給業者のカタログを調べることにより特定され得る。Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓカタログ，　２００５版（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ，　Ｉｐｓｗｉｃｈ，　ＭＡ　０１９３８－２７２３）は、引用により本明細書に組み込まれ、本発明では、そこに開示されたトリガー残基を含有する核酸を切断する任意の酵素、またはかかる酵素の類縁体の使用が想定される。有用な他の酵素としては、例えば、ｈＯＧＧ１およびその類縁体が挙げられる（Ｒａｄｉｃｅｌｌａ，　ＪＰら、Ｐｒｏｃ　Ｎａｔｌ　Ａｃａｄ　Ｓｅｉ　ＵＳＡ．，９４（１５）：８０１０－５，１９９７）。

【0200】

損傷塩基、無塩基残基などのトリガー残基を含有するオリゴヌクレオチドを合成するための方法は、当該技術分野で知られている。ＡＰエンドヌクレアーゼの基質である部位を含有するオリゴヌクレオチド、例えば、無塩基残基を含有するオリゴヌクレオチド合成するための方法は、当該技術分野で知られており、一般的に自動固相オリゴヌクレオチド合成に適している。ある一部の実施形態において、ウリジンを無塩基残基の所望の位置に含有するオリゴヌクレオチドを合成する。次いで、該オリゴヌクレオチドを、ＵＤＧなどのウラシルを除去する酵素で処理し、それにより、該オリゴヌクレオチド内のどこかにウリジンが存在した無塩基残基を生成させる。

【0201】
本発明のある一部の実施形態において、オリゴヌクレオチドプローブは、Ｎａｕｗｅｌａｅｒｔｓ，　Ｋ．ら，　Ｎｕｃ．Ａｃｉｄｓ．Ｒｅｓ．，３１（２３），２００３に記載された二糖ヌクレオシドを含む。ライゲーション後、伸長された二本鎖を、過ヨウ素酸塩（$NaIO_4$）を用いて切断した後、塩基（例えば、ＮａＯＨ）で処理して標識を除去し、遊離３’ＯＨおよび$P5-OPO_3H_2$基を生成させる。配列決定が３’→５’または５’→３’方向のどちらで行なわれるかに応じて、伸長された二本鎖をポリヌクレオチドキナーゼまたはホスファターゼで処理し、伸長可能な末端を生成させることが必要または望ましいことがある。したがって、本発明のある特定の方法では、伸長可能な末端は、ポリヌクレオチドキナーゼまたはホスファターゼでの処理によって生成される。
【0202】
二糖ヌクレオシドを含むポリヌクレオチドは、無塩基残基を含むとみなされる。例えば、一方のヌクレオチドの３’ＯＨと次のヌクレオチドの５’リン酸基との間に挿入されたリボース残基を含有するポリヌクレオチドは、無塩基残基を含むとみなされる。
【0203】
キャッピング
場合によっては、伸長可能な末端を有するプローブの全部未満が、伸長、ライゲーションおよび切断の各サイクルにおいて成功裏のライゲーション反応に関与する。かかるプローブが継続されるサイクルに関与する場合、各ヌクレオチド同定工程の精度が進行的に低下し得ることは認識されよう。本発明者らは、ホスホロチオレート連結を含有する伸長プローブの使用により、高効率でのライゲーションが可能になることを示したが、本発明のある特定の実施形態では、ライゲーションを行なわない伸長可能な末端が後続のサイクルに関与するのを抑制するためにキャッピング工程を含める。３’－Ｏ－Ｐ－Ｓ－５’ホスホロチオレート結合を含有する伸長プローブを用いる５’→３’方向における配列決定の場合、キャッピングは、例えばライゲーションまたは検出工程後に、ライゲートされない伸長可能な末端をＤＮＡポリメラーゼで伸長させ、伸長不可能な部分（例えば、ジデオキシヌクレオチドまたはヌクレオチドなどの連鎖停止ヌクレオチド）にブロッキング部分を結合させることにより行なわれ得る。３’－Ｓ－Ｐ－Ｏ－５’ホスホロチオレート結合が伸長プローブを用いる３’→５’方向の配列決定の場合、キャッピングは、例えばライゲーションまたは検出工程後に、例えば、鋳型をホスファターゼで処理することにより行なわれ得る。他のキャッピング方法もまた使用され得る。
【0204】
Ｈ．オリゴヌクレオチドプローブファミリーを用いる配列決定
上記の配列決定法を集合的に「方法Ａ」といい、該方法では、任意の特定の伸長プローブ結合された標識と、プローブの近位末端（すなわち、伸長された二本鎖の伸長可能なプローブ末端ライゲートされた末端）の１つ以上のヌクレオチドの正体との間に直接的で既知の対応がある。したがって、新たにライゲートされた伸長プローブの標識を
同定することは、鋳型内の１つ以上ヌクレオチドを同定するのに充分である。本発明は、集合的に「方法ＡＢ」と称し、これもまた、伸長、ライゲーションおよび、好ましくは切断の連続的サイクルを伴い、ヌクレオチド同定に対する異なるアプローチが採用された、さらなる配列決定法を提供する。
【0205】
本発明は、少なくとも２つの識別可能に標識されたオリゴヌクレオチドプローブファミリーのコレクションを使用する配列決定法ＡＢを提供する。各プローブファミリーは、標識に基づいた名称、例えば、「赤色」、「青色」、「黄色」、「緑色」に割り当てられる。上記の方法のように、伸長は、初期オリゴヌクレオチドおよび鋳型によって形成された二本鎖から開始される。初期オリゴヌクレオチドは、オリゴヌクレオチドプローブをその末端にライゲートして伸長された二本鎖を形成させ、次いで、これを、ライゲーションの連続的サイクルによって反復的に伸長させることにより伸長される。伸長された二本鎖のたった１回の伸長が１サイクルで起こるように、プローブは伸長不可能な部分を、末端位

置に（二本鎖の成長核酸鎖にライゲートされたヌクレオチドと反対のプローブ末端に）有する。各サイクル中、成功裏にライゲートされたプローブ上の、またはこれと会合させた標識が検出され、伸長不可能な部分が除去または修飾されて、伸長可能な末端が生成する。標識の検出により、プローブが属するプローブファミリー名が特定される。

【0206】

伸長、ライゲーションおよび検出の連続的なサイクルにより、一連の標識名の序列がもたらされる。標識は、連続的な位置の鋳型にハイブリダイズする成功裏にライゲートされたプローブが属するプローブファミリーに対応する。プローブは、ライゲーション後、鋳型内の異なるヌクレオチドと反対の位置に位置する近位末端を有する。したがって、プローブファミリー名の順序と鋳型内のヌクレオチドの順序との間に対応がある。

【0207】

切れやすい連結が、伸長プローブ内の近位ヌクレオシドと隣接するヌクレオシド間に位置する本発明の実施形態では、プローブファミリーの序列リスト名は、伸長されたオリゴヌクレオチドプローブが、各サイクルで１つのヌクレオチドによって伸長されるため、単一の初期オリゴヌクレオチドから開始される伸長、ライゲーション、検出および切断の連続的サイクルによって得られ得る。切れやすい連結がその他の２つのヌクレオシド間に位置する場合は、プローブファミリーの序列リスト名は、配列決定法Ａで記載のような、結合反応において異なる位置にハイブリダイズする初期オリゴヌクレオチドが使用される複数の配列決定反応から得られる結果から合成される。

【0208】

新たにライゲートされたプローブがどのプローブファミリーに属するかがわかることは、それ自体では、鋳型内のヌクレオチドの正体を決定するのに充分でない。それどころか、それどころか、プローブファミリー名が決定されることは、ヌクレオチドのある特定の組合せが、プローブの少なくとも一部分の配列である可能性を排除するが、各ヌクレオチドの正体に少なくとも２つの可能性が残る。したがって、さらなる情報の非存在下で、プローブファミリー名がわかることにより、新たにライゲートされたプローブ内のヌクレオチドと反対の位置に位置する鋳型内のヌクレオチドの正体に少なくとも２つの可能性が残されたままになる。したがって、伸長、ライゲーション、検出（および、任意選択で切断）任意の１サイクルは、それ自体では、任意の鋳型内のヌクレオチドが同定されない。しかしながら、鋳型の配列の１つ以上の可能性の排除は可能にし、それにより配列に関する情報が提供される。本発明のある特定の実施形態において、後述のようなプローブおよびプローブファミリーの適切な設計により、鋳型の配列が、さらに決定され得る。したがって、本発明のある特定の実施形態において、配列決定法ＡＢは、２つの段階：プローブファミリーの序列リスト名を得る第１の段階、および序列リストをデコード化して鋳型の配列を決定する第２の段階を含む。

【0209】

特に記載のない限り、配列決定法ＡおよびＡＢでは、一般的に、プローブの合成、鋳型の調製、ならびに伸長、ライゲーション、切断および検出の工程の実施に、類似した方法を用いる。

【0210】

配列決定方法ＡＢのためのオリゴヌクレオチド伸長プローブおよびプローブファミリーの特徴

配列決定法ＡＢにおける使用のためのプローブファミリーは、各プローブファミリーが、異なる配列の複数の標識されたオリゴヌクレオチドプローブを配列内の各位置に含み、プローブファミリーが、該位置に異なる塩基を有する少なくとも２つのプローブを含むことを特徴とする。各プローブファミリー内のプローブは同じ標識を含む。好ましくは、プローブは切れやすいヌクレオシド間結合を含む。切れやすい連結は、プローブ内の任意の場所に位置し得る。好ましくは、プローブは、リガーゼによって伸長可能でない部分を一方の末端に有する。好ましくは、プローブは、切れやすい連結と、リガーゼによって伸長可能でない部分との間の位置で標識されており、その結果、伸長可能なプローブ末端への

プローブのライゲーション後、切れやすい連結の切断により、伸長可能なプローブ末端にライゲートされる非標識部分、および非標識部分にはもはや結合されない標識された部分がもたらされる。

【0211】

各プローブファミリー内のプローブは、好ましくは、少なくともｊ個のヌクレオシドＸを含み、ここで、ｊは少なくとも２であり、このとき各Ｘは、プローブファミリー内のプローブにおいて少なくとも２重に縮重している。各プローブファミリー内のプローブは、さらに、少なくともｋ個のヌクレオシドＮを含み、ここで、ｋは少なくとも２であり、Ｎは、任意のヌクレオシド表す。一般に、ｊ＋ｋは１００以下であり、典型的には３０以下である。ヌクレオシドＸは、プローブ内の任意の場所に位置し得る。ヌクレオシドＸは、連続的な位置する必要はない。同様に、ヌクレオシドＮは、連続的な位置する必要はない。換言すると、ヌクレオシドＸおよびＮは散在させ得る。それにもかかわらず、ヌクレオシドＸは、５’→３’配列を有するとみなされ得る。ヌクレオシドは連続的である必要はないと理解され得る。例えば、構造X_AＮＸ$_C$ＮＮＸ$_C$ Ｎのプローブ内のヌクレオシドＸは、配列ＡＧＣを有するとみなされ得る。同様に、ヌクレオシドＮは、配列を有するとみなされ得る。

【0212】

ヌクレオシドＸ同一であっても異なっていてもよいが、独立して選択されない、すなわち、各Ｘの正体は、プローブ内の１つ以上他のヌクレオシドＸの正体によって拘束される。したがって、一般的に、ヌクレオシドＸのある特定の組合せのみが、任意の特定のプローブおよび任意の特定のプローブファミリー内のプローブにおいて存在する。換言すると、各プローブにおいて、ヌクレオシドＸの配列は、長さｊの可能な全ての配列の部分集団のみを表し得る。したがって、Ｘの１つ以上のヌクレオチドの正体により、他方のヌクレオシドの１つ以上の可能な正体が制限される。

【0213】

ヌクレオシドＮは、好ましくは、独立して選択され、Ａ、Ｇ、ＣまたはＴ（あるいは、任意選択で縮重低減性ヌクレオシド）であり得る。好ましくは、ヌクレオシドＮの配列は、１つ以上のＮが縮重低減性ヌクレオシドであり得る以外は、長さｋの可能な全ての配列を表し、したがって、プローブは、２つの部分を含有し、そのうち、ヌクレオシドＮからなる部分は非拘束部分とよばれ、ヌクレオシドＸからなる部分は、拘束部分とよばれる。上記のように、該部分は、連続的なヌクレオシドである必要はない。拘束部分および非拘束部分を含有するプローブは、本明細書において部分拘束プローブという。好ましくは、拘束部分内の１個以上のヌクレオシドは、プローブの近位端、すなわち、伸長可能なプローブ末端にライゲートされるヌクレオシドを含有する末端（これは、種々の本発明の実施形態において、オリゴヌクレオチドプローブ５’または３’末端のいずれかであり得る）に存在する。

【0214】

任意のオリゴヌクレオチドプローブの拘束部分は、ある特定の配列のみを有するものであり得るため、プローブの拘束部分内のヌクレオシドの１つ以上の正体がわかることは、他方のヌクレオシドの１つ以上に関する情報を提供する。該情報は、他方のヌクレオシドの１つ以上を正確に同定するのに充分である場合もそうでない場合もあるが、拘束部分の他方のヌクレオシドの１つ以上の正体の１つ以上の可能性を排除するには充分である。配列決定法ＡＢのある特定の好ましい実施形態において、プローブの拘束部分の１つのヌクレオシドの正体がわかることは、拘束部分の他方のヌクレオシドの各々を正確に同定する、すなわち、拘束部分を含むヌクレオシドの正体および順序を決定するのに充分である。

【0215】

上記の配列決定法のように、鋳型に相補的な伸長プローブ内の最も近位ヌクレオシドは、初期オリゴヌクレオチドの伸長可能な末端にライゲートされ（伸長、ライゲーションおよび検出の第１のサイクル）、伸長、ライゲーションおよび検出の後続のサイクルでは、伸長されたオリゴヌクレオチドプローブの伸長可能な末端にライゲートされる。検出によ

り、新たにライゲートされたプローブが属するプローブファミリー名が決定される。プローブの拘束部分内の各位置は、少なくとも２重に縮重しているため、プローブファミリー名は、それ自体では、拘束部分内の任意のヌクレオチドが同定されない。しかしながら、拘束部分の配列は、長さｊの可能な全ての配列の部分集団の１つであるため、プローブファミリーが同定されることにより、拘束部分の配列のある特定の可能性が排除される。プローブの拘束部分は、その配列決定部分を構成する。したがって、属するプローブファミリーが同定されることによってプローブの拘束部分の１個以上のヌクレオシドの
正体の１つ以上の可能性が排除されることにより、伸長プローブハイブリダイズする鋳型内のヌクレオチドの正体１つ以上の可能性が排除される。本発明の好ましい実施形態において、部分拘束プローブは、切れやすい連結を任意の２つのヌクレオシド間に含む。

【0216】

ある特定の実施形態において、部分拘束プローブは、一般構造（Ｘ）$_{j}$（Ｎ）$_{k}$を有し、式中、Ｘは、ヌクレオシドを表し、（Ｘ）$_{j}$は各位置で少なくとも２重に縮重しており、そのため、Ｘは、異なる塩基対合特異性を有する任意の少なくとも２ヌクレオシドであり得、Ｎは任意のヌクレオシドを表し、ｊは少なくとも２であり、ｋは１～１００であり、少なくとも１つのＮまたはプローブ末端のＸ以外のＸは検出可能な部を含む。好ましくは、（Ｎ）$_{k}$独立して、各位置で４重に縮重しており、そのため、各プローブにおいて、（Ｎ）$_{k}$は、（Ｎ）$_{k}$の１つ以上の位置が縮重低減性ヌクレオチドで占められ得る以外は、長さｋの可能な全ての配列を表す。（Ｘ）$_{j}$内のヌクレオシドは同一であっても異なっていてもよいが、独立して選択されない。換言すると、各プローブにおいて、（Ｘ）$_{j}$は、長さｊの可能な全ての配列の部分集団のみを表し得る。したがって、（Ｘ）$_{j}$の１つ以上のヌクレオチドの正体により、他方のヌクレオシドの１つ以上の可能な正体が制限される。したがって、プローブは２つの部分を含有し、その（Ｎ）$_{k}$は非拘束部分であり、（Ｘ）$_{j}$は拘束部分である。

【0217】

本発明のある特定の好ましい実施形態において、部分拘束プローブは、構造５’－（Ｘ）$_{j}$（Ｎ）$_{k}$Ｎ$_{B}$＊－３’または３’－（Ｘ）$_{j}$（Ｎ）$_{k}$Ｎ$_{B}$＊－５’（式中、Ｎは任意のヌクレオシド表し、Ｎ$_{B}$はリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、（Ｘ）$_{j}$は、各位置で少なくとも２重に縮重しているプローブの拘束部分であり、（Ｘ）$_{j}$内のヌクレオシドは同一であっても異なっていてもよいが、独立して選択されない、少なくとも１つのヌクレオシド間結合は切れやすい連結であり、ｊは少なくとも２であり、ｋは１～１００である、ただし、検出可能な部分は、任意のヌクレオシドＮまたはプローブ末端のＸ以外のＸ上に、Ｎ$_{B}$の代わりまたはこれに加えて存在し得るものとする）を有する。切れやすい連結は、（Ｘ）$_{j}$内の２つのヌクレオシド間、（Ｘ）$_{j}$内の最も遠位ヌクレオチドと（Ｎ）ｋの最も近位ヌクレオシド間、（Ｎ）$_{k}$内のヌクレオシド間、または（Ｎ）ｋ内の末端ヌクレオシドとＮ$_{B}$間に存在し得る。好ましくは、切れやすい連結はホスホロチオレート結合である。

【0218】

別のより好ましい本発明の実施形態では、プローブは、構造５’－（ＸＹ）（Ｎ）$_{k}$Ｎ$_{B}$＊－３’または３’－（ＸＹ）（Ｎ）$_{k}$Ｎ$_{B}$＊－５’（式中、Ｎは任意のヌクレオシド表し、Ｎ$_{B}$はリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、ＸＹはプローブの拘束部分であり、ここで、ＸおよびＹは、同一または異なるが独立して選択されないヌクレオシドを表し、ＸおよびＹは少なくとも２重に縮重しており、少なくとも１つのヌクレオシド間結合は切れやすい連結であり、ｋは１～１００（両端を含む）である、ただし、検出可能な部分は、任意のヌクレオシドＮまたはプローブ末端のＸ以外のＸ上に、Ｎ$_{B}$の代わりまたはこれに加えて存在し得るものとする）を有する。好ましくは、切れやすい連結はホスホロチオレート結合である。構造５’－（ＸＹ）（Ｎ）$_{k}$Ｎ$_{B}$＊－３’を有するプローブは、５’→３’方向の配列決定に有用である。構造３’－（ＸＹ）（Ｎ）$_{k}$Ｎ$_{B}$＊－５’　を有するプローブは、３’→５’方向の配列決定に有用である。

【0219】
ある特定の好ましいプローブの構造を、以下により詳細に示す。５’→３’方向の配列決定には、構造５’－Ｏ－Ｐ－Ｏ－（Ｘ）$_{j}$（Ｎ）$_{k}$－Ｏ－Ｐ－Ｓ－（Ｎ）$_{i}$ N_B＊－３’（式中、Ｎは任意のヌクレオシドを表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、（Ｘ）$_{j}$は、各位置で少なくとも２重に縮重しているプローブの拘束部分であり、（Ｘ）$_{j}$内のヌクレオシドは同一であっても異なっていてもよいが、独立して選択されない、ｊは少なくとも２であり、（ｋ＋ｉ）は１～１００であり、ｋは１～１００であり、ｉは０～９９である、ただし、検出可能な部分は、（Ｎ）$_{i}$の任意のヌクレオシド上にN_Bの代わりまたはこれに加えて存在し得るものとする）を有する部分拘束プローブ。本発明のある特定の実施形態において、（Ｘ）$_{j}$は（ＸＹ）であり、ここで、ＸおよびＹは、少なくとも２重に縮重しており、同一または異なるが独立して選択されないヌクレオチドを表す。本発明のある特定の実施形態において、ｉは０である。
【0220】
５’→３’方向の配列決定のための他の好ましいプローブは、構造５’－Ｏ－Ｐ－Ｏ－（Ｘ）$_{j}$－Ｏ－Ｐ－Ｓ－（Ｎ）$_{i}$ N_B＊－３’を有し、式中、Ｎは任意のヌクレオシドを表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、（Ｘ）$_{j}$は、各位置で少なくとも２重に縮重しているプローブの拘束部分であり、（Ｘ）$_{j}$内のヌクレオチドは同一であっても異なっていてもよいが、独立して選択されない、ｊは少なくとも２であり、ｉは１～１００である、ただし、（Ｎ）$_{i}$の任意のヌクレオシド上に、N_Bの代わりまたはこれに加えて存在し得るものとする。本発明のある特定の実施形態において、（Ｘ）$_{j}$は（ＸＹ）であり、ここで、位置ＸおよびＹは少なくとも２重に縮重しており、ＸおよびＹは、同一または異なるが独立して選択されないヌクレオシドを表す。５’→３’方向の配列決定のためのまた他の好ましいプローブは、構造５’－Ｏ－Ｐ－Ｏ－（Ｘ）$_{j}$－Ｏ－Ｐ－Ｓ－（Ｘ）$_{k}$（Ｎ）$_{i}$ N_B＊－３’を有し、式中、Ｎは任意のヌクレオシドを表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、（Ｘ）$_{j}$－Ｏ－Ｐ－Ｓ－（Ｘ）$_{k}$は、各位置で少なくとも２重に縮重しているプローブの拘束部分であり、（Ｘ）$_{j}$－Ｏ－Ｐ－Ｓ－（Ｘ）$_{k}$内の位置は少なくとも２重に縮重しており、同一であっても異なっていてもよいが、独立して選択されない、ｊおよびｋはともに少なくとも１であり、（ｊ＋ｋ）は少なくとも２（例えば、２、３、４または５）であり、ｉは１～１００である、ただし、検出可能な部分は、（Ｎ）$_{i}$の任意のヌクレオシド上に、N_Bの代わりまたはこれに加えて存在し得るものとする。
【0221】
本発明のある特定の実施形態において、ｊおよびｋはともに１である。
【0222】
３’→５’方向の配列決定では、構造５’－N_B＊（Ｎ）$_{i}$－Ｓ－Ｐ－Ｏ－（Ｎ）$_{k}$－Ｏ－Ｐ－Ｏ－（Ｘ）$_{j}$－３’（式中、Ｎは任意のヌクレオシドを表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、（Ｘ）$_{j}$は、各位置で少なくとも２重に縮重しているプローブの拘束部分であり、（Ｘ）$_{j}$内のヌクレオシドは同一であっても異なっていてもよいが、独立して選択されない、ｊは少なくとも２であり、（ｋ＋ｉ）は１～１００であり、ｋは１～１００であり、ｉは０～９９である、ただし、検出可能な部分は、（Ｎ）$_{i}$の任意のヌクレオシド上に、N_Bの代わりまたはこれに加えて存在し得るものとする）を有する部分拘束プローブ。本発明のある特定の実施形態において、（Ｘ）$_{j}$は（ＸＹ）であり、ここで、ＸおよびＹ少なくとも２重に縮重しており、同一または異なるが独立して選択されないヌクレオシドを表す。本発明のある特定の実施形態において、ｉは０である。
【0223】
３’→５’方向の配列決定のための他の好ましいプローブは、構造５’－N_B＊（Ｎ）$_{i}$－Ｓ－Ｐ－Ｏ－（Ｘ）$_{j}$－３’を有し、式中、Ｎは任意のヌクレオシドを表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、（Ｘ）$_{j}$は、

各位置で少なくとも２重に縮重しているプローブの拘束部分であり、（Ｘ）$_{j}$ 内のヌクレオシドは同一であっても異なっていてもよいが、独立して選択されない、ｊは少なくとも２であり、ｉは１～１００である、ただし、検出可能な部分は、（Ｎ）$_{i}$ の任意のヌクレオシド上に、N_B の代わりまたはこれに加えて存在し得るものとする。本発明のある特定の実施形態において、（Ｘ）$_{j}$ は（ＸＹ）であり、ここで、ＸおよびＹ少なくとも２重に縮重しており、同一または異なるが独立して選択されないヌクレオシドを表す。本発明のある特定の実施形態において、ｊは２～５であり、例えば、任意の部分拘束プローブにおいて２、３、４または５である。

【0224】

３’→５’方向の配列決定のためのまた他の好ましいプローブは、構造５’－N_B *（Ｎ）$_{i}$ －Ｓ－Ｐ－Ｏ－（Ｘ）$_{k}$ －Ｏ－Ｐ－Ｏ－（Ｘ）$_{j}$ －３’（式中、Ｎは任意のヌクレオシドを表し、N_B はリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、－（Ｘ）$_{k}$ －Ｏ－Ｐ－Ｏ－（Ｘ）$_{j}$ は、各位置で少なくとも２重に縮重しているプローブの拘束部分であり、－（Ｘ）$_{k}$ －Ｏ－Ｐ－Ｏ－（Ｘ）$_{j}$ 内のヌクレオシドは同一であっても異なっていてもよいが、独立して選択されない、ｊおよびｋはともに少なくとも１であり、（ｊ＋ｋ）は少なくとも２（例えば、２、３、４または５）であり、ｉは１～１００である、ただし、検出可能な部分は、（Ｎ）$_{i}$ の任意のヌクレオシド上に、N_B の代わりまたはこれに加えて存在し得るものとする）を有する。ある特定の実施形態において、ｊ＝１およびｋ＝１である。

【0225】

切れやすい連結が（Ｘ）$_{j}$ の最も近位ヌクレオシドと（Ｘ）$_{j}$ の次に最も近位ヌクレオシドとの間に位置する本発明の実施形態では、プローブファミリーの序列リスト名は、伸長されたオリゴヌクレオチドプローブが、各サイクルで１つのヌクレオチドによって伸長されるため、単一の初期オリゴヌクレオチドから開始される伸長、ライゲーション、検出および切断の連続的サイクルによって得られ得る。切れやすい連結が、その他の２つのヌクレオシド間に位置する本発明の実施形態では、プローブファミリーの序列リスト名は、配列決定法Ａで記載のような、結合反応において異なる位置にハイブリダイズする初期オリゴヌクレオチドが使用される複数の配列決定反応から得られる結果から合成される。

【0226】

上記のもの以外の任意の多数の構造を有するプローブが、配列決定法ＡＢに使用され得ることは理解されよう。例えば、プローブは、ＸＮＹ（Ｎ）$_{k}$（式中、拘束されたヌクレオシドＸおよびＹは隣接してない）、またはＸＩＹ（Ｎ）$_{k}$（式中、Ｉは普遍塩基である）などの構造を有するものであり得る。（Ｎ）$_{k}$ Ｘ（Ｎ）$_{l}$ 、（Ｎ）$_{i}$ Ｘ（Ｎ）$_{j}$ Ｙ（Ｎ）$_{k}$ Ｚ（Ｎ）$_{l}$ 、（Ｎ）$_{i}$ Ｘ（Ｎ）$_{j}$ ＹＩＺ（Ｎ）$_{l}$ および（Ｎ）$_{i}$ Ｘ（Ｎ）$_{j}$ Ｙ（Ｎ）$_{k}$ Ｚ（Ｉ）$_{l}$ は、さらなる可能性を表す。上記のプローブのように、これらのプローブは、切れやすい連結、検出可能な部分、および一方の末端にリガーゼによって伸長可能でない部分を含む。好ましくは、プローブは、リガーゼによって伸長可能でない部分と反対のプローブ末端のヌクレオチドに結合された検出可能な部分含まない。任意のこれらの構造または他のものを有するプローブを含むプローブファミリーは、各プローブファミリーが異なる配列の複数の標識されたプローブを含み、配列内の各位置において、プローブファミリーが、該位置に異なる塩基を有する少なくとも２つのプローブを含むという基準を満たす。好ましくは、各プローブ内のヌクレオシドの総数は１００以下、例えば３０以下である。

【0227】

オリゴヌクレオチド伸長プローブファミリーのコード化。本発明の配列決定法は、コード化されたプローブファミリーを利用するものである。「コード化」は、ある特定の標識を、規定の１組の配列の１つを有する部分を含むプローブと会合させ、その結果、該規定の組の配列のメンバーである配列を有する部分を含むプローブが該標識で標識されるスキームをいう。一般に、コード化では、複数の識別可能な標識の各々を１つ以上プローブと会合させ、その結果、各識別可能な標識が異なる群のプローブと会合され、各プローブが

たった１つの標識（これは、検出可能な部分の組合せを含み得る）によって標識される。好ましくは、各プローブ群のプローブは、各々、同じ規定の組の配列のメンバーである配列を有する部分を含む。該部分は、単一のヌクレオシドであってもよく、多くのヌクレオシド長、例えば、２、３、４、５またはそれ以上のヌクレオシド長であってもよい。該部分の長さは、完全長のプローブ小画分のみを構成するものであってもよく、プローブ全体を構成するものであってもよい。規定の組の配列は、単一の配列のみを含むものであってもよく、該部分の長さに応じて、任意の数の異なる配列を含むものであってもよい。例えば、該部分が単一のヌクレオシドである場合、規定の組の配列は、最大４つの要素（Ａ、Ｇ、Ｃ、Ｔ）　を有し得る。部分が２つのヌクレオシド長である場合、規定の組の配列は、１６個までの要素（ＡＡ、ＡＧ、ＡＣ、ＡＴ、ＧＡ、ＧＧ、ＧＣ、ＧＴ、ＣＡ、ＣＧ、ＣＣ、ＣＴ、ＴＡ、ＴＧ、ＴＣ、ＴＴ）　を有し得る。一般に、規定の組の配列は、可能な配列の総数よりも少数の要素を含み、コード化では、１つより多くの規定の組の配列を用いる。

【0228】

本明細書に記載した配列決定法Ａでは、一般的に、近位ヌクレオシドプローブ内の（すなわち、伸長可能なプローブ末端にライゲートされたヌクレオシド）と標識の正体との間に直接的な対応がある単一のコード化を有する１組のプローブを利用する。近位ヌクレオシドは、鋳型内でハイブリダイズするヌクレオチドに相補的であり、そのため、新たにライゲートされたプローブの近位ヌクレオシドの正体により、伸長された二本鎖の位置と反対側に位置する鋳型内のヌクレオチドの正体が決定される。一般的な意味において、本明細書に記載の他方の配列決定法に有用なプローブは、構造Ｘ（Ｎ）$_k$ を有し、式中、Ｘは近位ヌクレオシドであり、各ヌクレオシドＮは４重に縮重しており、そのため、長さｋの可能な全ての配列が、該プローブを構成するオリゴヌクレオチドプローブ分子のプールに提示される。したがって、例えば、一部のオリゴヌクレオチドプローブ分子がＡを位置ｋ＝１に含有する場合、あるものはＧを位置ｋ＝１に含有し、あるものはＣを位置ｋ＝１に含有し、あるものはＴを位置ｋ＝１に含有し、他の位置ｋについても同様であり、（Ｎ）$_k$ 内のＸに隣接するヌクレオシドは位置ｋ＝１を占めるとみなされる；（Ｎ）$_k$ 内の次のヌクレオシドは位置ｋ＝２を占めるとみなされるなどである。しかしながら、任意の所与のオリゴヌクレオチドプローブにおいて、Ｘは、たった１つの塩基対合特異性を表し、これは、典型的には、ある特定のヌクレオシドの正体、例えば、Ａ、Ｇ、ＣまたはＴに対応する。したがって、Ｘは、典型的には、ある特定のプローブを構成するプローブ分子のプール内で均一にＡ、Ｇ、ＣまたはＴである。図２は、構造Ｘ（Ｎ）$_k$ を有するプローブの適当なコード化を示す。このコード化によれば、Ｘ＝Ｃを有するプローブは標識「赤色」に割り当てられる；Ｘ＝Ａを有するプローブは標識「黄色」に割り当てられる；Ｘ＝Ｇを有するプローブは標識「緑色」に割り当てられる；およびＸ＝Ｔを有するプローブは標識「青色」に割り当てられる。したがって、プローブの配列決定部分とその標識間に１対１対応が存在する。

【0229】

新たにライゲートされた伸長プローブの標識の正体が、伸長プローブの最も近位ヌクレオシドの正体に対応する上記のアプローチは、標識の正体が伸長プローブ最も近位ヌクレオシドのみの正体に対応するのではなく、伸長プローブ内の最も近位の２つ以上のヌクレオシドの配列に対応し、その結果、鋳型内の多数のヌクレオチドの正体が、伸長、ライゲーションおよび検出の１サイクルで決定され得る（典型的には、その後、切断を行なう）コード化を包含するように拡張され得ることは認識されよう。しかしながら、かかるコード化は、依然として標識をオリゴヌクレオチド伸長プローブ内の単一の配列に会合させるものであり得、そのため、対向する位置にある鋳型内の相補的なヌクレオチドの正体が同定され得る。例えば、上記のように、１サイクルで２つのヌクレオチドを同定するためには、各々が対応する標識を有する（すなわち、１６種類の識別可能な標識）１６種類の異なるオリゴヌクレオチドプローブが必要とされ得る。

【0230】

配列決定法ＡＢでは、標識をプローブと会合させる代替的なアプローチを用いる。標識の正体とプローブの配列決定部分の配列間の１対１対応ではなく、同じ標識を、異なる配列決定部分を有する多数のプローブに割り当てる。プローブは部分的に拘束されており、プローブの拘束部分がその配列決定部分である。したがって、同じ標識が、各々が異なる配列拘束部分を有し、該配列が規定の１組の配列の１つである複数の異なるプローブに割り当てられる。上記のように、同じ標識を含むプローブは、「プローブファミリー」を構成する。該方法では、各々が異なる配列の拘束部分を有し、該配列が規定の１組の配列の１つである複数のプローブを含む複数のかかるプローブファミリーを用いる。

【0231】

複数のプローブファミリーをプローブファミリーの「コレクション」という。プローブファミリーのコレクション内の各プローブファミリー内のプローブは、該コレクション内の他のプローブファミリーを標識するために使用される標識と識別可能な標識で標識されている。各プローブファミリーは、好ましくは、その独自の規定の組の配列を有する。好ましくは、各プローブファミリー内のプローブの拘束部分は同じ長さであり、好ましくは、プローブファミリーのコレクション内のプローブファミリーの拘束部分は同じ長さである。好ましくは、プローブファミリーのコレクション内のプローブファミリーの規定配列の組の組合せは、拘束部分の長さの可能な全ての配列を含む。好ましくは、プローブファミリーのコレクションは、４つの識別可能に標識されたプローブファミリーを含むか、または外ファミリーからなる。好ましくは、プローブの拘束部分は２ヌクレオシド長である。

【0232】

広範なさまざまな異なるようにコード化された識別可能に標識されたプローブファミリーのコレクションが上記の基準を満足し、本発明の方法を実施するために使用され得る。しかしながら、ある特定のプローブファミリーのコレクションが好ましい。部分拘束プローブを含む４つの識別可能に標識されたプローブファミリーの好ましいコレクションの例示的なコード化を図２５Ａに示す。図２５Ａに示すように、拘束部分は、プローブ内の２つの最も３’側のヌクレオシドからなる。プローブファミリーは、「赤色」、「黄色」、「緑色」および「青色」に標識される。各プローブファミリー内のプローブは、その配列が規定の１組の配列の１つであり、該規定の組は各プローブファミリーで異なる拘束部分を含む。例えば、プローブの近位端であるみなされる各配列の３’末端からから始めると、「赤色」プローブファミリーの規定の組の配列は｛ＣＴ、ＡＧ、ＧＡ、ＴＣ｝である；「黄色」のプローブファミリーの規定の組の配列は｛ＣＣ、ＡＴ、ＧＧ、ＴＡ｝である；「緑色」のプローブファミリーの規定の組の配列は｛ＣＡ、ＡＣ、ＧＴ、ＴＧ｝である；「青色」のプローブファミリーの規定の組の配列は｛ＣＧ、ＡＡ、ＧＣ、ＴＴ｝である。各規定の組は、その他の組の１つに存在する任意のメンバーを含まず、好ましい特徴である。また、プローブファミリーのコレクション内のプローブファミリーの規定配列の組の組合せは、長さ２の可能な全ての配列、すなわち、可能な全てのジヌクレオシドを含む。このプローブファミリーのコレクションの別の特徴（好ましいが必要ではない）は、プローブの拘束部分内の各位置が、４重に縮重している、すなわちＡ、Ｇ、ＣまたはＴのいずれかで占められ得ることである。このプローブファミリーのコレクションの別の特徴（好ましいが必要ではない）は、規定配列の組の各々において、たった１つの配列が、任意の特異的ヌクレオシドを任意の位置に、例えば、最も近位の位置または任意の他の位置に有することである。規定配列の組の各々において、たった１つの配列が、任意の特異的ヌクレオシドを拘束部分内の２位以降に（最も近位ヌクレオシドを１位とみなす）を有することは特に好ましいが必要ではない。例えば、赤色プローブファミリーの規定の組の配列において、たった１つの配列がＴを２位に有する；たった１つの配列がＧを２位に有する；たった１つの配列がＡを２位に有する；たった１つの配列がＣを２位に有する。

【0233】

図２５Ａに示したものなどの任意の特定のコード化が与えられると、プローブファミリーの１つのプローブの拘束部分内の１個以上のヌクレオシドの正体がわかることにより、

該プローブの拘束部分内の他方のヌクレオチドに関する情報が提供される。最も一般的な意味において、プローブファミリー内のプローブの拘束部分内の１個以上のヌクレオシドの正体がわかることにより、該プローブファミリーの規定の組の配列は、該正体を該位置を有するヌクレオシドを有する配列を含まないため、他方の位置の１つにおけるヌクレオシドの１つ以上の可能な正体を排除するのに充分な情報が提供される。典型的には、プローブファミリー内のプローブの拘束部分内の１個以上のヌクレオシドの正体がわかることにより、複数のヌクレオシド、例えば、その他のヌクレオシドの各々の１つ以上の可能な正体を排除するのに充分な情報が提供される。好ましいコード化では、プローブファミリー内のプローブの含有された部分の１個以上のヌクレオシドの正体がわかることにより、プローブ内のその他のヌクレオシドの各々の１つ以外すべての可能性が排除される。例えば、図２５Ａに示すコード化されたプローブファミリーの場合、プローブが赤色ファミリーのメンバーであることがわかっている場合、また、最も近位ヌクレオシドがＣであることもわかっている場合、隣接するヌクレオシドはＴのはずである。同様に、プローブが緑色ファミリーのメンバーであることがわかっている場合、また、最も近位ヌクレオシドがＧであることもわかっている場合、隣接するヌクレオシドはＴのはずである。したがって、拘束部分の１つのヌクレオシドの正体がわかることは、他方のヌクレオシドの１つ以外すべての可能性を排除するのに充分であり、そのため、他方のヌクレオシドの正体が完全に特定化される。しかし、プローブの拘束部分の少なくとも１つのヌクレオシドの正体がわからないと、拘束部分の各位置のヌクレオシドがＡ、Ｇ、ＣまたはＴであり得るため、属する　プローブファミリー名がわかったことに基づいて、プローブ内の特異的ヌクレオシドの正体に関する情報を取得することはまったくできない。図２５Ｂは、プローブファミリーの好ましいコレクション（上側パネル）および配列決定法ＡＢを用いたライゲーション、検出および切断のサイクル（下側パネル）を示す。

【0234】

本発明者は、図２５Ａに示すような、２ヌクレオシド長であって、プローブファミリーのコレクションの好都合な特徴を有する拘束部分を含む２４個のプローブファミリーのコレクションを設計した。これらのプローブファミリーは、プローブが属するプローブファミリー名がわかること、およびプローブ内の１つのヌクレオシドの正体がわかることが、拘束部分の他方のヌクレオシドを正確に同定するのに充分であるという点で最大の情報を与える。これは、すべてのプローブおよび各拘束部分内のすべてのヌクレオシドについてそうである。プローブファミリーの２４個の好ましいコレクションの各々のコード化スキームを表１に示す。表１では、１～２４の範囲のコード化ＩＤを各プローブファミリーのコレクションに割り当てている。各コード化により、配列決定法ＡＢにおける使用のための一般構造（ＸＹ）N_k の好ましいプローブファミリーのコレクションの拘束部分が規定され、それによりコレクションそれ自体が規定される。表１において、コード化ＩＤの下の欄の値１は、該コード化に従って、それぞれ第１および第２の欄に示されたヌクレオシドＸおよびＹを含むプローブが、第１のプローブファミリーに割り当てられることを示す；（ｉｉ）コード化ＩＤの下の欄の値２は、該コード化に従って、それぞれ第１および第２の欄に示されたヌクレオシドＸおよびＹを含むプローブが、第２のプローブファミリーに割り当てられることを示す；（ｉｉｉ）コード化ＩＤの下の欄の値３は、該コード化に従って、それぞれ第１および第２の欄に示されたヌクレオシドＸおよびＹを含むプローブが、第３のプローブファミリーに割り当てられることを示す；ならびに（ｉｖ）コード化ＩＤの下の欄の値４は、該コード化に従って、それぞれ第１および第２の欄に示されたヌクレオシドＸおよびＹを含むプローブが、第４のプローブファミリーに割り当てられることを示す。値１、２、３および４は各々、標識を表す。例えば、コード化９により図２５Ａに示すプローブファミリーのコレクションが規定され、この場合、１は青色を表し、２は緑色を表し、３は赤色を表し、４は黄色を表す。標識に対する値の割り当ては随意であり、例えば、１は、充分等しく緑色、赤色または黄色を表し得ることは認識されよう。値１、２、３および４と標識との関連を変更することによって各プローブファミリー内のプローブの組は変更され得ず、単に、異なる標識が各プローブファミリーと関連することに

なり得るだけである。

【0235】

表１：オリゴヌクレオチドプローブファミリーコード化

【0236】

【表1】

		コード化ＩＤ																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
X	Y																								
A	A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C	A	2	4	3	2	2	4	3	2	2	3	4	3	2	3	3	4	2	3	4	4	2	4	3	4
G	A	4	3	2	3	3	2	4	4	3	2	2	4	4	2	4	3	4	2	3	2	3	2	4	3
T	A	3	2	4	4	4	3	2	3	4	4	3	2	3	4	2	2	3	4	2	3	4	3	2	2
A	C	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
C	C	1	1	1	1	1	1	1	1	4	4	3	4	4	4	4	3	3	4	3	3	3	3	4	3
G	C	3	4	4	4	4	3	3	3	1	1	1	1	3	3	3	4	1	1	1	1	4	4	3	4
T	C	4	3	3	3	3	4	4	4	3	3	4	3	1	1	1	1	4	3	4	4	1	1	1	1
A	G	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
C	G	4	2	4	4	4	2	4	4	1	1	1	1	1	1	1	1	4	2	2	2	4	2	2	2
G	G	1	1	1	1	2	4	2	2	4	4	4	2	2	4	2	2	2	4	4	4	1	1	1	1
T	G	2	4	2	2	1	1	1	1	2	2	2	4	4	2	4	4	1	1	1	1	2	4	4	4
A	T	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
C	T	3	3	2	3	3	3	2	3	3	2	2	2	3	2	2	2	1	1	1	1	1	1	1	1
G	T	2	2	3	2	1	1	1	1	2	3	3	3	1	1	3	1	3	3	2	3	2	3	2	2
T	T	1	1	1	1	2	2	3	2	1	1	1	1	2	3	1	3	2	2	3	2	3	2	3	3

好ましいプローブファミリーのコレクションを規定する表１の使用をさらに説明するため、コード化１７を考察する。このコード化によれば、拘束部分ＡＡ、ＧＣ、ＴＧおよびＣＴを有するプローブが標識１（例えば、赤色）に割り当てられ、拘束部分ＣＡ、ＡＣ、ＧＧおよびＴＴを有するプローブが標識２（例えば、黄色）に割り当てられ、拘束部分ＴＡ、ＣＣ、ＡＧおよびＧＴを有するプローブが標識３（例えば、緑色）に割り当てられ、拘束部分ＧＡ、ＴＣ、ＣＧおよびＡＴを有するプローブが標識４（例えば、青色）に割り当てられている。得られるプローブファミリーのコレクションを図２６に示す。

【0237】

図２７Ａ～２７Ｃは、プローブファミリーの２４個の好ましいコレクションを図式的に規定する代替法を表す。該方法では、図２７Ａのものなどのダイアグラムを利用する。かかるダイアグラム内の第１の欄は第１の塩基を表す。各標識は、４種類の異なる塩基配列に結合されている。その各々は、第１の欄の塩基を選択された標識の欄の塩基と並置させて示されている。例えば、項目「第１の塩基」の欄にＡがある場合、配列ＡＡを有する拘束部分を有するプローブは、プローブファミリー１（標識１）に割り当てられ、配列ＡＣ

を有する拘束部分を有するプローブは、プローブファミリー２（標識２）に割り当てられ、配列ＡＧを有する拘束部分を有するプローブは、プローブファミリー３（標識３）　に割り当てられ、配列ＡＴを有する拘束部分を有するプローブは、プローブファミリー４（標識４）に割り当てられる。プローブファミリーに対する割り当ては、Ｃ、ＧまたはＴで始まる拘束部分を有するプローブと同様にして行なわれる。したがって、図２７Ａに示すように塩基で空欄を埋めたダイアグラムは、図２７Ｂに示すようなコード化に翻訳され、ここでは、｛ＡＡ、ＣＣ、ＧＧ、ＴＴ｝の組の拘束部分を有するプローブはプローブファミリー１に割り当てられ、｛ＡＣ、ＣＡ、ＧＴ、ＴＧ｝の組の拘束部分を有するプローブはプローブファミリー２に割り当てられ、｛ＡＧ、ＣＴ、ＧＣ、ＴＡ｝の組の拘束部分を有するプローブはプローブファミリー３に割り当てられ、｛ＡＴ、ＣＧ、ＧＡ、ＴＣ｝の組の拘束部分を有するプローブはプローブファミリー４に割り当てられる。図２７Ｃは、プローブファミリーの２４個の好ましいコレクションの各々を作製するために、図２７Ａダイアグラムの陰影部分の代わりに挿入され得るダイアグラムを示す。配列決定法ＡＢにおいて好ましいプローブファミリーのコレクションを使用する方法は、以下にさらに記載する。

【0238】

表１に規定されるコード化されたプローブファミリーの２４個のコレクションは、配列決定法ＡＢにおける使用のためのプローブファミリーのコレクションの好ましい実施形態のみを表す。プローブファミリー名がわかることとともに、拘束部分内の１個以上のヌクレオシドの正体がわかることによって、１つ以上他のヌクレオシドに関する情報が提供される同じ基本原理を用いる広範なさまざまな他のコード化スキーム、プローブファミリーおよびプローブ構造が使用され得る。プローブファミリーの好ましいコレクションと比較すると、プローブファミリーのやや好ましいコレクションは、一般的に、（ｉ）少なくとも一部プローブに関して、プローブファミリー名がわかることによってもたらされる情報の量およびヌクレオシドの正体が少ない；または（ｉｉ）少なくとも一部のプローブに関して、プローブファミリー名がわかることによってもたらされる情報の量がより多いため、やや好ましい。

【0239】

一般に、プローブファミリーのやや好ましいコレクションは、配列決定法ＡＢを、好ましいプローブファミリーのコレクションが使用される様式と同様にして行うために使用され得る。しかしながら、デコード化に必要とされる該工程が異なり得る。例えば、状況によっては、候補配列を互いに比較することが、配列の少なくとも一部分を決定するのに充分であり得る。

【0240】

プローブが２ヌクレオシド長である拘束部分を含むプローブファミリーのやや好ましいコレクションの一例を図２８に示す。このコード化によれば、｛ＡＡ、ＡＣ、ＧＡ、ＧＣ｝の組の拘束部分を有するプローブはプローブファミリー１に割り当てられ、｛ＣＡ、ＣＣ、ＴＡ、ＴＣ｝の組の拘束部分を有するプローブはプローブファミリー２に割り当てられ、｛ＡＧ、ＡＴ、ＧＧ、ＧＴ｝の組の拘束部分を有するプローブはプローブファミリー３に割り当てられ、｛ＣＧ、ＣＴ、ＴＧ、ＴＴ｝の組の拘束部分を有するプローブはプローブファミリー４に割り当てられる。このプローブファミリーのコレクションでは、プローブファミリー名がわかることにより、プローブファミリー名を決定するためにその標識が検出された新たにライゲートされた伸長プローブ内の近位ヌクレオシドと反対の位置に位置する鋳型内のヌクレオチドの正体についてのある特定の可能性が排除される。例えば、プローブファミリー名が１である場合、新たにライゲートされた伸長プローブ内の近位ヌクレオシドはＡまたはＧのはずであり、そのため、相補的鋳型内のヌクレオチドはＴまたはＣのはずである。拘束部分内の各位置において少なくとも２つの可能性があるため、ヌクレオチドは正確には同定され得ないが、好ましいプローブファミリーのコレクションが使用される場合とは対照的に、一部の可能性を排除するのには充分な情報が１サイクルから得られる。

【0241】
本発明のある特定の実施形態において、拘束部分が３ヌクレオシド長である部分拘束プローブが使用される。その拘束部分が長さ３可能な全ての配列を含む（これは、好ましい）プローブを含めるため、プローブファミリーのコレクションは、$4^3=64$種の異なるプローブを含むものであるのがよい。図２９Ａは、拘束部分３ヌクレオシド長（トリヌクレオシド）を有するプローブを含むプローブファミリーのコレクション拘束部分を作製するために使用され得るダイアグラムを示す。この図は、Ａ、Ｇ、ＣおよびＴで表示された４組の列、ならびにプローブファミリー名１、２、３および４の４つの欄を示す。各組みの４列は、内部にヌクレオシドの正体を有するボックスと反対である。トリヌクレオシドのプローブファミリーを決定するため、トリヌクレオシドの最後のヌクレオシドを含むボックスをまず選択する。該ボックスに隣接する４つの列内で、トリヌクレオシド内の第１のヌクレオシドを特定する文字が標示された列を選択する。該列内で、トリヌクレオシドの第２のヌクレオシドを含む欄を選択する。該トリヌクレオシドは、該欄の上部に標示されたプローブファミリーに割り当てられる。例えば、以下の手順に従って、トリヌクレオシド「ＴＣＧ」をプローブファミリーに割り当てる。最後のヌクレオシドが「Ｇ」であるため、「Ｇ」を含むボックスと対向する位置に位置する４列の組、すなわち、第３の組の列に注目する。第１のヌクレオシド「Ｔ」であるため、考慮事項を４つの組の最後の列にさらに限定する。プローブファミリー割り当ては、真ん中のヌクレオシドを含む欄の表題によって決定される。真ん中のヌクレオシドは「Ｃ」であるため、トリヌクレオシドはプローブファミリー１に割り当てられる。類似したプロセスにより、プローブファミリー割り当て：ＡＡＡ＝１；ＡＴＡ＝２；ＡＧＡ＝３；ＧＴＡ＝４；ＧＡＧ＝１；ＴＧＧ＝２などがもたらされる。このプロセスは、可能な全てのトリヌクレオシドがプローブファミリーに割り当てられるまで継続される。
【0242】
図２９Ｂは、３ヌクレオシド長の拘束部分を有するプローブを含むプローブファミリーのコレクションさらなる拘束部分を構築するための手順を示す。該手順は、かかるコレクションを拘束部分が２ヌクレオシド長でありコレクションが４つのプローブファミリーを含む上記のプローブファミリーの２４個の好ましいコレクションの各々から構築するために使用される。プローブファミリーの好ましいコレクションを示す例示的なダイアグラムを図の上側部分に示す。このダイアグラムの欄を、上側のダイアグラムの各欄に割り当てられた色に従って、図の下側部分の欄に直接マッピングしている。したがって、上側のダイアグラムの欄は、左から右に向かって青色、緑色、黄色および赤色である。下側のダイアグラムの欄１のエントリーは、上から下に向かって青色、緑色、黄色および赤色であり、４ヌクレオシドの各組は、上側のダイアグラム欄に対応する。下側ダイアグラムの第２、３および４欄は、欄１の４ヌクレオシドの各組を下方に進行的に移動させることにより作製される。
【0243】
「プローブファミリー」は、各々が同じ標識を有する複数の異なるプローブを含む単一の「スーパープローブ」であるとみなされ得ることは認識されよう。この場合、プローブを構成するプローブ分子は、一般的に、該プローブの任意の部分において実質的に同一の分子の実質的に同一の分子集団ではない。用語「プローブファミリー」の使用は、任意の限定的な効果を有することを意図するものではなく、かかる「スーパープローブ」を構成し得るプローブの特徴の記載の便宜上のために用いる。
【0244】
デコード化
上記のように、少なくとも２つの識別可能に標識されたプローブファミリーを含むプローブファミリーのコレクションを用いる伸長、ライゲーション、検出および切断の連続的サイクルにより、プローブファミリーの序列リスト名が、単一の配列決定反応、または序列リスト内の鋳型内の異なる部位から開始される多重配列決定反応で決定されたプローブファミリー名の合成のいずれかからもたらされる。実施されるサイクル数は、所望の配列

の長さおよそ同等であるはずである。序列リストは、相当な量の情報を含むが、直接的に目的の配列が得られる形態ではない。おそらく目的の配列を表す配列を得るため、さらなる工程（１つまたは複数）（その少なくとも１つは、さらなる配列に関する情報の少なくとも１つの項目の収集を伴う）を行なわなければならない。おそらく目的の配列を表す配列は、本明細書において「正確な」配列といい、正確な配列をプローブファミリーの序列リストから抽出するプロセスは、を「デコード化」という。上記の「序列リスト」内の要素は、リストの作製中またはその後のいずれかで再編成され得ることは認識されよう。ただし、リスト内の要素と鋳型内のヌクレオチド間の対応を含む情報の内容は保持されるものとし、また、再編成、再分化および／または置換（ｐｅｒｍｕｔａｔｉｏｎ）は、デコード化プロセス中に適切に考慮されるものとする（後述）。したがって、用語「序列リスト」は、上記のようにして作製された再編成、再分化および／または置換された変形例の序列リストを包含することが意図される（ただし、かかる再分化および／または置換された変形例は実質的に同じ情報内容を含むものとする）。

【0245】

序列リストは、さまざまなアプローチを用いてデコード化され得る。一部のこれらのアプローチは、プローブファミリーの序列リスト名からの１組の少なくとも１つの候補配列の作製を伴う。候補配列の組により、目的を達成するのに充分な情報が提供され得る。好ましい実施形態において、おそらく目的の配列を表す配列を候補配列の中から、または候補配列と比較される１組の配列から選択するために、１つ以上のさらなる工程が行なわれる。例えば、アプローチの一例において、少なくとも１つの候補配列の少なくとも一部分を、少なくとも１つの他の配列と比較する。正確な配列は、比較に基づいて選択される。本発明のある特定の実施形態において、デコード化は、該方法を反復すること、および元のプローブファミリーのコレクションと異なるようにコード化されたプローブファミリーのコレクションを用いて第２のプローブファミリーの序列リスト名を得ることを伴う。第２のプローブファミリーの序列リストからの情報は、正確な配列を決定するために使用される。ある一部の実施形態において、別のコード化されたプローブファミリーのコレクションを用いて伸長、ライゲーションおよび検出の１サイクルという少ないサイクルから得られる情報は、正確な配列の選択の可能にするのに充分である。換言すると、別のコード化されたプローブファミリーを用いて同定された第１のプローブファミリーにより、どの候補配列が正確であるかを決定するのに充分な情報が提供される。

【0246】

他のデコード化アプローチは、任意の利用可能な配列決定法、例えば、１サイクルの配列決定法Ａによって鋳型内の少なくとも１つのヌクレオチドを特異的に同定することを伴う。１つ以上のヌクレオチド（１つまたは複数）に関する情報が、プローブファミリーの序列リスト名をデコード化するための「鍵」として用いられる。あるいはまた、鋳型の配列決定される部分は、その配列が未知である領域に加えて、既知配列の領域を含むものであり得る。配列決定法ＡＢが、未知配列と既知配列の少なくとも１つのヌクレオチドとの両方を含む鋳型の部分に適用される場合、既知配列が、プローブファミリーの序列リスト名をデコード化するための「鍵」として用いられ得る。以下のセクションに候補配列を作製するプロセスを記載する。その後のセクションに、既知配列との比較、第２の組の候補配列との比較、および既知ヌクレオチド正体の利用によって正確な配列を選択するための候補配列の使用を記載する。

【0247】

候補配列の作製

鋳型の配列決定される領域は、伸長、ライゲーションおよび切断の連続的サイクルによって生成される伸長された二本鎖に相補的であることは認識されよう。したがって、伸長された二本鎖の候補配列を作製することは、鋳型の配列決定される領域の候補配列を作製することに等価である。実際には、鋳型の配列決定される領域の候補配列を作製し得るか、または伸長された二本鎖の候補配列を作製し、その相補配列を得て鋳型の配列決定される領域の候補配列を決定し得る。後者のアプローチをここに記載する。候補配列をプロー

ブファミリー名のリストから作製するため、プローブファミリーのリストの第１のメンバーを考慮する。該プローブファミリーと関連する拘束部分の組により、配列内の最初のヌクレオチドの可能性が、拘束部分の長さに相当する長さに限定される。例えば、拘束部分がジヌクレオチドである場合、伸長された二本鎖内の最初のジヌクレオチドの可能な配列は、該プローブファミリーに含まれるプローブ内に存在する拘束部分に限定される（したがって、鋳型の配列決定される領域内の第１のジヌクレオチドの可能な配列は、該プローブファミリーに含まれるプローブ内に存在する拘束部分に相補的な組合せに限定される）。最初のジヌクレオチドの可能性は、典型的にはコンピュータによって記録される。同様に、伸長された二本鎖内の第２のジヌクレオチド（すなわち、第１のジヌクレオチドから１ヌクレオチドずれたジヌクレオチド）の可能な配列が、第２のプローブファミリーに含まれるプローブ内に存在する拘束部分に限定される（したがって、鋳型内の第２のジヌクレオチド、すなわち、第１のジヌクレオチドから１ヌクレオチドずれたジヌクレオチドの可能な配列が、第２のプローブファミリーに含まれるプローブ内に存在する拘束部分に相補的な組合せに限定される）。第２のジヌクレオチドの可能な配列もまた記録される。続くジヌクレオチドの可能な配列（ｐｏｓｓｉｂｉｌｉｔｙ）も同様に、決定対象の配列の所望の長さに相当するまで、またはリスト内のプローブファミリーがなくなるまでジヌクレオチドの可能な配列が記録される。

【0248】

可能な配列の記録プロセスの代表例を図３０に示す。図において、プローブファミリー名のリストは、図２５Ａに示すプローブファミリーコレクションを用いて作製されたものと想定している。図３０の左端の欄はプローブファミリーのリストを示し、上から下の順に、黄色、緑色、赤色、青色である。リスト内の各プローブファミリーに対応するジヌクレオチドの配列可能性を図の右側に示す。ヌクレオチド位置を配列可能性の上部に示す。配列は１位から始まり、そのため、第１のジヌクレオチドは１位と２位を占める；第２のジヌクレオチドは２位と３位を占めるなどである。黄色プローブファミリーでは、該可能性は、図３０に示すようにＣＣ、ＡＴ、ＧＧおよびＴＡである。緑色プローブファミリーでは、該可能性は、ＣＡ、ＡＣ、ＧＴおよびＴＧであるなどである。各ジヌクレオチドの可能な配列の記録プロセスは、所望の配列長さに達するまで継続される。

【0249】

可能性の組を作製した後、第１の仮定を、候補配列内の第１のヌクレオチドの正体において行ない、これは、図３０において１位と表示する配列の５’位置であると仮定するものである。第１の仮定は、該ヌクレオチドがＡである、該ヌクレオチドがＧである、該ヌクレオチドがＣであるまたは該ヌクレオチドがＴであるとするものであり得る。

【0250】

各ジヌクレオチドの可能な配列は、隣接するジヌクレオチドが重複する、すなわち、第１のジヌクレオチドの第２のヌクレオチドはまた、第２のジヌクレオチドの第１のヌクレオチドであるため、隣接するジヌクレオチドの可能な配列によって限定されることが観察される。例えば、第１のヌクレオチドをＣと仮定した場合、第１のジヌクレオチドはＣＣとなるはずである。第１のジヌクレオチドがＣＣであるならば、第２のジヌクレオチドは、Ｃをその第１の位置に有するはずである。Ｃをその第１の位置に有する第２のジヌクレオチドの唯一可能な配列はＣＡであるため、第２のジヌクレオチドがＣＡのはずであることは明白である。したがって、最初のの３つのヌクレオチドの配列は、ＣＣＡのはずである。同様に、第３のジヌクレオチドの可能な配列は、第２のジヌクレオチドの可能な配列によって限定される。第２のジヌクレオチドがＣＡであるならば、第３のジヌクレオチドは、その第１の位置にＡを有することが唯一の可能性であるため、ＡＧのはずである。したがって、最初の４つのヌクレオチドの配列はＣＣＡＧのはずである。このプロセスを継続すると、最初の５つヌクレオチドに対して５’－ＣＣＡＧＣ－３’の配列がもたらされる。したがって、ＣＣＡＧＣは第１の候補配列である。

【0251】

第２の候補配列は、第１のヌクレオチドがＡであると仮定することによりもたらされる

。この仮定により、ＡＴが第１のジヌクレオチドとなる。ＴＧは、ＡＴが第１のジヌクレオチドの配列と整合する第２のジヌクレオチドの唯一の可能な配列である。ＧＡは、ＴＧが第２のジヌクレオチドの配列と整合する第３のジヌクレオチドの唯一の可能な配列である。ＡＡは、ＧＡが第３のジヌクレオチドの配列と整合する第４のジヌクレオチドの唯一の可能な配列である。これらのジヌクレオチドを完全長の候補配列に合成すると、ＡＴＧＡＡが得られる。同様に、第１のヌクレオチドがＧであるという仮定により、候補配列ＧＧＴＣＧが得られ、第１のヌクレオチドがＴであるという仮定により、候補配列ＴＡＣＴＴが得られる。したがって、各々、仮定された配列の第１のヌクレオチドの異なるヌクレオチドで始まる４つの候補配列がもたらされる。

【0252】

仮定は、その他のヌクレオチドの１つではなく第１のヌクレオチドで行なわなければならないとする要件はない。例えば、仮定は、充分等しく第４のヌクレオチドの正体において行なわれたものであり得、この場合、候補配列は、鋳型に沿って「逆行方向」（すなわち、３’→５’方向）に移動させることによりもたらされ得る。例えば、第４のヌクレオチドがＴであると仮定することは、第４のジヌクレオチドはＴＴのはずであり、第３のジヌクレオチドはＣＴのはずであり、第２のジヌクレオチドはＡＣのはずであり、第１のジヌクレオチドはＣＣのはずであることを意味する（ヌクレオチドは、５’→３の向きに記載されるが、その正体は、配列において３’→５’に移動させることによりもたらされる）。あるいはまた、仮定は、配列の中央の任意のヌクレオチドにおいて行なわれ得、ジヌクレオチドの正体は、５’→３’および３’→５の両方向に移動させることによりもたらされる。ヌクレオチドの１つに対する仮定の非存在下では、各位置がＡ、Ｇ、ＣまたはＴで占められ得るため、各ヌクレオチドの正体が完全に決定されない状態になることは認識されよう。

【0253】

好ましいプローブファミリーのコレクションを用いる場合、任意の単一のヌクレオチド（例えば、第１のヌクレオチド）の正体を仮定すると、唯一の候補配列が生成される。しかしながら、プローブファミリーのやや好ましいコレクションを用いる場合、１つより多くのヌクレオチドの正体を仮定することが必要であり得る。すなわち、第１のヌクレオチドの正体を仮定することでは、残りの配列は完全には特定されない。例えば、プローブファミリーのやや好ましいコレクションは、その規定配列がＡＡおよびＡＣであるメンバーファミリーを含むものであり得る。かかる場合では、第１のヌクレオチドがＡであると仮定することにより、第２のヌクレオチドに対して２つの可能性が残る。プローブファミリーのやや好ましいコレクションを用いる配列決定を以下にさらに論考する。拘束部分が非連続的なヌクレオチドからなる場合、上記のアプローチは、若干の修正を伴ってやはり使用され得ることは認識されよう。

【0254】

候補配列と既知配列との比較による配列同定

一般的に、上記のようにして、伸長された二本鎖の候補配列が決定された場合、鋳型の配列決定される領域に対応する候補配列は、その相補配列を得ることにより得られる。場合によっては、候補配列自体が、目的を達成するのに充分な情報を提供する。例えば、配列決定の目的が、単にある特定の配列可能性を排除することである場合、候補配列とその可能な配列とを比較することで充分であり得る。例えば、図３０に示す候補配列は、配列決定対象の領域はポリＡテイルの一部でないという決定を可能にし得るものである。より長い配列により、配列決定対象の領域がベクターの一部でないことが確認され得る。

【0255】

多くの場合、正確な配列を明確に決定することが望ましい。好ましい本発明の実施形態によれば、正確な配列は、鋳型の配列決定される領域の候補配列を１組の既知配列と比較することにより同定される。既知配列の組は、例えば、ある特定の目的の生物体の１組の配列であり得る。例えば、ヒトＤＮＡを配列決定する場合、候補配列を、Ｈｕｍａｎ　Ｄｒａｆｔ　Ｇｅｎｏｍｅ　Ｓｅｑｕｅｎｃｅと比較し得る。公的に入手可能なヒトゲノム

配列情報源の案内については、ＵＲＬ　ｗｗｗ．ｎｃｂｉ．ｎｉｈ．ｇｏｖ／ｇｅｎｏｍｅ／ｇｕｉｄｅ／ｈｕｍａｎ／のウェブサイトを参照のこと。別の例として、感染因子（例えば、被験体から単離された細菌またはウイルス）由来の核酸が配列決定される場合、該細菌またはウイルスのバリアント株の配列を含むデータベースが検索され得る。完全または部分配列のいずれかを含む多くのかかる生物体指定データベースが、当該技術分野で知られており、配列決定作業が加速されると、より多くが利用可能となる。一部の代表的な例としては、マウス（例えば、ウェブサイトＵＲＬ　ｗｗｗ．ｎｃｂｉ．ｎｌｍ．ｎｉｈ．ｇｏｖ／ｇｅｎｏｍｅ／ｓｅｑ／ＭｍＨｏｍｅ．ｈｔｍｌを参照）、ヒト免疫不全ウイルス（例えば、ウェブサイトＵＲＬ　ｈｉｖ－ｗｅｂ．ｌａｎｌ．ｇｏｖ／ｃｏｎｔｅｎｔ／ｈｉｖ－ｄｂ／ｍａｉｎｐａｇｅ．ｈｔｍｌを参照）、マラリア種Ｐｌａｓｍｏｄｉｕｍ　ｆａｌｃｉｐａｒｕｍ（例えば、ウェブサイトＵＲＬ　ｗｗｗ．ｔｉｇｒ．ｏｒｇ／ｔｄｂ／ｅｄｂ２／ｐｆａｌ／ｈｔｍｌｓ／ｉｎｄｅｘ．ｓｈｔｍｌを参照）などのデータベースが挙げられる。もちろん、生物体指定の組の配列を用いる必要はない。広範なさまざまな生物体およびウイルス由来の配列を含むＧｅｎＢａｎｋ（ウェブサイトＵＲＬ　ｗｗｗ．ｎｃｂｉ．ｎｌｍ．ｎｉｈ．ｇｏｖ／Ｇｅｎｂａｎｋ／）などのデータベースを検索してもよい。データベースは、鋳型を誘導した生物体またはウイルス由来の任意の配列を含むものである必要はない。一般に、配列はゲノム配列、ｃＤＮＡ配列、ＥＳＴｓなどであり得る。多数の配列が検索され得る。

【0256】

単純に検索を行なうことは、目的を達するのに充分であり得る。例えば、ウイルス系核酸が患者から単離された場合、候補配列を該ウイルスの１組の既知配列と比較することにより、該ウイルス系核酸が、該ウイルス由来の配列を含むか含まないかいずれであるかが、配列のマッチングを調べられない場合であっても、決定され得る。マッチングの存在により、患者が該ウイルスに感染していることが確認され得、一方、マッチングの欠如は、患者が該ウイルスに感染していないことを示し得る。

【0257】

ある特定の実施形態において、既知配列の組は、より狭い範囲の配列を含み、これは、配列決定が行なわれる目的に特別に調整されたものであり得る。したがって、配列決定される核酸に関する情報は、既知配列の組を選択するために使用され得る。例えば、鋳型がある特定の遺伝子の配列を示すものであることがわかっている場合、既知配列は、所与の目的の遺伝子座の遺伝子、変異型および野生型配列の異なる対立遺伝子を表すものなどであり得る。候補配列のどれが正確であるかを決定するためには、候補配列を単一の既知配列と比較することだけが必要であり得る。例えば、本発明のある特定の実施形態において、鋳型は、目的の領域を含むＤＮＡ増幅することにより得られる（例えば、目的の領域に隣接するプライマーを用いて）。目的の領域は、変異または多型（例えば、ある特定の疾患と関連する変異または多型）が存在し得る部位を包含する。鋳型が、ある特定の目的の領域由来の配列を示すものであることがわかっている場合、候補配列を該領域の単一の参照配列と、例えば、野生型または変異型形態の配列と比較するだけでよい。換言すると、鋳型の配列の一部または全部がわかっている場合は、複数の既知配列との比較を行なうことは必要とされ得ない。それどころか、既知配列の一部または全部を含む候補配列が適宜選択される。例えば、ＢＲＣＡ１およびＢＲＣＡ２遺伝子内の変異は、乳癌リスクの増大と関連していることがわかっており、被験体がかかる変異を保有している否かの決定において重要な関心となる。鋳型がＢＲＣＡ１遺伝子由来の配列を含むことがわかっている場合、例えば、該遺伝子の一部分を包含する目的の領域と隣接するプライマーが鋳型のクローン性の集団を作製するために使用された場合、正確な配列を決定するためには、候補配列を野生型または変異型ＢＲＣＡ１配列と比較するだけでよい。

【0258】

より一般的な場合では、候補配列を既知配列の組と比較することにより、任意の候補配列類似する任意の既知配列が同定される。ただし、候補配列は充分な長さのものであり、データベースが１つより多くの候補配列と同一のであるか非常に類似する配列を含む尤度

は非常に小さいものとする。換言すると、候補配列が充分に長い場合、その１つより多くが既知配列の組に提示されることは考えにくい。候補配列は、「マッチング」しているとみなされる任意の配列と比較される。典型的には、マッチングが存在することが確立される必要とされる同一性の程度の閾値を設定することが望ましい。例えば、既知配列は、候補配列と既知配列が少なくとも５０％、少なくとも６０％、少なくとも７０％、少なくとも８０％、少なくとも９０％、少なくとも９５％、少なくとも９９％またはまさに１００％同一である場合、マッチングしているとみなされ得る。典型的には、同一性割合は、少なくとも１０ヌクレオチド長、例えば１０～１５ヌクレオチド、１５～２０ヌクレオチド、２０～２５ヌクレオチド、２５～３０ヌクレオチドなどの領域で評価する。該領域の長さは、さまざまな異なる基準、例えば、限定されないが、複数の既知配列内の配列の数、複数の既知配列の正体または供給源などに応じて選択され得る。例えば、候補配列をＧｅｎＢａｎｋなどの大きなデータベースの配列と比較する場合、より少ない配列を含むデータベースが利用される場合は、長鎖の使用が望ましいことがあり得る。本発明のある特定の実施形態では、配列を、必ずしも互いに隣接していない複数の異なる領域において比較する。好ましくは、合わせた領域の長さは、少なくとも１０ヌクレオチド長、例えば１０～１５ヌクレオチド、１５～２０ヌクレオチド、２０～２５ヌクレオチド、２５～３０ヌクレオチドなどである。場合によっては、既知配列の組内の多数の配列がマッチングし得る。配列は、例えば、鋳型を誘導したものと同じ生物体に見られる相同遺伝子、異なる生物体由来の相同遺伝子、、偽遺伝子、ｃＤＮＡおよびゲノム配列などを示すものであり得る。

【0259】

一般に、既知配列の組内の配列にもっとも類似する候補配列が適宜選択される。あるいはまた、例えば、配列決定法が高エラー率に供されたかもしれないと思われる理由がある場合、適宜データベースから対応する配列を選択することが好ましいことがある。例えば、エラー率が所定の閾値より高いことがわかっている場合、正確な配列としてデータベースから配列を選択することが好ましいことがある。

【0260】

マッチングの尤度が多数の候補配列に見られることを確実にするために必要とされる長さは、さまざまな考慮事項、例えば、限定されないが、既知配列の具体的な組、許容されるマッチングの閾値などに依存する。一般に、典型的な生物体のゲノムにおいて、１回で提示される配列の長さは約２５～２６ヌクレオチドにすぎない。したがって、およそこの長さの候補配列の作製が、正確な配列を同定するのに充分である。一般に、候補配列は、少なくとも１０ヌクレオチド長、好ましくは少なくとも１５、少なくとも２０ヌクレオチド長、例えば、２０～２５、２５～３０、３０～３５、３５～４０、４５～５０またはさらに長鎖であるのがよい。

【0261】

第１の組の候補配列と第２の組の候補配列との比較による配列同定

本発明のある特定の実施形態において、デコード化は、第１のコード化に従ってコード化されたプローブファミリーの第１のコレクションを用いて第１のプローブファミリーの序列リスト作製し、これから第１の組の候補配列を作製し、次いで、第２のプローブファミリーの序列リストを同じ鋳型から、第２のコード化に従ってコード化されたプローブファミリーの第２のコレクションを用いて作製し、これから第２の組の候補配列を作製することにより行なわれる。新たに合成されたＤＮＡ鎖を２つの配列決定反応の間で鋳型から取り出すか、または同一の配列の鋳型を、プローブファミリーの第２のコレクションを用いて配列決定する。候補配列の組を比較する。どのプローブファミリーのコレクションが使用されるかとは無関係に、候補配列の１つが正確な配列であり、その他は正確でない（または、よくても部分的に正確である）ことは認識されよう。したがって、どの候補配列の組も正確な配列を含むが、ほとんどの場合、候補配列の任意の所与の組のその他の候補配列は、候補配列の別の組に見られるものと異なる。したがって、候補配列の２つの組を単純に比較することにより、正確な配列が決定され得る。２種類の異なるようにコード化

されたプローブファミリーのコレクションを用いて等しい長さの候補配列を作製する必要はない。本発明の好ましい実施形態において、プローブファミリーの第２のコレクションを用いて作製された候補配列は２ヌクレオチドという短いものであり得、または同等に、プローブファミリーの第２のコレクションを用いて作製されたプローブファミリーの序列リストは、１要素（すなわち、ライゲーションおよび検出の１サイクル）という短いものであり得る。

【0262】

図３１Ａ～３１Ｃは、２種類の識別可能に標識された好ましいプローブファミリーを用いた候補配列の作製およびデコード化の一例を示す。図３１　Ａは、第１のコード化に従ってコード化されたプローブファミリーの好ましいコレクションを示す。図３１Ｂは、プローブファミリー黄色、緑色、赤色、青色（これは、「２３１４」と表示され得、ここで、赤色＝１、黄色＝２、グリーン＝３および青色＝４である）の序列リストからの４種類の候補配列の作製を示し、その正確な配列をＣＡＧＧＣ（太字で示す）と仮定している。図３１Ｃは、第２のコード化に従ってコード化されたプローブファミリーの好ましいコレクションを示す。鋳型内の第１のジヌクレオチドはＣＡであるため、黄色プローブファミリーの最上のプローブが伸長の第１のサイクルにおいて伸長可能な末端にライゲートされる。これにより、第１のジヌクレオチドに対して下記の組の候補配列：ＣＡ、ＴＣ、ＧＧ、ＡＴがもたらされる。第１のプローブファミリーのコレクションを用いて作製された候補配列のうち、配列ＣＡＧＧＣだけが、任意のこれらのジヌクレオチドで始まる。したがって、これは正確な配列のはずである。一般に、第１および第２のプローブファミリーのコレクションは、下記の基準：第１と第２のプローブファミリーのコレクションを比較する場合、（ｉ）第１のコレクション内の各プローブファミリーの４種類のプローブのうち３種類が第２のコレクションの新たなプローブファミリーに割り当てられるべきである；および（ｉｉ）３種の再割り当されたプローブの各々が第２のコレクションの異なるプローブファミリーに割り当てられるべきであるを満たすべきであることが好ましい。

【0263】

既知ヌクレオチド正体を用いたプローブファミリーの序列リストのデコード化

上記のように、候補配列は、伸長された二本鎖または鋳型内の単一のヌクレオチドの正体を仮定することにより作製され得る。使用される具体的なプローブファミリーコレクションに応じて、一般的に、少なくとも４種類の候補配列を作製することが必要である。しかしながら、多数の候補配列の作製は、鋳型内の少なくとも１つのヌクレオチドの正体がわかっている場合は（したがって、伸長された二本鎖の場合も）回避され得る。その場合は、単一の候補配列を作製するだけでよい。候補配列を作製するための方法は、上記のものと同一である。鋳型内の少なくとも１つのヌクレオチドの正体は、任意の配列決定法、例えば、限定されないが、配列決定法Ａ、１組の識別可能に標識されたヌクレオチドおよびポリメラーゼを用いる初期オリゴヌクレオチドからのプライマー伸長などを用いて決定され得る。鋳型内の１つ以上のヌクレオチドが、まず、配列決定法ＡＢ以外の配列決定法を用いて配列決定され得、次いで、初期オリゴヌクレオチドおよび任意の伸長産物が除去され得、同じ鋳型が配列決定法ＡＢを用いる配列決定に供され得ることは認識されよう（または逆も同様）。

【0264】

別のアプローチは、その配列が決定される部分に加えて既知の正体の１つ以上の既知ヌクレオチドを含む鋳型を単純に配列決定することである。例えば、鋳型の初期オリゴヌクレオチドが結合する領域と未知配列が始まる領域との間の部分に、１つ以上の既知の正体のヌクレオチドが含まれ得る。鋳型のこの部分を配列決定法ＡＢに供することにより、配列内の１つ以上のヌクレオチドの正体が予め決定され、したがって、単一の候補配列を作製するために使用され得、これは正確な配列である。

【0265】

したがって、上記の方法は、（ｉ）どの正体が既知ヌクレオチドの正体と一致するか、およびその近位ヌクレオチドが、既知の正体のヌクレオチドに隣接するヌクレオチドに対

向してライゲートされたプローブの拘束部分の可能な配列を調べることにより、正体を、既知の正体のヌクレオチドに隣接する鋳型内のヌクレオチドに割り当てる工程；（ｉｉ）どの正体が、その近位ヌクレオチドが後続のヌクレオチドに対向してライゲートされたプローブの拘束部分の可能な配列と一致するかを調べることにより、正体を後続のヌクレオチドに割り当てる工程；ならびに（ｉｉｉ）　配列が決定されるまで工程（ｉｉ）を反復する工程を含む。伸長された二本鎖と鋳型の配列決定される領域との間に正確な対応があるため、これらの工程は、同じ工程を伸長された二本鎖において行なうことと等価であることを理解されたい。

【0266】

やや好ましいプローブファミリーを用いる配列決定

やや好ましいプローブファミリーのコレクションは、配列決定法ＡＢを、好ましいプローブファミリーのコレクションが使用される様式と同様にして行なうために使用され得る。しかしながら、結果はいくつかの点で異なり得る。例えば、配列のある特定部分は候補配列から、さらなる情報を必要せずに完全に同定され得る。図３２は、図２８に示すようにしてコード化されたプローブファミリーのやや好ましいコレクションを用いる配列決定の一例を示す。配列の決定は、一般的に、好ましいプローブファミリーのコレクションで記載したようにして行なう。目的の鋳型は配列「ＧＣＡＴＧＡ」を有し、これは、プローブファミリーの序列リストでは「１２３４１」となる。１位のヌクレオチドをＡと仮定すると、「ＡＣＡＴＧＡ」が候補配列として得られる。しかしながら、好ましいプローブファミリーのコレクションの場合とは異なり、標識「１」は、第１のヌクレオチドとしてＡを有する２つの異なるジヌクレオチド、すなわち「ＡＡ」および「ＡＧ」と関連するため、第２のヌクレオチドに対して２つの可能性がある。したがって、１位のヌクレオチドがＡであると仮定すること、「ＡＣＡＴＧＣ」が第２の候補配列として得られる。１位のヌクレオチドがＧであると仮定すると、「ＧＣＡＴＧＡ」が候補配列として得られ、また、「ＧＣＡＴＧＣ」が候補配列として得られる。標識「１」は、ＣまたはＴを１位に有するジヌクレオチドとはなんら関連しないため、「Ｃ」または「Ｔ」で始まる候補配列は得られない。図３２は、互いにアライメントさせた４つの候補配列を示す。すべての候補配列の中央の４つのヌクレオチドがＣＡＴＧであることが観察される。したがって、正確な配列は、ＣＡＴＧを２～５位に含むものであるはずである。もし、これらのヌクレオチドが目的のものでない場合は、さらなるデコード工程を行なう必要はない。

【0267】

上記のように、プローブファミリーのコレクションは、４種類の異なるプローブファミリーからなるものである必要はく、２つよりより多く4^N（ここで、Ｎは拘束部分の長さである）までの任意の数からなるものであり得る。しかしながら、４つより少ないファミリーを用いる場合、４つより多くの候補配列作製することが必要であり得、一方、４つより多くのプローブファミリーを用いる場合は、さらなる標識が必要とされる。これらおよび他の理由により、４つのプローブファミリーからなるコレクションが好ましい。

【0268】

候補配列を互いに比較することによる配列同定

本発明のある特定の実施形態において、目的の配列の一部または全部は、候補配列を互いに比較することにより決定され得る。一般に、かかる比較は、その全長において候補配列のどれが正確であるかを決定するのに充分でないことがあり得る。しかしながら、候補配列の２つ以上が該配列の一部分において同一であるか充分類似している場合、この情報は、鋳型の上記の部分内のヌクレオチドの配列を明確に同定するのに充分であり得る。

【0269】

所望の場合、鋳型は、同定された配列を有するさらなる部分を得るため、さらに１回以上、別のコード化されたプローブファミリーを用いて配列決定され得る。これらの部分を組み合わせ、所望の長さの配列が合成され得る。

【0270】

プローブファミリーを用いたエラー補正。多くの場合、同じＤＮＡ　配列の一部または

全部を提示する多数の鋳型を配列決定し、該配列をアライメントすることが望ましい。鋳型が目的の領域の一部しか含まない場合、重複断片を合成することによって、より長い配列を得る。例えば、生物体のゲノムを配列決定する場合、典型的には、ＤＮＡを断片化し、充分な断片を、各ＤＮＡ鎖がいくつかの（例えば、４～１２個の）異なる断片内に提示されるように配列決定する。重複配列を合成してより長い配列にするためのコンピュータソフトウエアが当業者に知られている。

【0271】

慣用的な配列決定法が用いられる場合、ほとんどの場合、ある領域において、断片の１つ（異常断片という）がその他のものと該領域内の単一の位置で異なる場合以外は、多数の断片が完璧にアライメントされる。孤立性の差が配列決定エラーを表すか否か、または真性の差（例えば、一塩基多型）が該位置に存在するか否かを調べることは、問題となり得る。

【0272】

本発明は、配列決定法ＡＢを用いてエラーチェックを行なうこと新規な方法を提供する。該方法によれば、同じＤＮＡ鎖を示す断片を含む鋳型が、上記のような識別可能に標識されたプローブファミリーのコレクションを用いて配列決定され、各鋳型のプローブファミリーの序列リストがもたらされる。プローブファミリーの序列リストをアライメントする。いくつかのリストが、単一の位置でその他の断片異なる１つのリスト以外、リスト内の所定の長さ、例えば、１０、１５、２０または２５それ以上の要素において完璧にアライメントされたら、その差は、配列決定エラーに起因する。実際に多型が存在する場合、異常断片から得られた序列プローブリストは、２つ以上の隣接する位置で、その他の断片から得られた序列プローブリストと異なる。

【0273】

例えば、表１のコード化４を用いたプローブファミリーの好ましいコレクションを使用する配列決定法ＡＢを、配列５’－ＣＡＧＡＣＧＡＣＡＡＧＴＡＴＡＡＴＧ－３’を含む鋳型に適用すると、下記のプローブファミリーの序列リスト：「２３３２４３２２１３２４４４１４２」が得られ、これを以下に示す。

【0274】

【化1】

```
2332432213244414 2
CAGACGACAAGTATAATG
```

実際にＳＮＰが存在する場合（例えば、ＣＡＧＡＣＧＡＧＡＡＧＴＡＴＡＡＴＧ、式中、下線のヌクレオチが多型性部位を表す）、リスト内の２つの連続する要素に変化がもたらされる：２３３２４３３３１３２４４４１４２式中、下線は、ＳＮＰの結果として生じた変化を示す。プローブファミリーの序列リストとＳＮＰを含む配列間の対応を以下に示す。

【0275】

【化2】

```
2332433313244414 2

CAGACGAGAAGTATAATG
```

しかしながら、ライゲートされた伸長プローブと会合した標識の同定におけるエラーは、プローブファミリーの序列リストにおける単一のエラーおよびその点の前方（ｆｏｒｗａｒｄ）から得られる候補配列における変化をもたらす。例えば、７回目にライゲートされ伸長プローブ２３３２４３３２１３２４４４１４２（式中、下線の数字は誤同定された標識を表す）と会合した標識の決定におけるエラーによって、得られる候補配列はＣＡＧＡＣＧＡＧＴＴＣＡＴＡＴＴＡＣに変化する（式中、下線部分は、配列決定エラーの結果として生じた変化を示す）。プローブファミリーの序列リストと配列間の対応を以下に示す。

【0276】
【化3】

```
 2332432132444142
CAGACGAGTTCATATTAC
```

３塩基、４標識スキームを用いる場合、ＳＮＰを含む断片により、異常断片のプローブファミリーの序列リストにおいて３連続の差がもたらされるが、配列決定エラーでは、１つの差しかもたらされない。例えば、図２９に示すようにしてコード化されたプローブファミリーのコレクションを用いる場合、配列ＣＡＧＡＣＧＡＣＡＡＧＴＡＴＡＡＴＧのプローブファミリー正体の序列リストを以下に示す。

【0277】
【化4】

```
 2322224132412244
CAGACGACAAGTATAATG
```

ＳＮＰを含む異常断片、例えば、ＣＡＧＡＣＧＡＧＡＡＧＴＡＴＡＡＴＧは、ＳＮＰを含まない断片から得られる序列リストと比べて３つの連続する位置が異なるプローブファミリーの序列リストをもたらし得、これを以下に示す。

【0278】
【化5】

```
 2322213332412244
CAGACGAGAAGTATAATG
```

配列決定エラーは、たった１つの差をプローブファミリーの序列リスト内にもたらし得、エラー点の前方と完全に異なる候補配列が作製され得る。

【0279】
したがって、断片（異常断片）から作製されたプローブファミリーの序列リストを、同じＤＮＡ鎖を示すが、他方の序列リストと単一の孤立性の位置で異なる他の断片から作製されたプローブファミリーの序列リストとアライメントさせる場合、差を含む序列リストが配列決定エラーを表すことがあり得る（プローブファミリーの誤同定）。断片（異常断片）から作製されたプローブファミリーの序列リストを、同じＤＮＡ鎖を示すが、他方の序列リストと２つ以上の連続する位置で異なる他の断片から作製されたプローブファミリーの序列リストとアライメントさせる場合、異常断片がＳＮＰを含むものであることがあり得る。好ましくは、プローブファミリーの序列リストのアライメントされる部分は、少なくとも３または４要素の長さ、好ましくは少なくとも６、８またはそれ以上の要素の長さである。好ましくは、アライメントされる部分は、少なくとも６６％同一、少なくとも７０％同一、少なくとも８０％同一、少なくとも９０％同一、またはそれ以上、例えば１００％同一である。

【0280】
同様に、断片の候補配列を、配列の第１の部分において同じＤＮＡ鎖を示すが、他の断片の候補配列と配列の第２の部分において実質的に異なる他の断片の候補配列とアライメントさせる場合、配列決定エラーが起こることがあり得る。ある断片の候補配列を、配列の２つの部分において同じＤＮＡ鎖を示すが単一の位置で異なる他の断片の候補配列とアライメントさせる場合、異常断片がＳＮＰを含むものであることがあり得る。好ましくは、候補配列のアライメントされる部分は、少なくとも４ヌクレオチド長である。好ましくは、アライメントされる部分は、少なくとも６６％同一、少なくとも７０％同一、少なくとも８０％同一、少なくとも９０％同一、またはそれ以上、例えば１００％同一である。

【0281】
したがって、本発明は、（ａ）配列決定法ＡＢを用いて複数の鋳型を配列決定する工程、ここで、鋳型は、単一の核酸配列の重複断片を表す；（ｂ）工程（ａ）で得られた配列

アライメントする工程；および（ｃ）配列が第１の部分と全体的に実質的に同一であり、第２の部分と実質的に異なる場合（各部分は少なくとも３ヌクレオチド長を有する）、配列間の差が配列決定エラーを表すと決定する工程を含む、一塩基多型を配列決定エラーと識別する方法を提供する。本発明は、さらに、（ａ）単一の核酸配列の重複断片を表す複数の鋳型を用いて配列決定法ＡＢを行なうことにより、プローブファミリーの複数の序列リスト得る工程；（ｂ）工程（ａ）で得られたプローブファミリーの序列リストをアライメントし、該リストが少なくとも９０％同一であるアライメント領域を得る工程；および（ｃ）該リストがアライメント領域内のたった１つの位置で異なる場合、プローブファミリーの序列リスト間の差は、配列決定エラーを表すと決定する工程；または（ｄ）該リストがアライメント領域内の２つ以上の隣接する位置で異なる場合、プローブファミリーの序列リスト間の差は一塩基多型を表すと決定する工程を含む、一塩基多型を配列決定エラーと識別する方法を提供する。

【0282】

非局在化情報収集

当該技術分野でよく知られているように、「ビット」（２進数）は、基数を２とする、換言すると、１または０のいずれかである１桁をいい、デジタルデータの最小単位を表す。ヌクレオチドは任意の４種類の異なる正体を有し得るため、ヌクレオチドの正体の指定には２ビットが必要であることは認識されよう。例えば、Ａ、Ｇ、ＣおよびＴは、それぞれ００、０１、１０および１１で表され得る。識別可能に標識されたプローブファミリーの好ましいコレクションにおけるプローブファミリー名の指定では、４つの識別可能に標識されたプローブファミリーがあるため、２ビットが必要である。

【0283】

最も慣用的な配列決定の形態および配列決定法Ａでは、各ヌクレオチドは、別個の単位として同定され、１つのヌクレオチドに対応する情報が一度に収集される。各検出工程では、２ビットの情報が単一のヌクレオチドから取得される。対照的に、配列決定法ＡＢでは、各検出工程において、２ビット未満の情報が複数のヌクレオチドの各々から取得されるが、プローブファミリーの好ましいコレクションが使用される場合は、やはり１回の検出工程で２ビットの情報が取得される。プローブファミリーの序列リスト内の各プローブファミリー名は、鋳型内の少なくとも２つのヌクレオチドの正体を表し、その正確な数は、プローブの配列決定部分の長さによって決定される。例えば、配列５’－ＣＡＧＡＣＧＡＣＡＡＧＴＡＴＡＡＴＧ－Ｓ’から、表１のコード化４に従ってコード化されたプローブファミリーのコレクションを用いて得られるプローブファミリーの序列リストを考察されたい。

【0284】

【化6】

23324322132444142
CAGACGACAAGTATAATG

プローブファミリー２は、ジヌクレオチドＣＡが、プローブファミリー２のプローブ内に存在する指定される部分の１つであるため、リスト内の第１のプローブファミリーである。プローブファミリー３は、ジヌクレオチドＡＧが、プローブファミリー３のプローブ内に存在する指定される部分の１つであるため、リスト内の第２のプローブファミリーである。上記のように、４つのプローブファミリーがあるため、各プローブファミリー正体は、２ビットの情報で表される。したがって、各検出工程では、２つのヌクレオチドに関する２ビットの情報が収集され、平均１ビットの情報が各ヌクレオチドからもたらされる。

【0285】

したがって、本発明は、多数の伸長、ライゲーションおよび検出のサイクルを含む、配列決定方法であって、検出工程が、平均２ビットの情報を同時に、任意の個々のヌクレオチドの２ビットの情報を取得することなく、鋳型内の少なくとも２つのヌクレオチドの各

々から取得することを含む配列決定方法を提供する。本発明は、さらに、（ａ）伸長、ライゲーション、検出および切断の連続的サイクルを行なう工程、ここで、検出工程が、平均２ビットの情報を同時に、任意の個々のヌクレオチドの２ビットの情報を取得することなく、鋳型内の少なくとも２つのヌクレオチドの各々から取得することを含む；ならびに（ｂ）工程（ａ）で得られた情報を少なくとも１ビットのさらなる情報と組み合わせて配列を決定する工程を含む、オリゴヌクレオチドプローブファミリーの第１のコレクションを用いて、鋳型ポリヌクレオチド内のヌクレオチドの配列を決定する方法を提供する。種々の本発明の実施形態において、少なくとも１ビットのさらなる情報は、鋳型内のヌクレオチドの正体、候補配列を少なくとも１つの既知配列と比較することにより得られる情報；およびオリゴヌクレオチドプローブファミリーの第２のコレクションを用いて該方法を反復することにより得られる情報からなる群より選択される項目を含む。

【0286】

したがって、該方法では、個々のヌクレオチドから２ビットの情報は取得されないが、平均２ビットの情報が鋳型から各サイクルで収集され、プローブファミリーの好ましいコレクションが使用される場合は、非局在化様式で収集される。２または３つのプローブファミリーのコレクションが使用される場合は、２ビット未満の情報が各サイクル中で収集される。

【0287】

非局在化情報の収集はいくつかの利点を有し、例えば、上記のものなどのエラーチェック法の適用が可能になる。また、各鋳型内のヌクレオチドは、好ましい実施形態では、非局在化情報の収集によって、１回より多くのインテロゲーションが行なわれる（ｉｎｔｅｒｒｏｇａｔｅ）ため、特定のヌクレオチドと会合したフルオロフォアの検出における系統的偏重（ｓｙｓｔｅｍａｔｉｃ　ｂｉａｓ）の回避が補助され得る。

【0288】

本明細書に記載のプローブファミリーおよびプローブファミリーのコレクションは、プローブの伸長、ライゲーションおよび切断の連続的サイクルを伴う方法に加え、さまざまな配列決定法に使用され得る。本発明はまた、上記のような配列および構造を有し、プローブが任意選択で切れやすい連結を含まないプローブファミリーおよびプローブファミリーのコレクションを提供する。例えば、プローブは、ホスホジエステル主鎖連結のみを含むものであり得、および／またはトリガー残基含まないものであり得る。本発明のある一部の実施形態において、プローブファミリーは、伸長およびライゲーションの連続的サイクルを用いるが、各サイクル中で切断を伴わない配列決定を行うために使用される。例えば、プローブファミリーは、ＷＯ２００５０２１７８６および当該技術分野において他に記載されたものなどのライゲーション系の方法に使用され得る。プローブファミリーをかかる方法で使用するためには、プローブ上の標識は、例えば、ＷＯ２００５０２１７８６に開示のように、核酸の切れやすい連結を切断することなく除去され得るように、切断可能なリンカーによって結合されているのがよい。かかる方法は、例えば、ＷＯ２００５０２１７８６に記載されたライゲーションカセットではなくプローブファミリーを用い、多数の反応を並行して、または連続的に行ない、次いで、プローブファミリーのリストを合成することにより、プローブファミリーの序列リスト作製するために使用され得る。リストは、上記のようにしてデコード化される。

【0289】

Ｉ．キット

本発明の種々の実施形態を行なためのさまざまなキットが提供され得る。キットのある特定のものは、ホスホロチオレート結合を含む伸長オリゴヌクレオチドプローブを含む。キットは、１種類以上の初期オリゴヌクレオチドをさらに含むものであり得る。キットは、ホスホロチオレート連結の切断に適した切断試薬（例えば、$AgNO_3$）および該切断を行なうための適切なバッファーを含むものであり得る。キットのある特定のものは、トリガー残基、例えば、損傷塩基または無塩基残基を含有するヌクレオシドを含む伸長オリゴヌクレオチドプローブを含む。キットは、１種類以上の初期オリゴヌクレオチドをさら

に含むものであり得る。キットは、ヌクレオシドと隣接する無塩基残基間の連結の切断に適した切断試薬および／または損傷塩基をポリヌクレオチドから除去するのに適した試薬（例えば、ＤＮＡグリコシラーゼ）を含むものであり得る。ある特定のキットは、二糖ヌクレオチドを含むオリゴヌクレオチドプローブを含み、切断試薬として過ヨウ素酸塩を含む。ある特定の実施形態において、キットは、識別可能に標識されたオリゴヌクレオチドプローブファミリーのコレクションを含む。

【0290】

キットは、ライゲーション試薬（例えば、リガーゼ、バッファーなど）および具体的な本発明の実施形態を行なうための取扱い説明書を含むものであり得る。使用され得るその他の酵素、例えば、ホスファターゼ、ポリメラーゼに適切なバッファーを含めてもよい。場合によっては、これらのバッファーは同一であり得る。キットはまた、鋳型を固定するための支持体、例えば、磁気ビーズを含むものであり得る。ビーズは、ＰＣＲ増幅を行なうためにプライマーにより官能性付与されていてもよい。他の任意選択的な成分としては、洗浄溶液；ＰＣＲ増幅のための鋳型の挿入用ベクター；ＰＣＲ試薬、例えば、増幅プライマー、熱安定性ポリメラーゼ、ヌクレオチドなど；エマルジョン調製のための試薬；ゲル調製のための試薬などが挙げられる。

【0291】

ある特定の好ましいキットには、ホスホロチオレート結合を含む蛍光標識されたオリゴヌクレオチドプローブが、該プローブの異なる末端ヌクレオチドに対応するプローブが異なるスペクトルにより分離可能な蛍光染料を担持するように提供される。より好ましくは、４種類のかかるプローブが提供され、これは、４種類のスペクトルにより分離可能な蛍光染料の各々と、プローブの４種類の可能な末端ヌクレオチドとの間の１対１対応を可能にする。

【0292】

キットは、対合末端または断片ライブラリーを生成するのに好適なオリゴヌクレオチドおよび／またはベクターを含有する場合がある。キットは、ライブラリーのメンバーである鋳型分子の相補的な共通部分である１つ以上のブロッキングオリゴヌクレオチドを含有する場合がある。

【0293】

識別物、例えば、バーコード、高周波ＩＤタグなどをキット内またはキット上に存在させてもよい。識別物は、例えば、品質管理、在庫管理、追跡、ワークステーション間の移動などの目的でキットを固有に識別するために使用され得る。

【0294】

キットは、一般的に、ある種の個々の試薬が個別に収容され得るように、１つ以上の容器（ｖｅｓｓｅｌ）または収容器（ｃｏｎｔａｉｎｅｒ）を含む。キットはまた、個々の収容器を、市販用に比較的コンパクトに（ｃｌｏｓｅ　ｃｏｎｆｉｎｅｍｅｎｔ）封入するための手段、例えば、取扱い説明書、発泡スチレンなどのパッケージング材料などが封入され得るプラスチックボックスを含むものであり得る。

【0295】

Ｊ．Ｐａｒａｌｌｅｌ配列決定および自動配列決定システム

Ｍａｃｅｖｉｃｚは、特定の配列を有する単一鋳型種の配列決定を開示しているが、種々の配列を有する複数の鋳型の同時配列決定と並行してこの方法を実施する可能性については述べていない。本発明者等は、高いスループットで配列決定を効率的に実施するためには、上述のように、複数の担体（例えば、ビーズ）を、それぞれの担体に特定の配列の鋳型が付着するように調製し、それぞれの担体に付着した鋳型に対して、本明細書に記載の方法を同時に実施することが望ましいことを認識した。本手法のある実施形態において、複数のこのような担体は、スライドなどの平面の基材の中または上に配列される。ある実施形態において、担体は、ゲルなどの半固形培地の中または上に配列される。担体は、無作為に配列される場合があり、すなわち、基材上の各担体の位置は既定されていない。担体は、規則的な間隔を置いて位置する必要もなければ、列や行などの規則正しい配置に

配置する必要もない。好ましくは、担体の多くまたはほとんどから個々のシグナルを検出することが可能な密度で、担体が配列される。ある好ましい実施形態において、担体は、主として単一の焦点面に分布する。同じ配列の鋳型が付着した複数の担体が、例えば品質管理の目的で含まれる場合もある。配列決定反応は、担体のそれぞれに付着した鋳型において、並行して実施される。

【0296】

シグナルは、種々のイメージング様式を含めた種々の手段のいずれかを使用して収集される場合がある。好ましくは、検出前に基材（例えば、基材上に配置された半固形担体に埋め込まれたビーズ）上に配列される微粒子に対して配列決定が実施される実施形態の場合、画像化装置の分解能は１μｍ以下である。例えば、ＣＣＤカメラを取り付けた走査型顕微鏡、または十分な分解能を有するマイクロアレイスキャナーを使用できると考えられる。あるいは、蛍光検出のために装備される顕微鏡に取り付けたフローセルまたは流体工学ワークステーションにビーズを通過させることもできる。その他のシグナル収集方法には光ファイバー束が含まれる。適切な画像収集処理ソフトウェアが使用される場合もある。

【0297】

本発明のある実施形態において、配列決定はマイクロ流体デバイスで行われる。例えば、鋳型が付着したビーズをデバイスに充填し、その中に試薬を流す場合がある。例えばＰＣＲを使用した鋳型合成もデバイスで行うことができる。米国特許第６，６３２，６５５号では、好適なマイクロ流体デバイスの例が記載されている。

【0298】

本発明は、配列情報を複数の鋳型から並行して、すなわち、実質的に同時に収集するために使用され得るさまざまな自動配列決定システムを提供する。好ましくは、鋳型は、実質的に平面状の基材上でアレイ状である。図２１は、本発明のシステムの一例の写真を示す。上側の写真に示すように、本発明のシステムは、ＣＣＤ　カメラ、蛍光顕微鏡、可動ステージ、Ｐｅｌｔｉｅｒフローセル、温度制御装置、液体操作装置および専用のコンピュータを備える。これらの構成要素の種々の置き換えがなされ得ることは認識されよう。例えば、代替的に画像捕捉装置が使用され得る。このシステムのさらなる詳細は実施例９に示す。

【0299】

本発明の自動配列決定システムならびに関連する画像処理方法およびソフトウエアは、さまざまな配列決定法、例えば、本明細書に記載のライゲーション系の方法ならびに他の方法、例えば、限定されないが、合成方法による配列決定、例えば、合成による蛍光インサイチュで配列決定（ＦＩＳＳＥＱ）など（例えば、を参照　Ｍｉｔｒａ　ＲＤら．，Ａｎａｌ　Ｂｉｏｃｈｅｍ．，３２０（１）：５５－６５，２００３）の両方を行うために使用され得ることは認識されよう。本明細書に記載のライゲーション系の配列決定法の場合のように、ＦＩＳＳＥＱは、半固相支持体内または該支持体上に直接固定化された鋳型上、半固相支持体内または該支持体上の微粒子上に固定化された鋳型上、基材に直接結合された鋳型上などで行なわれ得る。

【0300】

本発明のシステムの重要な態様の１つは、フローセルである。一般に、フローセルは、内部を流体が流動し得る流入ポートおよび流出ポートを有するチャンバを含む。種々のフローセルならびにその製造のための材料および方法の論考については、例えば、米国特許第６，４０６，８４８号および同第６，６５４，５０５号ならびにＰＣＴ公開公報ＷＯ９８０５３３００を参照のこと。流体の流動により、種々の試薬をフローセル内に位置する実体（例えば、鋳型、微粒子、解析物など）に添加し、これらから除去することが可能になる。

【0301】

好ましくは、本発明の配列決定システムにおける使用に適したフローセルは、流体がその表面を流動するような基材、例えば、スライドなどの実質的に平面状の基材が取り付け

られる位置、および照光、励起、シグナル取得などを可能にする窓を備える。本発明の方法によれば、微粒子などの実体は、典型的にはフローセル内に配置する前に、基材上にアレイ状にする。

【0302】

本発明のある特定の実施形態において、フローセルは垂直に配向させ、これにより、気泡をフローセルの上面から散逸させることが可能になる。フローセルは、流体経路がフローセルの下部から上に流れるように、例えば、流入ポートがセルの下部に存在し、流出ポートがセルの上に存在するように配設する。導入され得る気泡は浮揚性であるため、これは、照光窓を障害することなく速やかに流出ポートに浮揚する。気泡を液体の表面まで、その密度が該液体のものより低いことによって上昇させるこのアプローチを、本明細書では「重量測定気泡移動」という。したがって、本発明は、重量測定気泡移動が可能になるように配向させたフローセルを含む配列決定システムを含むを提供する。好ましくは、自身に直接もしくは間接的に結合された微粒子（例えば、共有結合もしくは非共有結合により基材に結合）、または基材に接着もしくは固定された半固相支持体内または該支持体上に固定化された微粒子を有する基材をフローセル内に垂直になるように、すなわち、基材の最大の平面状の表面が、設置面に対して垂直になるように取り付ける。好ましい実施形態では、微粒子は、支持体または基材内またはその上面に固定化されているため、互いに対して実質的に固定された位置に維持され、これにより逐次的な画像取得と画像レジストレーションが容易になる。

【0303】

図２４Ａ～Ｊは、種々の向きの本発明のフローセルまたはその一部分の概略図を示す。本発明のフローセルは、任意のさまざまな目的、例えば、限定されないが、解析方法（例えば、配列決定、ハイブリダイゼーションアッセイなどの核酸解析法；タンパク質解析法、結合アッセイ、スクリーニングアッセイなどに使用され得る。フローセルはまた、合成を行うため、例えば、コンビナトリアルライブラリーを作製するためなどに使用され得る。

【0304】

図２２は、別の本発明の自動配列決定システムの概略図を示す。フローセルは、自動温度制御ステージ（実施例９に記載のものと類似）上に取り付けられ、液体操作システム（例えば、マルチポートバルブを有するシリンジポンプなど）に接続されている。該ステージは、他の工程、例えば、伸長、ライゲーションおよび切断などが別のフローセルで行なわれている間に、１つのフローセルが画像化されるのを可能にするために、多数のフローセルを収容する。このアプローチは、高価な光学システム　の利用を最大限にするとともに、処理量を増大させる。

【0305】

流体ラインには、気泡を検出ため、および試薬の使用をモニターするための光学および／または伝導性センサーが設置されている。流体工学システム内の温度制御およびセンサーにより、試薬が、長時間の安定に適切な温度に維持されるが、フローセル内に侵入するとき作業温度またはで上昇せず、アニーリング、ライゲーションおよび切断工程中での温度変動が回避されることが確保される。試薬は、好ましくは、誤負荷を回避するためキット内に予め装填しておく。

【0306】

光学装置としては、４つのカメラが含まれ、各々が、４つのフィルターセットの１つから１つの画像を撮影する。光退色性効果を低減させるため、照光光学装置は、画像化される領域だけに照光されるように、視野周辺の多重照光が回避されるように設計されたものであり得る。画像化光学装置は、標準的な無限遠補正顕微鏡対物レンズおよび標準的なビームスプリッタおよびフィルターから構成されたものであり得る。標準的な２，０００×２，０００ピクセルＣＣＤカメラは、画像を取得するために使用され得る。該システムには、光学装置のための適切な機械的支持体が組み込まれれている。照光強度は、好ましくは、後の解析ソフトウエアによる使用のためにモニターされ、記録される。

【0307】
複数の画像（例えば、代表的な実施形態において、およそ１８００以上の非重複画像フィールド）を速やかに取得するため、該システムには、好ましくは、高速オートフォーカスシステムを用いる。画像自体の解析に基づくオートフォーカスシステムは、当該技術分野でよく知られている。これは、一般的に、１回のフォーカシング事象に少なくとも５フレームを必要とする。これは、フォーカシング画像を取得するために余分な照光が必要とされるという点で、低速および高価の両方である（光退色性が増大する）。本発明のある特定の実施形態において、択一的なオートフォーカスシステム、例えば、該機械的システムが応答し得るのと同等に高速にフォーカシングし得る独立した光学装置に基づくシステムが使用される。かかるシステムは当該技術分野で知られており、例えば、コンシューマＣＤプレイヤーに用いられるフォーカシングシステム（これは、ＣＤ回転時にリアルタイムでサブミクロンフォーカシングを維持する）が挙げられる。
【0308】
本発明のある特定の実施形態において、該システムは、遠隔操作される。特定のプロトコルを実行するためのスクリプトは、セントラルデータベース内に保存し、各配列決定実行のためにダウンロードされ得る。試料は、バーコードを付けて、試料追跡の完全性および試料と最終データとの関連付けが維持され得る。リアルタイムのセントラルモニタリングにより、プロセスエラーの迅速な解決が可能になる。ある特定の実施形態において、機器によって収集された画像は、直ちにセントラルマルチテラバイト保存システムおよび１つ以上のプロセッサバンクにアップロードされる。セントラルデータベースからの追跡データを用い、プロセッサ（１つまたは複数）により画像が解析され、配列データが作製され、任意選択で、例えば、機器の性能を調節するために、バックグラウンド蛍光レベルおよびビーズ密度などのメトリクスが処理される。
【0309】
制御ソフトウエアは、ポンプ、ステージ、カメラ、フィルター、温度制御を適正にシーケンス処理するため、ならびに画像データの注記付けおよび保存のために使用される。ユーザーインターフェースは、例えば、作業者が機器をセットアップし、維持するのを補助するために設けられ、好ましくは、スライドの負荷／取り出しおよび流体ラインの準備（ｐｒｉｍｉｎｇ）のためのステージを位置合わせする機能を含むものである。例えば、作業者に、種々の実行パラメータ、例えば、温度、ステージ位置、光学フィルター構成の現状、実行プロトコルの状態などを示すためのディスプレイ機能を含め得る。好ましくは、試薬ロットおよび試料ＩＤなどの追跡データを記録するデータベースへのインターフェースを含める。
【0310】
Ｋ．画像およびデータ処理方法
本発明は、少なくとも一部は、コンピュータ可読媒体上に保存されたコンピュータコード（すなわち、ソフトウエア）として行なわれ得るさまざまな画像およびデータ処理方法を提供する。さらなる詳細は、実施例９および１０に示す。また、一般に、配列決定法ＡおよびＢでは、ともに、一般的に、例えば、多重配列決定反応で収集されたデータの追跡管理、かかるデータの合成、候補配列の作製、配列比較の実施などを伴う該処理工程を行なうための適切なコンピュータソフトウエアが使用される。
【0311】
Ｌ．配列情報を保存するコンピュータ可読媒体
また、本発明は、本発明の配列決定法を適用することにより得られた情報を保存するコンピュータ可読媒体を提供する。情報としては、生データ（すなわち、さらなる処理または解析がされていないデータ）、処理または解析されデータなどが挙げられる。データには、画像、数値も含まれる。情報は、典型的には、検索が容易なように配設された、例えば、コンピュータメモリ内に保存されたデータベース、すなわち、情報（例えば、データ）のコレクションに保存され得る。情報としては、例えば、配列および配列に関する任意の情報（例えば、部分配列）、配列と参照配列との比較、配列解析の結果、ゲノム情報、

例えば、多型情報など（例えば、ある特定の鋳型が多型を含むか否か）または変異情報など、連鎖情報（すなわち、例えば、染色体内での核酸配列の別の核酸配列に対する物理的な位置に関する情報）、疾患関連情報（すなわち、疾患の存在または疾患に対する感受性を、被験体の身体的形質、例えば、被験体の対立遺伝子と相関させる情報）などが挙げられる。情報は、試料ＩＤ、被験体ＩＤなどと関連したものであり得る。試料、被験体などに関するさらなる情報は、例えば、限定されないが、試料の供給源、試料において行なわれた処理工程、情報の解釈、試料または被験体の供給源などに含まれ得る。本発明はまた、前述の任意の情報をコンピュータ可読形式に受信する、例えば、コンピュータ可読媒体に保存することを含む方法を含む。該方法は、該情報にに基づく診断的、予後的もしくは予測的情報を提供する工程、または第３者に、好ましくはコンピュータ可読媒体に保存された情報を単に提供する工程をさらに含み得る。

【0312】

以下の実施例は例示の目的で提供され、本発明を限定することは意図されない。

【0313】

（実施例１：ホスホロチオレート含有オリゴヌクレオチドの効率的な切断およびライゲーション）

この実施例では、３’－Ｓホスホロチオレート結合を含有する伸長オリゴヌクレオチドの効率的なライゲーションおよび切断を示す実験を記載する。

【0314】

材料および方法

ライゲーション配列決定プロトコル

鋳型調製：サイクル的オリゴヌクレオチドライゲーションおよび切断による配列決定の可能性の評価を示すため、および該方法のある特定の態様の変形の効果を検討するため、２組のモデルビーズ系鋳型集団を調製した。実施例に記載したような好ましい実施では、サイクル的オリゴヌクレオチドライゲーションおよび切断により、鎖は３’→５’方向に伸長される。したがって、ライゲーション効率を評価するため、モデル鋳型を、ビーズに５’末端において結合させ、同じ結合領域を３’末端に有するように設計した。１組は、二重ビオチン部分によってストレプトアビジンコート磁気ビーズ（１ミクロン）に結合させた短鎖（７０ｂｐ）オリゴヌクレオチドで構成されたものであった。これらの短鎖鋳型集団の各々は、同一のプライマー結合領域（４０ｂｐ）および特有の配列領域（３０ｂｐ）を３’末端に有するように設計した。この短鎖オリゴヌクレオチド鋳型集団をライゲーション配列決定鋳型１～７（ＬＳＴ１～７）と命名した。

【0315】

第２の組のビーズ系鋳型集団は、１８３ｂｐのスペーサー配列（ヒトｐ５３エキソン由来）を各鋳型集団内に挿入することにより誘導した長鎖のＰＣＲ生成ＤＮＡ断片（２３２ｂｐ）から設計した。鋳型は、二重ビオチン含有フォワードプライマー、および短鎖鋳型集団と同じ３０塩基の特有３’末端配列を含有するリバースプライマーを用いて増幅させた。鋳型は、水酸化ナトリウム含有バッファーを用いて一方の鎖を融解して離すことにより単鎖に作製した。これらの長鎖鋳型集団は、係属中の特許出願に記載された短鎖断片対末端ライブラリーから生成される種を模倣するように設計し、長鎖ＬＳＴ１～７と命名した。

【0316】

プライマーハイブリダイゼーション：２．５μＬの１００μＭ　ＦＡＭ標識プライマーを、１００μＬの１×クレノウバッファーと予備混合した。この溶液を、バッファーの除去後、鋳型が結合された磁気ビーズ（10^6／μＬ）のアリコート３０μＬに添加し、得られた溶液を充分混合した。鋳型／プライマーハイブリダイゼーションを行なわせた後（ハイブリダイゼーション反応は６５℃で２分間、４０℃で２分間および氷上で２分間行なった）、プライマー／バッファーを除去し、ビーズを、３×洗浄ＩＥバッファーを用いて洗浄し、次いで、３００μＬ（10^6／ｍＬ）をＴＥＮＴバッファー（１０ｍＭ　Ｔｒｉｓ，２ｍＭ　ＥＤＴＡ，３０ｍＭ　ＮａＯＡｃおよび０．０１％Ｔｒｉｔｏｎ　Ｘ－１０

０含有）に再懸濁した。
【0317】
ライゲーション１：次いで、ＬｉｇＳｅｑ－ＦＡＭがハイブリダイズした２．５×１０^{6}のＬＳＴ７ビーズを、３０分間３７℃で、１μＬの１００μＭ　ＬＳＴ７－１九量体，４μＬの５×Ｔ４リガーゼバッファー（Ｉｎｖｉｔｒｏｇｅｎ），１４μＬのH_2Oおよび１μＬのＴ４リガーゼ（１ｕ／μＬ，Ｉｎｖｉｔｒｏｇｅｎ）を含有する混合物中でインキュベートした。
【0318】
切断１：次いで、ビーズを１００μＬのＬＳＷａｓｈ１（１×ＴＥ，３０ｍＭ酢酸ナトリウム，０．０１％Ｔｒｉｔｏｎ　Ｘ１００含有）で３回洗浄し；この溶液の１０μＬアリコートを取り出し、解析のために保存した。次いで、ビーズ（ＩＸ）を１００μＬの３０ｍＭ酢酸ナトリウム中で洗浄した。５０μＬの５０ｍＭ　$AgNO_3$をこの溶液に添加し、得られた混合物を３７℃で２０分間インキュベートした。$AgNO_3$を除去し、ビーズを１回、１００μＬの３０ｍＭ酢酸ナトリウム中で洗浄した。次いで、ビーズを１００μＬのＬＳＷａｓｈ１で３回洗浄し、９０μＬの洗浄。（ＴＥＮＴバッファー）に再懸濁し、この溶液の１０μＬアリコートを取り出し、解析のために保存した。
【0319】
ライゲーション２：ＴＥＮＴバッファーの除去後、ビーズを１４μＬのH_2Oに再懸濁し、３７℃で３０分間、１μＬの１００μＭ　ＬＳＴ７－５九量体，４μＬの５×Ｔ４リガーゼバッファー（Ｉｎｖｉｔｒｏｇｅｎ）および１μＬのＴ４リガーゼ（１ｕ／μＬ，Ｉｎｖｉｔｒｏｇｅｎ）を含有する混合物とともにインキュベートした。
【0320】
切断２：ビーズを、１００μＬのＬＳＷａｓｈ１（１×ＴＥ，３０ｍＭ酢酸ナトリウム，０．０１％Ｔｒｉｔｏｎ　Ｘ１００）中で３回洗浄し、４５μＬのＷａｓｈ１Ｅに再懸濁した。この混合物の１５μＬアリコートを取り出して解析のために保存した。次いで、ビーズを１００μＬの３０ｍＭ酢酸ナトリウムで１回洗浄し、５μＬの２０ｍＭ酢酸ナトリウムに再懸濁した。５０μＬの５０ｍＭ　$AgNO_3$をビーズに添加し、混合物を３７℃で２０分間インキュベートした。$AgNO_3$の除去後、ビーズを、１００μＬの３０ｍＭ酢酸ナトリウムで１回洗浄した。次いで、ビーズを、１００μＬのＬＳＷａｓｈ１中で３回洗浄し、３０μＬのＷａｓｈ１Ｅに再懸濁した。この混合物　２０μＬアリコートを取り出して解析のために保存した。
【0321】
結果
この実験は、図８を参照すると、より良好に理解されよう。図８の上側のセクションは、実験手順の全体的な概要を示す。初期オリゴヌクレオチド（プライマー）を、ビオチン結合によってビーズ結合された鋳型（ＬＳＴ７で示す）にハイブリダイズさせた。初期オリゴヌクレオチドには５’リン酸基を含め、その３’末端をＦＡＭで蛍光標識した。２種類の９量体（九量体）オリゴヌクレオチドプローブ（第１切断可能なオリゴおよび第２切断可能なオリゴ）を、内部にホスホロチオレート含有チミジン塩基（ｓＴ）（下線）を含むように合成した。第１の切断可能なプローブをプライマーの伸長可能な末端に、Ｔ４ＤＮＡリガーゼを用いてライゲートし、次いで、硝酸銀を用いて切断した。切断により、伸長プローブの末端の５ヌクレオチドが除去され、伸長可能な末端が、プローブのプライマーにライゲートされたままの部分上に生成した。次いで、第２の切断可能なプローブを伸長可能な末端にライゲートさせ、次いで、同様に切断させた。
【0322】
蛍光キャピラリー電気泳動ゲルシフトアッセイを、ライゲーションおよび切断の工程をモニタリングするために使用した。このアッセイでは、プライマーを鋳型鎖に、５’リン酸基が、接近する（ｉｎｃｏｍｉｎｇ）オリゴヌクレオチドプローブのライゲーション基質としての機能を果たし得る（フルオロフォアは、移動度型キャピラリーゲル電気泳動のレポーターとしての機能を果たす）ようにハイブリダイズさせる。各工程後、ビ

ーズのアリコートを解析のために取り出した。オリゴヌクレオチドプローブのライゲーション後、磁気ビーズを磁石を用いて回収し、ライゲートされたプライマーおよびプローブ（１つまたは複数）からなるライゲート種を、鋳型ビーズから熱変性によって放出させ、自動ＤＮＡ配列決定機器（ＡＢＩ　３７３０）を用いる蛍光キャピラリー電気泳動に供した。標識されたサイズ標準（リサミンラダー；　１５～１２０ヌクレオチドのサイズ範囲；クロマトグラムにおいて１組の橙色ピークとして出現、図８参照）。典型的なゲルシフトにおいて、潜在ピークとしては、ｉ）プライマーピーク（無伸長またはプライマー伸長の不足による）、ｉｉ）アデニル化ピーク（ＤＮＡリガーゼの作用による非生産的ライゲーション接合部の５’末端アデノシン残基の結合による－図８Ｆの機構を参照、また、Ｌｅｈｍａｎ，Ｉ．Ｒ．，Ｓｃｉｅｎｃｅ，１８６：７９０－７９７，１９７４も参照）、およびｉｉｉ）終了ピーク（オリゴプローブの結合による）が挙げられる。ライゲーション効率を評価するためのゲルシフトアッセイの使用の利点の１つは、ピーク下面積が、各種の濃度と直接相関することである。

【0323】

図８Ａは、Ｔ４　ＤＮＡリガーゼおよびホスホジエステル結合のみを含有する正確にマッチングしたプローブ（図８Ａの左に示す）を用いて行なわれる対照ライゲーションを示す。橙色ピークはサイズマーカーを表す。左の青色ピークは、ライゲーションの非存在下のプライマー位置を示す。正確にマッチングしたプローブライゲーションは、左へのシフトをもたらす（矢印）。図８Ｂは、同じ条件下で、内部に　チオレート含有Ｔ塩基を含有するプローブ（図８Ｂの左に示す）を用いて行なわれるライゲーションを示す。対照プローブで観察されるものと同一のシフトが見られた（矢印）。ライゲートされたホスホロチオレート含有プローブを含有するビーズ連結鋳型集団を、次いで、硝酸銀とともにインキュベートし、プローブ切断を誘導した。ゲルシフト解析により、左にシフトされた４ｂｐ切断産物（図８Ｃ）の標示によって効率的な切断を確認した。予測される切断産物を図８Ｃの左に示す。切断されたビーズ系鋳型集団を、次いで、第２の回目のライゲーションに曝露し、右にシフトされた１３ｂｐ伸長産物（図８Ｄ）の出現によって生産的ライゲーションが示された。予測される切断産物を図８Ｄの左に示す。２回目の切断により、予測された左にシフトされた８ｂｐ切断産物（図８Ｅ）によって示されるように、効率的な多数の切断工程がなされ得ることが確認された。これらの結果は、成功裏のライゲーションおよびホスホロチオレート結合を含有するプローブの切断を示す。

【0324】

これらの実験で、ライゲーションが１００％完了まで進行しなかったことは明白であるが、より高い完了度が、Ｔ４　ＤＮＡリガーゼを用いた他の実験において観察された（下記参照）。確かに、ライゲーションが完了まで進行することは望ましいが、必須要件ではない。例えば、上記のようなライゲーション工程後、任意の非ライゲート５’末端を５’－ホスファターゼで処理することにより有効に「キャッピングする」ことが可能である。しかしながら、その場合、ライゲーション可能な分子の自然減により、行なわれ得る連続ライゲーションの回数が制限され得る。所与の回数の連続ライゲーションについて、読出し長さは、各ライゲーション／切断サイクル後に残ったプローブの長さ、および
配列決定反応の回数（各々の後には、プライマーの除去およびプライマー結合部位の異なる部分に結合するプライマーのハイブリダイゼーションが起こり、これらは、所与の鋳型において行なわれ得、「リセット」回数ともよばれる）に依存する。これは、プローブの５’末端側に位置する切断可能な連結を有するより長鎖のプローブの使用の場合に論じられる。本発明者らの実験では、六量体プローブにより、八量体およびより長鎖のプローブよりも多量の非ライゲーション可能アデニル化産物をもたらす。したがって、八量体およびより長鎖プローブは、実質的に完了までライゲートされる（下記参照）。また、六量体プローブの５’末端への蛍光部分の付加により、ライゲーション効率が低下するようであるが、八量体プローブへの蛍光部分の付加は、影響はほとんどまたは全くない。これらの理由のため、八量体またはより長鎖のプローブは好ましいとみなされる。

【0325】

さらなる実験（後述）により、ホスホロチオレート結合および縮重低減性ヌクレオチドを含有するプローブのライゲーションおよび切断；ライゲートされた伸長プローブの３’末端特異性および選択性；ゲル内ライゲーションおよび切断；最小シグナル損失でのプライマーハイブリダイゼーションおよび除去の連続的サイクル；３’→５’伸長でのＴ４またはＴａｑリガーゼの１００％忠実度；ならびにライゲートされた伸長プローブの４色スペクトル分離可能性を示した。該方法を行なうための自動システムを構築した。

【0326】

（実施例２：縮重低減性ヌクレオチドを含有するホスホロチオレート含有オリゴヌクレオチドの効率的な切断およびライゲーション）

しかしながら、プローブ長さと競合する考慮事項は、伸長オリゴヌクレオチドの忠実度およびその後続のライゲーション効率に対する影響である。Ｔ４　ＤＮＡリガーゼの忠実度は、接合後、第５番目の塩基以降で急速に低下することが示されている（Ｌｕｏら．，Ｎｕｃｌｅｉｃ　Ａｃｉｄ　Ｒｅｓ．，２４：３０７１－３０７８および３０７９－３０８５，１９９６）。ミスマッチを新たなライゲーション接合部の５’側に導入した場合、ライゲーション効率は自然減により低下し得るが、バックグラウンドシグナルにおける位相の散逸または増大は生じない（合成方法によるポリメラーゼ系配列決定に見られる大きな障害）。

【0327】

プローブの組は、キャラクタライズされていないＤＮＡのデノボ（ｄｅ　ｎｏｖｏ）配列決定を可能にするため、好ましくは、任意のＤＮＡ配列にハイブリダイズできるものであるのがよい。しかしながら、標識されたプローブの組の複雑度は、４重縮重塩基の長さおよび数にともなって指数関数的に増大する。また、複合プローブの組は、あらゆるプローブ種のほぼ等しい提示を維持したまま、合成するのはより困難であり、生成することよりも困難である。また、これは、各種を一定濃度に維持するのに、より高濃度のプローブ混合物を必要とする。この複雑度に対処するための方法の一例は、４重縮重塩基の代わりに普遍塩基、例えば、デオキシイノシンなどがある特定の位置に組み込まれたヌクレオチドの使用である。

【0328】

４重縮重塩基（Ｎ；等モル量のＡ、Ｃ、Ｇ、Ｔ）および普遍塩基イノシン（Ｉ）を、八量体内の種々の位置に有する１２種類のオクタヌクレオチドプローブを設計した（イノシンは、Ｂ－ＤＮＡ内の任意の４つの標準塩基と二座に配位した水素結合をすることができる；イノシン塩基対の安定性の順は、Ｉ：Ｃ＞Ｉ：Ａ＞Ｉ：Ｔ≒Ｉ：Ｇである）。これらのプローブ設計の評価の目的の１つは、依然としてイノシン塩基の存在下での効率的なライゲーションを支持したまま、どれだけ低い八量体複雑度が達成され得るかを調べることであった。

【0329】

最初の研究では、いくつかのオリゴヌクレオチドプローブを、ビーズ系鋳型（長鎖－ＬＳＴ１）にＴ４　ＤＮＡリガーゼを用いてライゲートさせた。ライゲーションされると、フルオロフォア標識プライマー（３’ＦＡＭプライマー）は、ライゲートされたオリゴヌクレオチドプローブの量に比例して右にシフトする。設計プローブＮＩ８－９は最高レベル完了を示し、プローブの効率的なライゲーションにより、＞９９％のプライマー集団が右にシフトした（図９参照）。これらの反応は２５℃で行なった；反応温度を３７℃に上げると、ライゲーションは、いくぶん効率が下がり、完了率はより変動的であった。

【0330】

データをより詳細に調べると、接合部の３’側の最初の５つのヌクレオチド内により少数のイノシン塩基を有するプローブ（下線）は、より高いライゲーション効率を示すことが示された。ライゲーション効率に対する配列状況の潜在的効果をさらに検討し、評価するため、ライゲーション接合部の３’側の最初の５つの塩基内にたった１つのイノシン残基を有する４種類の設計オリゴヌクレオチドプローブを、すべての鋳型においてスクリーニングした。図１０は、Ｔ４　ＤＮＡリガーゼを使用し、多数の鋳型上の選択したプロー

ブ組成物でのゲルシフトアッセイを用いて評価したときのライゲーション完了を示す。これらの最初の実験のデータにより、ライゲーション効率、したがって完了は、イノシン残基がライゲーション接合部３’側の最初の５つの位置（下線）に配置された場合、変動的であり配列依存性であることが示された。八量体の効率的なライゲーションは、一貫して観察されたが、設計オリゴヌクレオチドプローブＮＩ８－９では、本明細書に示すように、試験したすべての鋳型において＞９９％完了であった。

【0331】

なんら理論に拘束されることを望まないが、このデータ（アデニル化中間体の存在を含む）は、Ｔ４　ＤＮＡリガーゼのためのコアＤＮＡ結合部位内の好ましくないイノシン塩基対により、ＤＮＡタンパク質複合体が不安定化され、酵素結合および後続のライゲーションが充分に低減されるという結論を裏付ける。しかしながら、興味深い疑問は、かかるイノシン塩基対の不安定化が、ライゲートされるオリゴヌクレオチドプローブの忠実度に影響し得るのかどうかということであった。

【0332】

（実施例３：プローブライゲーションの忠実度）

Ｔａｑ　ＤＮＡリガーゼなどの細菌ＮＡＤ依存性リガーゼは、ライゲーション接合部において高い配列忠実度を有し、３’側のミスマッチは本質的にニック閉鎖（ｎｉｃｋ－ｃｌｏｓｕｒｅ）活性を有しないが、５’側のミスマッチはある程度許容性（ｔｏｌｅｒａｔｅｄ）であることが報告されている（Ｌｕｏら．，Ｎｕｃｌｅｉｃ　Ａｃｉｄ　Ｒｅｓ．，２４：３０７１－３０７８および３０７９－３０８５，１９９６）。他方、Ｔ４　ＤＮＡリガーゼは、いくぶんストリンジェンシーが低く、ミスマッチが接合部の３’および５’の両側で許容されることが報告されている。したがって、Ｔ４　ＤＮＡリガーゼを用いてプローブライゲーションの忠実度を、本発明者らのシステムの状況におけるＴａｑ　ＤＮＡリガーゼとの比較において評価することは、興味深かった。

【0333】

本発明者らは、標準的なＡＢＩ配列決定手法を用いてライゲートされるオリゴヌクレオチドの配列忠実度を評価する２つの方法を開発した。第１の方法は、ライゲーション産物をクローニングし、配列決定するように設計した。この方法では、ライゲーション伸長産物をアダプター配列に結合し、クローニングし、細菌内で形質転換した。個々のクローンを採取し、配列決定して、ライゲーション接合部における各位置でのミスマッチ頻度の定量的評価を得た。第２の方法は、ライゲーション産物を直接配列決定するように設計した。該アプローチでは、単鎖ライゲーション産物をビーズ系鋳型から変性させ、相補プライマーを用いて直接配列決定した。精度の低い位置は配列図（ｔｒａｃｅ）において多数の重複ピークを示し、該位置における配列忠実度を示す定性的評価が得られる。

【0334】

第１の方法を用いて、Ｔ４およびＴａｑ　ＤＮＡリガーゼによるプローブライゲーションの相対忠実度を評価した。単一のビーズ系鋳型集団（ＬＳＴ１）を普遍配列決定プライマーにハイブリダイズさせ、これを初期オリゴヌクレオチドとして用いた。次いで、溶液系ライゲーション反応を、縮重オリゴヌクレオチドプローブ（Ｎ７Ａ、３ＡＮＮＮＮＮＮＮ５’、２０００ｐｍｏｌ）の存在下で３７℃、３０分間、Ｔ４　ＤＮＡリガーゼ（１５Ｕ／１×１０^{6}　ビーズ）またはＴａｑ　ＤＮＡリガーゼ（６０Ｕ／１×１０^{6}　ビーズ）のいずれかを用いて行なった（図１１、パネルＡ）。ライゲーション産物をクローニングし、配列決定して、そのライゲーション接合部の３’側（１～８位）の各ＤＮＡリガーゼの位置ごとの忠実度を評価した（図１１、パネルＢおよびＣ）。その結果、Ｔ４　ＤＮＡリガーゼは、最初の５つ位置ではＴａｑ　ＤＮＡリガーゼと本質的に同じレベルの忠実度を有するが、６～８位では忠実度が低いことが示された。これらの結果は、３種類の縮重イノシン含有設計プローブ（３’－ＮＮＮＮＮＩＩＩ－５’、３’－ＮＮＮＮＮＩＮＩ－５’および３’－ＮＮＮＩＮＮＮＩ－５’）での、７種類すべての鋳型（ＬＳＴ１～７）のライゲーション接合部におけるＤＮＡ配列を評価した後続のクローニング実験によってさらに支持された。これらの研究により、Ｔ４　ＤＮＡリガーゼはライゲーション接合

部において６～８位では低い配列忠実度を有することが確認されたが、最初の５つ位置では試験したすべての鋳型において高い忠実度が示される（データ示さず）。

【0335】

直接配列決定法を使用し、縮重イノシン含有プローブを用いてＴ４　ＤＮＡリガーゼの忠実度を評価した。オリゴヌクレオチドプローブは、Ｔ４　ＤＮＡリガーゼおよびビーズ系鋳型を含むライゲーション反応物において、２５℃および３７℃で評価した。オリゴヌクレオチドプローブライゲーション効率は、ゲルシフトアッセイを用いて評価した（図１２、パネルＡ）。オリゴヌクレオチドプローブライゲーションにおけるＴ４　ＤＮＡリガーゼの忠実度を評価するため、ＡＢＩ３７３０ｘｌ　ＤＮＡ解析装置を用いたライゲーション反応の直接配列決定を行なった（図１２、パネルＢ）。正確にマッチングしたオリゴプローブと２種類の代表的な縮重イノシン含有オリゴプローブ（ＮＩ８－９およびＮＩ８－１１）のライゲーションにより、＞９９％完了および非常に低頻度のミスマッチ（配列図における多重ピークの非存在）が得られた。このデータにより、プローブが効率的にライゲートされることによっても高い配列忠実度がもたらされることが示される。

【0336】

さらなる実験において、単一のビーズ系鋳型集団（ＬＳＴ１）を、５’リン酸基を含む普遍配列決定プライマーにハイブリダイズさせ、これを初期オリゴヌクレオチドとして用いた。溶液系ライゲーション反応を３７℃で３０分間、Ｔ４　ＤＮＡリガーゼ（１Ｕ／２５０，０００ビーズ）を用い、縮重イノシン含有オリゴヌクレオチドプローブ（３’ＮＮＮＮＮｉｉｉ５’、３’ＮＮＮＮＮＩＮｉ５’または３’ＮＮＮｉＮＮＮｉ５’、６００ｐｍｏｌ）の存在下で行なった。ライゲーション産物をクローニングし、コロニーを採取し、配列決定した。配列忠実度は、ライゲーション接合部における各位置に提示されたクローンの数を計算することにより決定した。結果を図１２のパネルＣ～Ｆに表で示す。これらの研究は、Ｔ４　ＤＮＡリガーゼを用いた縮重イノシン含有プローブの３’→５’ライゲーションが、最初の１～５位で高レベルの忠実度を有することを示す。

【0337】

（実施例４：ゲル内ライゲーションおよび切断）

サイクル的オリゴヌクレオチドライゲーション方法を検討、開発および最適化するための最初の実験は、上記のように、溶液中でビーズ系鋳型を用いて行なった。第２の組の実験では、ライゲーションおよび切断を、スライド上のポリアクリルアミドゲル内に包埋したビーズ系鋳型において行なった。

【0338】

スライドは、各々が自身に結合された単鎖ＤＮＡ鋳型のクローン性の集団を有する数百万個のビーズを５％ポリアクリルアミドと混合し、ガラススライド上で重合を行なわせることにより調製した。テフロン（登録商標）マスクを用いてビーズ含有ポリアクリルアミド溶液を封入した。図１４（上）は、Ｃｙ３標識プライマーがハイブリダイズされた鋳型が結合されたビーズがポリアクリルアミドゲル内に固定化されたスライドの一部分の蛍光画像を示す（このスライドは、異なる実験に使用したが、ここで使用したスライドの典型である）。図１４（下）は、ポリアクリルアミド溶液を封入するためのテフロン（登録商標）マスクを設けたスライドの概略図を示す。

【0339】

反応体をスライド内に、適切な溶液中への手作業によるスライドの浸漬、または自動層流型フローセル内へのスライドの配置のいずれかによって導入した。最初の研究により、効率的なゲル内ライゲーションが実際に、かかるスライド上のポリアクルルアミドマトリックス内に固定化されたビーズに結合された鋳型において行なわれ得ることが確認された。図１５に示す実験では、単鎖ＤＮＡ鋳型ビーズをアクリルアミドおよびＤＡＴＤを含むスライド上に固定化した。重合後、３’フルオロフォア標識された普遍５’リン酸化プライマー（Ｓｅｑプライマー）をゲル内に拡散させ、ハイブリダイズさせた（パネルＡ）。スライドを洗浄して非結合ｓｅｑプライマーを除去し、Ｔ４　ＤＮＡリガーゼ（１０Ｕ）およびオリゴヌクレオチドプローブを含むライゲーションカクテルを重層し、３７℃で３

０分間インキュベートした。次いで、スライドを、過ヨウ素酸ナトリウム（０．１Ｍ）を含有するバッファー中でインキュベートし、アクリルアミドポリマーを消化させ、ビーズ系鋳型集団を放出させた。ライゲーション産物を熱により鋳型鎖から変性させ、回収し、上記のゲルシフトアッセイを用いて解析した。ゲル内ライゲーション反応をＴ４　ＤＮＡリガーゼの非存在下で行ない、非ライゲート配列決定プライマーに代表的な単一のピークが示された（パネルＢ）。ライゲーション反応は、八量体プローブを用い、Ｔ４　ＤＮＡリガーゼの存在下で行ない、効率的なゲル内オリゴヌクレオチドライゲーションが示され、＞９９％のビーズ系鋳型集団が効率的にライゲートされた（パネルＣ）。

【0340】

（実施例５：４色検出）

検出効率を最大にするには、各可能な塩基付加産物に対応する異なる標識を有する１組のオリゴヌクレオチドプローブを用いることが望ましい。これを、図１５に概略を示し、適切な励起および発光フィルターを備えた本発明者らの自動配列決定機器においてモデル化した。プローブの特異性および選択性の課題に取り組むため、３組の八量体プローブを設計した。第１の組は、４種類の特有の鋳型集団に相補的で、異なる３’塩基および５’色素標識を有する４種類の八量体を含むものとした。第２の組は、特有の３’塩基および５’色素を有する７種類の特有の八量体を含むものとした。第３の組は、各々が異なる５’色素標識で同定される特有の３’末端塩基を有する４種類の縮重イノシン含有八量体設計プローブに対応させた。

【0341】

４色スペクトル正体を確認するため、プローブ組番号１を用いて、４種類の特有の鋳型集団（図１６参照）を検出した。ポリアクリルアミド内に包埋されたビーズに結合された４種類の特有の単鎖鋳型集団を含むスライドを調製した（パネルＡ）。各ビーズは、自身に結合された鋳型のクローン性の集団を有した。５’リン酸基を含有する普遍配列決定プライマーをインサイチュでハイブリダイズさせ、ライゲーション反応を、４種類の特有のフルオロフォアプローブ（Ｃｙ５、ＣＡＬ　６１０、ＣＡＬ　５６０、ＦＡＭ；各１００ｐｍｏｌ）およびＴ４　ＤＮＡリガーゼ（１０Ｕ／スライド）を含むオリゴヌクレオチドプローブ混合物を用いて行なった。スライドを３７℃で３０分間インキュベートし、洗浄して非結合プローブを除去した。スライドを明るい光で画像化し、白色光塩基画像（パネルＢ）を得た。蛍光励起には、４種類の帯域通過フィルター（ＦＩＴＣ、Ｃｙ３、ＴｘＲｅｄおよびＣｙ５）を用いた。蛍光画像捕捉をライゲーションの前後で行なった。個々の集団を擬似カラー表示し（パネルＣ）、画像値のスペクトル正体をプロットし、最小シグナル重複を確認した（パネルＤ）。

【0342】

（実施例６：ゲル内でのライゲーション特異性および選択性の実証）

３’末端特異性を確認するため、プローブ組番号２を用いて、単一の鋳型集団のインテロゲーションを行なった（図１７参照）。ポリアクリルアミドゲル内に包埋された自身に結合され単一の鋳型集団（ＬＳＴ１．Ｔ）を有するビーズを有するスライドを調製し、普遍配列決定プライマーとインサイチュでハイブリダイズさせた（パネルＡ）。ゲル内ライゲーション反応を、Ｔ４　ＤＮＡリガーゼ（１０Ｕ／スライド）を使用し、単一の３’塩基だけが異なる４種類の５’末端一標識されたプローブで構成されたオリゴヌクレオチドプローブ混合物を用いて行なった。スライドを３７℃で３０分間インキュベートし、洗浄して非結合プローブ集団を除去した。スライドを白色光で画像化し、塩基画像を得（パネルＢ）、蛍光励起には、４種類の帯域通過フィルター（ＦＩＴＣ、Ｃｙ３、ＴｘＲｅｄおよびＣｙ５）を用いた。蛍光画像捕捉をライゲーションの前後で行ない、単一のＦＡＭ系プローブ集団（青色スポット）がＴ４　ＤＮＡリガーゼを用いたゲル内ライゲーション後に存在し、スペクトル重複がないことを確認した（パネルＣ、Ｄ）。このデータは、Ｔ４　ＤＮＡリガーゼを用いたプローブ特異性がストリンジェントであり、ライゲーション接合部の最初の３’末端塩基によって決定されることを示す。

【0343】

３’末端特異性および選択性をさらに実証するため、プローブ組番号２を用いて、単一の塩基差を含み異なる量で存在するビーズ系鋳型集団の混合物を同定した。図１８のパネルＡに示すような、各々が自身に結合された４つの鋳型集団（各々は一塩基多型を有する（ＬＳＴ１；Ａ、Ｇ、ＣまたはＴ））の１つを有するビーズの混合物を有するスライドを調製した。ビーズを、スライド上のポリアクリルアミドゲル内に包埋した。ビーズ系鋳型集団は、パネルＤに概略を示すように、種々の異なる頻度で用いた。スライドをインサイチュで普遍配列決定プライマーとハイブリダイズさせた。ゲル内ライゲーション反応を、Ｔ４　ＤＮＡリガーゼ（１０Ｕ／スライド）および等モル量（各１００ｐｍｏｌ）の４種類の５’末端標識プローブ（単一の３’塩基だけ異なる）を含有するオリゴヌクレオチドプローブ混合物を用いて行なった。スライドを３７℃で３０分間インキュベートし、洗浄して非結合プローブ集団を除去した。スライドを白色光で画像化し、塩基画像を得（パネルＢ）、蛍光励起には、４種類の帯域通過フィルター（ＦＩＴＣ、Ｃｙ３、ＴｘＲｅｄおよびＣｙ５）を用いた。個々のプローブ画像を重ね撮りし、擬似カラー表示した（パネルＣ）。蛍光画像を、ビーズ呼び出し（ｃａｌｌｉｎｇ）ソフトウエアを用いて一覧にした。結果をパネルＤに示す。観察されたライゲーション頻度（Ｏｂｓ）は、予測した頻度（Ｅｘｐ）と相関したことが確認される。このデータは、ライゲーション後、多数の鋳型の存在下での高いプローブ特異性およびプローブ選択性を示し、ライゲーションによって、一塩基多型（ＳＮＰ）、すなわち、集団の異なる個体のゲノムＤＮＡ鎖内の単一のヌクレオチド塩基内に生じた改変を検出できる能力を示す。

【0344】

（実施例７：４色縮重イノシン含有伸長プローブを用いたゲル内でのライゲーション特異性および選択性の実証）

別の組の実験をプローブ組番号３を用いて行ない、プローブライゲーションの特異性および選択性を、４色縮重イノシン含有オリゴヌクレオチドプローブプールを用いてを評価した。結果を図１９に示す。ビーズ系スライドを上記のようにして調製したが、４種類の特有の単鎖鋳型集団をビーズ上に異なる量で存在せ、これを、次いで、普遍配列決定プライマーとインサイチュでハイブリダイズさせた（パネルＡ）。ゲル内ライゲーション反応を、Ｔ４　ＤＮＡリガーゼ（１０Ｕ／スライド）の存在下、５種類の縮重塩基（Ｎ；複雑度4^5＝１０２４）、２種類の普遍塩基（Ｉ、イノシン）および単一の既知ヌクレオチドを、特異的５’フルオロフォア（Ｇ－Ｃｙ５、Ａ－ＣＡＬ　６１０、Ｔ－ＣＡＬ５６０、Ａ－ＦＡＭ；各６００ｐｍｏｌ）に対応する３’末端に有するように設計された八量体からなるプローブプールを用いて行なった。スライドを３７℃で３０分間インキュベートし、洗浄して非結合プローブ集団を除去した。スライドを白色光で画像化し、塩基画像を得（パネルＢ）、蛍光励起には、４種類の帯域通過フィルター（ＦＩＴＣ、Ｃｙ３、ＴｘＲｅｄおよびＣｙ５）を用いた。個々のプローブ画像を重ね撮りし、擬似カラー表示した（パネルＣ）。蛍光画像を一覧にし、各ライゲーション産物の頻度を、ビーズ呼び出しソフトウエアを用いて表で示した（パネルＤ）；ビーズシグナル値の上部９０％表す未処理生データおよびフィルター処理データのスペクトル散布図をパネルＥに示す。このデータは、観察されたライゲーション頻度（Ｏｂｓ）が、各鋳型の既知濃度に基づいて予測した頻度（Ｅｘｐ）と相関したことを示す。これにより、縮重した普遍塩基含有プローブプールをＴ４　ＤＮＡリガーゼとともに使用すると、特異的および選択的なゲル内ライゲーションがもたらされ得ることが確認される。

【0345】

（実施例８：　ゲル内での初期オリゴヌクレオチドのハイブリダイゼーションおよび除去の反復サイクルの実証）

自動フローセル内に取り付けた顕微鏡スライド上のゲル内に固定化された鋳型において実験を行ない（下記参照）、初期オリゴヌクレオチドのアニーリングおよびストリッピングの多数のサイクルが、スライド上のゲル内に包埋されたビーズに結合された鋳型に適用され得、シグナル損失は最少であることを確認した。４４塩基蛍光標識初期オリゴヌクレオチドを使用した。図２０に示すように、１０サイクルで生じたシグナル損失は最少であ

った。初期オリゴヌクレオチドを、図２０ではプライマーという。上記のように、合成手順によるポリメラーゼ系配列決定の大きな欠点の１つは正（ｐｏｓｉｔｉｖｅ）および負（ｎｅｇａｔｉｖｅ）の両方の位相の散逸が個々の鋳型鎖において起こる傾向である。正の位相の散逸は、ヌクレオチドが成長鎖内に誤って組み込まれ、したがって、該特定の鎖の塩基配列が残りの鋳型から得られる配列より前で実行（ｒｕｎ　ａｈｅａｄ）され、ｎ＋１塩基呼び出し分位相ずれが引き起こされた場合に起こる。負の位相の散逸は、より一般的であり、鎖が充分に伸長されず、バックグラウンド塩基呼び出しがもたらされ、成長鎖より後ろ（ｎ－１）で実行された場合に起こる。伸長産物効率的にストリッピングできること、および差次的に位置する初期オリゴヌクレオチドをハイブリダイズすることにより鋳型を「リセット」できることで、シグナル自然減がほとんどまたは全くなしで、非常に長い読出し長さが可能になる。

【0346】

（実施例９：自動配列決定システム）

この実施例では、１つ以上の鋳型から配列情報を収集するために使用され得る代表的な本発明の自動配列決定システムを記載する。好ましくは、鋳型を、実質的に平面状の基材、例えば、ガラス顕微鏡スライド上に配置させる。例えば、鋳型は、基材上でアレイ状であるビーズに結合されたものであり得る。該システムの写真を図２１に示す。該システムは、Ｏｌｙｍｐｕｓエピ蛍光顕微鏡本体（横向きに設置）を主部とし、自動オートフォーカシングステージおよびＣＣＤカメラを備える。回転式ホルダ内の４つのフィルタキューブにより、さまざまな励起および発光波長での４色検出が可能である。ペルチェ（ｐｅｌｔｉｅｒ）温度制御を伴うフローセルは、スライドなどの基材を収容するように開放および閉鎖が可能であり（ゲルなどの半固相支持体を含む領域の縁部周囲を密閉するためのガスケットを有する）、ステージ上に設置されている。フローセルの垂直配向は、本発明のシステムの重要な態様であり、気泡がフローセルの上部から散逸するのを可能にする。セルは、各洗浄工程前にすべての試薬が噴出されるように、空気が充分充填されたものであり得る。フローセルは、４種類の差次的に標識されたプローブ混合物、切断試薬、任意の他の所望の試薬、酵素並行化バッファー、洗浄バッファーおよび単一のポートを介するフローセルへの空気の送達を可能にする２つの９－ポートＣａｖｒｏシリンジポンプを有する流体取り扱い部と接続されている。システムの操作は完全に自動化されており、多数のＩ／Ｏポートを有する専用のコンピュータを用いて、制御ソフトウエアによりプログラミング可能である。Ｃｏｏｋｅ　Ｓｅｎｓｉｃａｍカメラには１．３メガピクセル冷却ＣＣＤが組み込まれているが、より低いまたはより高い感度を有するカメラもまた使用され得る（例えば、４メガピクセル、８メガピクセルなども使用され得る）。フローセルには、０．２５ミクロンステージが用いられており、形状は１ミクロンである。

【0347】

（実施例１０：画像取得および処理方法）

この実施例では、自身に結合された標識された核酸を有するビーズのアレイからの画像の取得および処理のための代表的な方法を記載する。正確な素性の同定およびアライメントは、各取得画像の信頼できる解析に重要である。素性は、まず、各ビーズについて最も強いピクセル以外は排除するにより同定される。所与の画像のピクセル値をヒストグラムにプロットする；バックグラウンドに対応するピクセルを排除し、残りのピクセル値を分取する。均一な画像では、すべてのビーズがほぼ同じ強度であり、アルゴリズムにより、ピクセル値の下部８０～９０％を除外する。次いで、上部１０～２０％値を有するピクセルをスキャンし、４ピクセル半径内で極大のものを同定する。次いで、該領域内の平均強度および周辺の平均強度を記録する。これらの値により正規分布を形成し、次いで、その値が該分布から外れるピクセルを除く。最初に無視したピクセルの割合、該円形領域の大きさ、および正規分布内の可能なビーズを排除した切り捨て値を、すべてパラメータ化し、必要であれば調整し得る。該組の各画像について素性行列を作製することによりアライメントを行なう。次いで、得られた行列を、最も高頻度のｘ、ｙ座標オフセットについて検索し、最適なアライメントを同定する。

【0348】
ビーズ画像を、伸長プローブ添加の前に、Ｃｙ５チャネル（配列決定プライマーに対応）に収集する。これらの画像を用いて、各ビーズについて位置的座標および蛍光単位（ＲＦＵ値）としての生シグナル強度の両方をマーキングする（ｍａｒｋｉｎｇ）素性マップを作製する。各後続の二本鎖伸長後、画像の組を、Ｃｙ３－標識ヌクレオチドを添加する前と後の両方で取得する。これらの画像を元のＣｙ５　画像に対してアライメントし、次いで、ＲＦＵ値をビーズの各々に割り当て、記録する。各塩基付加の非標識（伸長前）および標識（蛍光付加）画像間の強度を差を差し引くことによりベースライン補正を適用する。これらのベースラインを差し引いた値を、次いで、各素性でＣｙ５画像において見られた強度によって標準化し、ビーズが伸長された、またはされていないとみなす（すなわち、ビーズは、該ビーズに結合された二本鎖が伸長された場合、伸長されたとみなす）基準を形成する。これらの方法を用い、約１，３００画像／スライドで１つの画像あたり数千の素性が解析され、１回の実験で５～１０００００００鋳型種の解析がもたらされ得る。アルゴリズムは、さらなる効率増強のために、後日でも容易にＭＡＴＬＡＢからＣ＋にポート移動（ｐｏｒｔ）され得るように設計した。
【0349】
（実施例１１：ビーズアライメントおよび追跡および配列でコード化）
この実施例では、自身に結合された標識され核酸を有するビーズのアレイからの画像処理および取得されデータからの配列決定のための代表的な方法を記載する。
【0350】
画像解析は、ビーズサイズに適合した直径を有するゼロインテグラルサーキュラートップハットカーネル（ｚｅｒｏ－ｉｎｔｅｇｒａｌ　ｃｉｒｃｕｌａｒ　ｔｏｐ－ｈａｔ　ｋｅｒｎｅｌ）を用いて画像を回転させることにより開始する。これは、自動的にバックグラウンドをゼロに標準化すると同時に、個々のビーズの中心を極大によりを同定する。最大値を位置し、他の極大から孤立性のものが、アライメント点として使用される。これらのアライメント点は、各画像について時系列でコンピュータ計算される。各対の画像について、アライメント点を比較し、変位ベクトルを、すべての共通アライメント点の平均変位に基づいてコンピュータ計算する。これにより、サブピクセル分解によるペアワイズ画像変位が提供される。
【0351】
Ｎ個の画像では、Ｎ＊（Ｎ－１）／２のペアワイズ変位があるが、これらのうちＮ－１だけは、残りが非依存的な組から計算され得るため、非依存的である。例えば、画像１と２間および画像１と３間の変位を測定すると、画像２と３間の変位が示唆される。画像２と３間の測定された変位が示唆された変位と同じでない場合、測定値は不一致である。この不一致の大きさは、どれだけ良好にアライメントアルゴリズムが機能しているかを測るための測定基準として使用され得る。本発明者らの最初の試験は、各寸法において、一般的に、０．１ピクセル未満である不一致を示す（図２３参照）。
【0352】
いったん、時系列の画像がアライメントされたら、個々のビーズを追跡するのに２つの方法がある。ビーズ密度が、別のビーズと接触していないビーズの大部分で低い場合、個々の各ビーズの光学質量中心が同定され得、ビーズ周囲の領域が積分され、ビーズ強度がコンピュータ計算される。ビーズ密度が非常に高いため、ビーズの大部分が接触している場合は、その周囲にある暗いバックグラウンドバンドによって個々のビーズを同定することは可能でない。しかしながら、サブピクセル分解のためアライメントされたすべての画像で、隣接するピクセルの相関性を経時的にコンピュータ計算することにより、同じビーズに属するピクセルを同定することが可能である。高度に相関するピクセル対は、確信的に同じビーズに割り当てられ得る。同様の手法がＤＮＡ配列決定ゲル内でのレーン追跡に適用され、良好な結果が得られている（Ｂｌａｎｃｈａｒｄ，Ａ．Ｐ．Ｓｅｑｕｅｎｃｅ－ｓｐｅｃｉｆｉｃ　ｅｆｆｅｃｔｓ　ｏｎ　ｔｈｅ　ｉｎｃｏｅｐｏｒａｔｉｏｎ　ｏｆ　ｄｉｄｅｏｘｙｎｕｃｌｅｏｔｉｄｅｓ　ｂｙ　ａ　ｍｏｄｉｆｉｅｄ　Ｔ７　ｐｏ

ｌｙｍｅｒａｓｅ，Ｃａｌｉｆｏｒｎｉａ　Ｉｎｓｔｉｔｕｔｅ　ｏｆ　Ｔｅｃｈｎｏｌｏｇｙ，１９９３）。いったんビーズが全４色時系列により追跡されたら、配列は、どの色がプローブオリゴヌクレオチドのどの最も３’側の塩基に対応するかがわかることによりデコード化される。

【0353】

（実施例１１：処理量計算）

一般に、配列決定システムの処理量は、主に、装置が１日あたりにもたらすことができる画像の数および１つの画像あたりの配列データのヌクレオチド（塩基）の数によって規定される。装置は、好ましくは、カメラが常時作動中で維持されるように設計され、計算は、１００％カメラ利用に基づいて行なう。１つの塩基の正体を決定するのに各ビーズが４色で画像化される実施において、１つのカメラで４画像、２つのカメラで２画像、または４つのカメラで１画像のいずれかが使用され得る。４つのカメラでの画像化により、他の選択肢よりも劇的に高い処理量が可能になり、好ましいシステムでは、該アプローチが用いられる。

【0354】

本発明者らの最初の試験は、５０ピクセル／ビーズのピクセル密度（５．４平方ミクロンを表す）は、標準的な画像解析に適度な（ｃｏｍｆｏｒｔａｂｌｅ）密度を提供することを示す。４メガピクセルＣＣＤ　カメラ（現在では、一般的）を用いることにより、単一のＣＣＤフレームで約８０，０００個のビーズの画像化ができる（本発明者らの現在の画像データに基づく）。別個のカメラでの４種類の画像の捕捉およびフローセル上の次のフィールドへの移動に１．５秒より多くかからない。７５％のビーズにより有用な情報が得られる場合、本発明者らは、およそ８０，０００ビーズ* ０．７５／１．５＝４０，０００塩基／秒の生配列データのデータを収集することができる。

【0355】

１００％カメラ利用の維持における大きな問題の１つは、ライゲーション／切断化学反応の１サイクルを行なうのにかかる時間全フローセルの画像化に必要とされる時間に適合させることである。伸長、切断およびライゲーションの１サイクルにかかる時間の適度な推定は１．５時間（５，４００秒間）である。５，４００秒間は１，８００画像フィールドまたは約１５ｍｍ×４５ｍｍの面積に適応し、これは、フローセルに適度なサイズである。４種類のカメラを用いるシステムの処理量の伸長な推定は、１５ｍｍ×４５ｍｍフローセルで４０，０００塩基／秒である。これは、２８実行／日で約６５０塩基読出し長さ（２０塩基／秒）の処理量に基づくと、およそ２，０００　ＡＢＩ３７３０ｘ１配列決定装置に相当し、本発明者らは、これらの装置を用いて達成した。ビーズ密度を２００，０００／画像に２．５倍増大させると、１００，０００塩基／秒への処理量の全体的な増加が可能になり、これは、ほぼ５，０００　ＡＢＩ３７３０ｘ１装置に相当する。この処理量レベルでの全出力／日は、約８．６Ｇｂ／日であり、１２×ヒトゲノム配列決定を完了するのに必要とされる時間は、約４．２日となり得る。

【0356】

本発明の配列決定本明細書に記載の方法は、さまざまな異なる配列決定システム、画像捕捉および処理方法などを用いて実施され得ることに注意されたい。例えば、米国特許第６，４０６，８４８号および同第６，６５４，５０５号ならびにＰＣＴ公開公報ＷＯ９８０５３３００の論考を参照のこと。

【0357】

（実施例１２：その上面で鋳型合成を微粒子の調製方法）

この実施例では、鋳型分子のクローン性の集団が各微粒子に結合されるように鋳型が増幅され得る（例えば、ＰＣＲにより）ように自身に結合された増幅プライマーを有する微粒子（この実施例では磁気ビーズ）のプロトコル調製を記載する。一般に、増幅ビーズは、クローンＰＣＲ反応に必要とされる１つのプライマーが自身に結合されている。このプライマーは、共有結合によりカップリングさせてもよく、または例えば、ビオチン標識し、ビーズ表面上のストレプトアビジンに結合させてもよい。ビーズは、標準的なＰＣＲ反

応（例えば、マイクロタイタープレートのウェル、チューブ内など）、実施例１３に記載のエマルジョンＰＣＲ反応などに使用され、自身に結合された鋳型分子のクローン性の集団を有するビーズが得られ得る。

【0358】

材料

１×ＴＥ：１０ｍＭ　Ｔｒｉｓ（ｐＨ８）１ｍＭ　ＥＤＴＡ

１×ＰＣＲバッファー：（ＴｈｅｒｍｏＰｏｌバッファー，ＮＥＢ）

２０ｍＭ　Ｔｒｉｓ－ＨＣｌ（ｐＨ８．８）

１０ｍＭ　ＫＣｌ

１０ｍＭ$(NH_4)_2SO_4$

２ｍＭ　$MgSO_4$

０．１％Ｔｒｉｔｏｎ　Ｘ－１００

１Ｍ　ベタイン（　１×ＰＣＲ－Ｂバッファーの場合のみ添加）

１×結合および洗浄バッファー

５ｍＭ　Ｔｒｉｓ　ＨＣｌ（ｐＨ７．５）

０．５ｍＭ　ＥＤＴＡ

１　ＭＮａＣｌ

ＤＮＡ捕捉プライマー（２０量体，５００μＭストック）

二重ビオチン－（ＨＥＧ）５－Ｐ１：５’－二重ビオチン－（ＨＥＧ）５－ＣＴＡ　ＡＧＧ　ＴＡＧ　ＣＧＡ　ＣＴＧ　ＴＣＣ　ＴＡ－３’

（ＨＥＧ）５＝ヘキサエチレングリコールリンカー、１８炭素含有スペーサー、使用され得るいくつかの異なるスペーサー部分の１種類。例えば、ビーズの表面から離れるオリゴのＰ１プライマー部分を増大させるため、スペーサーを含めることが有用である。本明細書に記載の任意のプライマーには、かかるスペーサー部分が組み込まれ得る。

Ｄｙｎａｌストック磁気ビーズ（１μｍ直径）＝１０ｍｇ／ｍｌ（７～１２×10^6ビーズ／μｌ）。

【0359】

方法

１．５０μｌビーズ（約４５０×10^6　ビーズ）を除去。

２．２００μｌ　１×ＴＥバッファーを添加、充分混合。磁石で分離。

３．１×ｗｉｔｈ　２００μｌ　１×ＴＥバッファー洗浄。磁石で分離。

４．１００μｌ　Ｂ／Ｗバッファー中に再懸濁。

５．３μｌのＰ１オリゴ（５００μＭストック＝１５００ｐｍｏｌ）を添加。

６．室温で＞３０分間回転。

７．２００μｌ　１×ＴＥバッファーで３回洗浄。

８．５０μｌ（初期容量）１×ＴＥバッファー中に再懸濁。

９．使用前にＤＮＡ捕捉ビーズを４℃保存または氷上に配置。ビーズは、１週間以内に使用するのがよい（ビーズは、＞１週間の保存期間で凝集する傾向がある）。

【0360】

（実施例１３：エマルジョン中の微粒子上でＰＣＲを行なうための方法）

この実施例では、エマルジョン中の微粒子上でＰＣＲを行ない、自身に結合されたクローン鋳型を有する微粒子を生成させるために使用され得る方法を記載する。微粒子（以下、ＤＮＡビーズをいう名称を用いる）を、まず、第１のプライマー（Ｐ１）により官能性付与させる。第２のプライマー（Ｐ２）を水相中に存在させ、そこでＰＣＲ反応を行なわせる。所望の場合は、低濃度をＰ１を水相中に含め得る（例えば、２０倍低い）。そうすることにより、鋳型の水相中での迅速な構築が可能になり、これは，さらなる増幅のための基材となる。Ｐ１が溶液中ですると、反応は、微粒子に結合されたＰ１の利用に向かって誘導される。Ｐ１＿Ｐ２　ｄｅｇｅｎ１０は、Ｐ１およびＰ２にハイブリダイズしてＰＣＲによる増幅をもたらす配列およびオリゴヌクレオチド集団に4^{10}の複雑度を与える１０縮重塩基鎖（オリゴヌクレオチド合成中に組み込まれる）を有するオリゴヌクレオチ

ド鋳型（１００ｂｐ）である。
Ｉ．エマルジョンプロトコル（１μｍビーズ）
１．油相の調製：
Ｓｐａｎ　８０（７％）
Ｔｗｅｅｎ　８０（０．４％）
低分子鉱油中で調製
新たに作製した油相のみ使用
全油相＝４５０μｌ
２．水相の調製：（２×１０^{9}滴が作製されたと概算，１１５μＬ／滴）
【0361】
【化7】

試薬（ストック）	（μｌ）反応液あたり	最終
dH_2O	156.0	---
$MgCl_2$ バッファー (10X)	32.0	1X
dNTP (100 mM ea)	11.3	各3.5 mM
$MgCl_2$ (1M)	7.3	23mM
ベタイン (5 M)	32.0	0.5M
P1 (プライマー1)(10 μM)	1.6	11.25pmol
P2 (プライマー2)(200 μM)	40.0	5625 pmol
P1_P2 degen10 (100 pM)	6.6	5.9x10^7/ul
DNA ビーズ (8M/μl)	25.0	150M/エマルジョン
白金 Taq (5 U/μl)	9.0	0.28U/ul

全水性容量＝３２０μｌ
最終反応＝２５５μｌ　水相　：４５０μｌ　油相
３．エマルジョンの添加まで水相チューブを氷に移動。
４．４５０μｌ　油相を２ｍｌクリオバイアルに添加。
５．クリオバイアルＵＰＲＩＧＨＴをＩＫＡ　ボルテックスに取り付けた発泡アダプター内に配置。ボルテックスを２５００ｒｐｍに設定。
６．アリコート水相（３　アリコート，各８５μｌ＝２５５μｌ）を油相に入れて振とうする。単分散性水相を、先端をチューブ内に配置し、ゆっくりと水相を先端から振とう油相内に分配することにより撹拌２ｍｌクリオバイアルに添加。残留水相に２回添加を反復。
７．エマルジョンの振とうを２４分間　２５００ｒｐｍで継続、
８．エマルジョンの約１００μｌ　アリコートを９６ウェルプレート内に移す（合計＝４ウェル）。また、残留水相のアリコート（６５μｌ）を、溶液系ＰＣＲ対照反応のために別のウェルに。プレートを密封し、次のセクションに概略を示すようにサイクルさせる。
Ｉｉ．エマルジョン増幅（１μｍ　ビーズ）
１．１μｍ　ビーズエマルジョン（プライマー使用Ｔｍｓ＝６２Ｃ）のＰＣＲサイクリングパラメータ：
プログラム：ＤＴＢ－ＰＣＲ
９４Ｃ，２分　ｎ＝１
９４Ｃ，１５秒
５７Ｃ，３０ｓ　ｎ＝１００
７０Ｃ，６０秒
５５Ｃ，５分　ｎ＝１
１０Ｃ，随意時間
２．サイクリング時間は約６時間である。
３．サイクリング後のエマルジョンを観察。良好なエマルジョンは均一な琥珀色に見え、分離水相は観察されない。「破断」したエマルジョン（溶液から分離）は、チューブの底部に異なる水相を有する。ビーズのこの集団はクローン性でないため、この段階での回収

は回避。
４．明るい視野での顕微鏡検査を用いてサイクル後エマルジョンを評価。サイクル的エマルジョンの２μｌアリコートを取り出し、ガラススライドに１滴落とす。エマルジョンアンプルに２２×６０ｍｍガラスカバースリップを重層。
５．２０×対物レンズを用いてエマルジョンを観察。ビーズは、好ましくは、単分散性であり、液滴の大部分が単一のビーズを含む。
注：エマルジョンアンプルが多数の多ビーズ液滴を含む場合、プールエマルジョン反応を単一の１．５ｍｌのエッペンドルフチューブに入れ、６０００ｒｐｍで１５秒間回転させる。チューブの底部に蓄積したビーズ懸濁液を取り出す。この集団は、遊離ビーズおよび単一ビーズ液滴より重い多ビーズ液滴の両方で構成され、したがって、短時間のスピン後、チューブの底部に沈降する、このビーズ集団はクローンでない、したがって、後続の処理前に回避するのがよい。工程４および５を反復することによりエマルジョンを再評価し、
エマルジョンアンプル中での単一のビーズ含有液滴の完全性を確認する。
６．次のセクションに概要を示すプロトコルを用いたエマルジョンの破壊（崩壊）
ＩＩＩ．エマルジョン破壊および融解（１μｍビーズ’）
ビーズ破壊洗浄（ＢＢＷ）バッファー
２％Ｔｒｉｔｏｎ　Ｘ－１００　２％Ｔｗｅｅｎ　２０；１０ｍＭ　ＥＤＴＡ
融解溶液　１００ｍＭ　ＮａＯＨ
１×ＴＥ：１０ｍＭ　Ｔｒｉｓ（ｐＨ８）１ｍＭ　ＥＤＴＡ
１×結合および洗浄（Ｂ／Ｗ）バッファー
５ｍＭ　Ｔｒｉｓ－ＨＣｌ（ｐＨ７．５）
０．５ｍＭ　ＥＤＴＡ
１Ｍ　ＮａＣｌ
１．各エマルジョンセット（４　アリコート）を単一の１．５ｍｌエッペンドルフチューブ中にプール。
２．８００μｌ　ＢＢＷバッファーを添加。反応チューブの１０秒間のボルテックスによりエマルジョンの破壊。
３．８０００ｒｐｍで２分間スピン。
４．上部８００μｌ（主に油相）の除去。チューブの底部でＤＮＡビーズをペレット化。
５．８００μｌ　ＢＢＷを添加、ボルテックスおよび８０００ｒｐｍで２分間スピン、上部６００μｌ除去。
６．
さらに２回６００μｌ　１×ＴＥで洗浄、磁石を用いて各洗浄液を交換。
８．５０μｌ　融解溶液をビーズペレットに添加、激しいペッティングにより再懸濁試料。融解溶液中で５分間室温でビーズのインキュベート。チューブ断続的に打つ。
９．チューブを磁石に配置して融解溶液を除去。１×１００μｌ　融解溶液で洗浄し、第２鎖の完全な除去を確保。
１０．ビーズペレットを２回１×ＴＥで洗浄、２０μｌ　ＴＥバッファー中に再懸濁して４Ｃで保存または２０μｌ　１×Ｂ／Ｗバッファー（次の工程が富化の場合）。ビーズが凝集しているようであれば、１×ＰＣＲ－　Ｂバッファーに交換。
１１．富化プロトコルを継続（任意選択的）。
【0362】
（実施例１４：自身に結合されたクローン鋳型集団を有する微粒子の富化方法）
　この実施例では、上面での鋳型増幅が例えばＰＣＲエマルジョンで成功裏に行なわれた微粒子富化方法を記載する。該方法は、自身に結合された捕捉オリゴヌクレオチドを有する大きな微粒子を利用する。捕捉オリゴヌクレオチドは鋳型内に存在するヌクレオチド領域に相補的なヌクレオチド領域を含む。
Ｉ　．エマルジョン富化（１ｕｍ）
Ａ．富化ビーズ（捕捉実体）の調製

富化ビーズ：
Ｓｐｈｅｒｏｔｅｃｈストレプトアビジンコートされたポリスチレンビーズ（約６．５ｕｍ）
ビーズストック（０．５％ｗ／ｖ）：３３，１２５　ビーズ／μｌ
Ｐｅｒ　プロトコル：（３３，１２５　ビーズ／μｌ）（８００μｌ）＝２６．５×１０^{6}　ビーズ
使用：
１１９０００００ビーズ／エマルジョン－エマルジョンクローン性の概算（２％）：約３Ｍ　鋳型－陽性ビーズ／エマルジョン。２～３富化ビーズ／概算鋳型陽性エマルジョンビーズ＝１００００００　富化ビーズ／エマルジョン反応を添加。
富化オリゴヌクレオチド（捕捉剤）：
Ｐ２－富化（３５量体，Ｔｍ＝７３Ｃ）
５’－二重ビオチン－１８－炭素スペーサー－ｔｔａｇｇａｃｃｇｔｔａｔａｇｔｔａｇｇｔｇａｔｇｃａｔｔａｃｃｃｔｇ　３’
（または）
Ｐ２－富化（例えば、３５量体まで，Ｔｍ＝５２Ｃ）
５’－二重ビオチン－１８－炭素スペーサー－ｇｇｔｇａｔｇｃａｔｔａｃｃｃｔｇ　３’
グリセロール溶液　－　６０％（ｖ／ｖ）
６ｍｌ　グリセロール
４ｍｌ　ヌクレアーゼ無含有H_2O
１．８００μｌのビーズを除去、１３，０００ｒｐｍで１分間の遠心分離によりＢ／Ｗバッファーに交換。１×の５００μｌ　Ｂ／Ｗバッファーでの洗浄、および１００μｌ　Ｂ／Ｗバッファーへの再懸濁。
２．２０μｌ　富化オリゴを添加。（５００μＭストック＝１０，０００ｐｍｏｌ／ｒｘｎ）。
３．ビーズ反応物を室温で１時間回転。
４．ビーズを３回、５００μｌ　１×ＴＥバッファーを用いて洗浄。１３，０００ｒｐｍで１分間遠心分離により洗浄間でビーズをペレット化。
５．ビーズを２５μｌ　Ｂ／Ｗバッファーに再懸濁。濃度＝１Ｍ　富化ビーズ／μｌ。注：４種類の富化エマルジョン集団を２０～３０μｌ　１×Ｂ／Ｗバッファーにプールし、約４０Ｍ　鋳型陽性ビーズを得る。次いで、多数のスライドに行ない得る。
Ｂ．富化手順
１．２０μｌの富化ビーズを、エマルジョン誘導型ビーズ（２０μｌ）を含有するチューブに添加。
穏和なピペッティングによりビーズ混合物を再懸濁（または概算鋳型陽性エマルジョンビーズごとに２～３　富化ビーズ増大を与える比を使用）。
２．ビオチン化Ｐ２－富化プライマーでコーティングされた富化ビーズを用いる場合、ビーズ混合物を６５Ｃで２分間インキュベート。チューブを氷に１０分間取り出す。
注：最初の実験は、１００－サイクルＰＣＲ（例えば、Ｐ２ＰＣＲ）に使用されるプライマー配列を含有する富化ビーズの使用は、富化すると、プライマーを含有するビーズを富化能力により効率的が低くなり得ることを示した。液滴中の２量体種誘導ビーズは鋳型がなかった。上記のＰ２－富化プライマーが負荷された富化ビーズを用いる場合、この短鎖プライマーのＴｍの低下によりビーズ混合物を５０Ｃ　２分間インキュベートする。
３．ビーズ混合物を、３００μｌ　６０％グリセロール溶液を含む１．５ｍｌエッペンドルフチューブに重層。
４．１３，０００ｒｐｍで１分間遠心分離
５．スピン後、陰性ビーズは、チューブの底部にペレット化する。結合された鋳型ビーズを含む富化ビーズは、グリセロール相上部に浮遊する。上相ビーズ集団を改修し、きれいな１．５ｍｌエッペンドルフチューブに移す。

注：チューブの底部ペレット化したビーズ（鋳型なしのビーズ）は洗浄し、鋳型陽性ビーズで概略を示した同じ洗浄レジメン後、磁石を用いて解析され得る。
６．ビーズを相から引き出すため、１ｍｌ　ヌクレアーゼ無含有Ｈ２０を添加し、グリセロール濃度を希釈する。穏和なピペッティングを用いてビーズ混合物を再懸濁する。１３，０００ｒｐｍで１分間スピンする。
７．スピン後、上清みを除去し、２回１００μｌ　ＴＥを用いて洗浄。
８．１００μｌ　融解溶液を添加し、ビーズペレットを洗浄。チューブを５分間室温で回転。
９．さらに１００μｌ　融解溶液を添加、鋳型ビーズを磁石を用いて単離。
１０．２回１００μｌ　ＴＥ洗浄により非磁石性富化ビーズを除去し、磁石でＤＮＡビーズを富化ビーズから離す。
１１．鋳型ビーズを１０～２０μｌ　１×ＴＥに再懸濁。ビーズ凝集しているようであれば、１×ＰＣＲ－Ｂバッファー中に含むで希釈。
１２．鋳型含有ビーズは、他の富化集団でプールされ得、次の実施例で記載のようにしてスライド上に負荷し得る。
【0363】
（実施例１５：半固相支持体に固定化された微粒子アレイ調製方法）
この実施例では、スライド上に位置する半固相支持体内に自身に結合された鋳型微粒子が固定化され（例えば、包埋され）スライドの調製を記載する。かかるスライドは、ポロニルスライドとよばれ得る。この実施例に使用される半固相支持体は、ポリアクリルアミドである。プロトコルの一例では、ポリメラーゼ分子を鋳型付近に捕捉して増幅を増強する方法を用いる。
【0364】
スライドの調製
Ａ．ガラススライド：結合シラン処理
結合シラン処理により、ガラススライド表面へのアクリルアミドゲル結合が容易になる。スライドは、使用前に結合シラン処理で前処理するのがよい。
注：
＊＊　結合シラン処理溶液を化学的結合で保存。
＊＊　結合シラン処理は刺激性である。溶液調製時は化学的に作業する。
＊＊　　ストック結合シラン処理溶液がきれないように確保する。
＊＊　ラックへの出し入れの移動時は、スライドの表面に触れないようにする。
【0365】
結合シラン処理溶液の調製：
１．１－Ｌ　プラスチック容器内に添加：
１　Ｌ　ｄＨ２０，１　撹拌バー
２２０ｕｌ濃酢酸を添加（ｐＨ３．５にする）。４ｍｌ結合シラン試薬混合溶液を＞１５分間、撹拌プレートを用いて添加。
スライド処理：
２．スライド（同じ方向に向ける）を裏返したプラスチック　３８４ウェルプレートに負荷。
３．ｄH_2Ｏでリンス処理によりスライド洗浄、ウェルをドレイン。
４，１００％エタノールでリンス。ウェルをドレイン。
５．再度ｄH_2Ｏでリンス、ウェルをドレイン、ベンチをを有する組織培養フード内に配置、ＵＶ光照射。スライドを洗浄して乾燥させる（約３０分）。
６．プレートをプラスチック容器内に配置し、スライドを結合シラン処理溶液で覆う。
７．溶液およびスライドを１時間反応させる。容器を断続的に撹拌し、ガラスへの結合シラン処理の均一なコーティングを確保。
８．インキュベーション後、スライドを３回ｄＨ２Ｏでリンス処理。
９．１回１００％エタノールでリンス処理。ウェルをドレイン。

１０．使用前にスライドを充分乾燥させる。
１１．結合シラン処理スライドをデシケータ内で乾燥。
【0366】
Ｂ．アクリルアミド系スライド（小さいマスク）
・　非捕捉プロトコル
１．すべての試薬氷上に配置。以下の冷却試薬を１．５ｍｌエッペンドルフチューブに添加：
【0367】
【化8】

試薬	amt (μl)	
	2 スライド	**1** スライド
1x TE	13	6.5
ビーズ (1-3M, １×ＴＥ中で希釈)	10	5
Rhinohide	1	0.5
40% アクリルアミド:Bis (19:1, F/S)	5	2.5
TEMED (5%, １×ＴＥ中)	2	1
APS (0.5%, 新たに作製)	3	1.5
合計	**34 μl**	**17 μl**

混合物を激しくピペッティングしてビーズをかき回す。ガラスカバースリップ下で１７μｌ／スライド負荷。
上下に重合。室温で６０分間.
きれいなかみそり刃でカバースリップを除く。
スライドを浸漬し。２回ＩＥバッファー中で１５分間洗浄（非結合ビーズを除去）。
包埋ビーズを有するスライドを４ＣでＩＥ保存。
２．フルオロフォア－標識配列決定プライマーを包埋ビーズ集団にハイブリダイズさせる。
洗浄１Ｅから１×ＰＣＲ－Ｂバッファーに、１×ＰＣＲ－Ｂバッファーを含有するＣｏｐｌｉｎ　ｊａｒ短時間浸漬させることによりスライドを平衡化させる。
３．１．５ｍｌエッペンドルフチューブ内で、１～６μｌ（１００μＭストック）プライマーを９９μｌ　１×ＰＣＲバッファーに添加する。アクリルアミドマトリックス上に、１００μｌ　プライマー溶液を滴下し、ガラスカバースリップを重層するか、ガスケットを封止する。
４．＜ＤＥＶＩＮ＞プログラムを用いてスライドを加熱することにより、プライマーを包埋されたビーズにハイブリダイズさせる。（６５Ｃで２分間、３０Ｃまで低速アニーリング）。スライド２回２分間洗浄ＩＥ中で洗浄。スライドは、ライゲーション系配列決定に供する準備ができている。
【0368】
・捕捉プロトコル
１．ｓｓＤＮＡ鋳型ビーズは、１Ｍ／μｌで調製する。［ポロニルスライド、４－５Ｍビーズ／スライド］。
２．ビーズ混合物を３０μｌ　１×ＰＣＲバッファー中に再懸濁。
３．１ｕｌの配列決定プライマー（１００μＭストック）を添加；充分混合
４．６５Ｃまで２分間加熱
５．氷上に５分間取り出す
６．８０μｌで１×ＴＥ洗浄
７．すべての固形分を磁石を用いて除去
８．以下に概要を示すようにして試薬を添加：
【0369】

【化9】

試薬	amt(μl) 2スライド
1x バッファー	1.5
10x バッファー	2.0
高濃度 (HC) 酵素	16.0
40% アクリルアミド:Bis (19:1, F/S)	14.4
Rhinohide	2.0
TEMED (5%, １×ＴＥ中)	2.0
APS (0.5%, 新たに作製)	1.5
合計	**39.4 μl**

混合物を激しくピペッティングしてビーズをかき回す。ガラスカバースリップ下で１７μｌ／スライド負荷。

９．重合、　好ましくは、上下にする（例えば、ＭＪ　Ｒｅｓｅａｒｃｈ　Ｔｅｔｒａｄ　ＰＣＲ装置で＜Ｐｏｌ－１＞サイクリングプロフィールを使用）

１０．かみそり刃でカバースリップを除くスライドを２回１Ｅバッファー中に１０分間浸漬および洗浄（非結合ビーズを除去するため）。

１１．ポロニルスライドはライゲーション系配列決定に供する準備ができている。

１２．包埋されたビーズを有するポロニルスライドは、ガスケット内に４Ｃで洗浄ＩＥ中に保存され得る。

【0370】

（実施例１６：固相支持体に結合された微粒子アレイ調製方法）

この実施例では、その上面に自身に結合された鋳型を有する微粒子が固相支持体に結合されたスライド調製を記載する。

【0371】

１．反応性ＮＨＳを有するポリマーテザーを用いて調製されたガラススライドを－２０Ｃで保存する（スライドH，製造番号１０７０９３６；Ｓｃｈｏｔｔ　Ｎｅｘｔｅｒｉｏｎ；Ｓｃｈｏｔｔ　Ｎｏｒｔｈ　Ａｍｅｒｉｃａ，Ｉｎｃ．，Ｅｌｍｓｆｏｒｄ，ＮＹ）

２．乾燥剤の存在下、使用前にスライドを室温に平衡化させる。

【0372】

３．５０ｍｌ　１×ＰＢＳ（３００ｍＭリン酸ナトリウム，ｐＨ８．７）中で５分間スライドを洗浄する。洗浄を２回繰り返す。

【0373】

４．スライドを溶液から取り出し、接着性ガスケットで覆う（試料負荷を可能にするため）。

【0374】

５．別のチューブで、アリコート１００～４０００００００タンパク質コートまたはＤＮＡコートビーズを１×ＰＢＳ，ｐＨ８．７に。ＤＮＡは、例えば、配列決定用のＤＮＡ鋳型であり得る。ＤＮＡは、例えば、ＮＨＳとの反応用のアミンリンカーを含むものであり得る。

【0375】

６．バッファー交換によってビーズ試料を３回１×ＰＢＳ，ｐＨ８．７で洗浄する。

【0376】

７．ビーズを１２５ｍｌ　１×ＰＢＳ，ｐＨ８．７中に再懸濁する。

【0377】

８．ビーズ溶液をスライドガスケット内に負荷し、スライド表面を均一にコートする。

【0378】

９．スライドを暗室チャンバ内に封鎖し、インキュベーションのために１～２時間室温で反応させる。

【0379】
１０．インキュベーション後、非結合ビーズ溶液を除去し、スライドを５０ｍｌ　１×ＴＥ（１０ｍＭ　Ｔｒｉｓ，１ｍＭ　ＥＤＴＡ，ｐＨ８）に移す。
【0380】
１１．１５分間／洗浄で定常的撹拌により、５０ｍｌ　１×ＴＥを用いて５回スライドを洗浄する。
【0381】
１２．スライドは、１×ＴＥ中に４Ｃで数週間保存され得る。
【0382】
１３．所望の場合は、ビーズ集団は、白色光（ＷＬ）を用いる明るい視野での画像解析により、または蛍光により、フルオロフォア系色素に結合された相補ＤＮＡオリゴヌクレオチドを用いて評価され得る。ＤＮＡ鋳型は、例えば、ライゲーション系配列決定を用いて配列決定され得る。
【0383】
図３３Ａは、自身にビーズが結合されたスライドの概略図を示す。ほんの少量の割合のＤＮＡ鋳型分子がスライドに結合されていることに注意されたい。１ミクロンビーズ（ＤｙｎａビーズＭｙＯｎｅ　ストレプトアビジンビーズ；Ｄｙｎａｌ　Ｂｉｏｔｅｃｈ，Ｉｎｃ．，製造番号６５０．０１）を使用した。しかしながら、広範なさまざまなビーズた使用され得る。
【0384】
図３３Ｂは、スライドに結合されたビーズ集団を示す。下側パネルは、白色光下でのスライドの同じ領域（左）および蛍光顕微鏡検査を示す。上側パネルは、ビーズ密度の範囲を示す。
【0385】
（実施例１６）　ゲル非含有ビーズに基づくアレイを使用した、オリゴヌクレオチド伸長およびライゲーションによる配列決定
本実施例では、ビオチンとストレプトアビジンの相互作用を介して基材（ガラススライド）に付着した微粒子のアレイの調製について記載し、ライゲーション、開裂および検出のサイクルによる配列決定の成功を実証する。ビオチン化鋳型が付着した微粒子は、エマルジョンＰＣＲを使用して調製し、後述の通り、半固形培地の非存在下で、ＰＥＧを含む連結を介して、ストレプトアビジンで官能基化した基材に付着させた。本方法では、増幅前にビオチン化プライマーを付着させたストレプトアビジンコートビーズを使用する。生産的な鋳型増幅が行われた粒子の増幅と濃縮の後、鋳型をビオチン化した。次いで、ビオチン化した鋳型が付着した微粒子を、ストレプトアビジンコートスライドとともにインキュベートした。したがって、本方法では、ビオチンとストレプトアビジンの連結を２回使用した。その他の手法では、プライマーを微粒子に連結させるか、あるいは増幅させた鋳型を基材に連結させる代替手段が使用されると考えられる。
【0386】
材料および方法：
ＢＡＣ　Ｅｃｏ　ｖ２．１ビーズの調製
ＭｙＯｎｅストレプトアビジンビーズ（１ミクロン）をビオチン化Ｐ１プライマーでコーティングし（図を参照）、エマルジョンＰＣＲにおいて、本発明者等のＢＡＣ－Ｅｃｏ（ｖ２．１）ライブラリーの鋳型が付着したビーズ群を作り出すために使用した。エマルジョンを破壊し、ビーズを標準的な方法でエクソヌクレアーゼにより精製および処理した。完全に伸長したＰＣＲ産物を有するビーズを、Ｐ２濃縮オリゴで被覆した濃縮ビーズに結合させることにより濃縮した（図を参照）。濃縮したビーズの溶液中の挙動を改善するために、これらのビーズをビオチン化Ｐ１オリゴとともにインキュベートして、ストレプトアビジンコーティングを露出したビーズの部分を被覆した。
【0387】
スライドへのＢＡＣ　Ｅｃｏ　ｖ２．１ビーズの沈着

ｓｓＤＮＡを含有する濃縮したＢＡＣ－Ｅｃｏ　ｖ２．１ビーズを、ストレプトアビジンでコーティングしたＯｐｔｉ－Ｃｈｅｍスライド（Ａｃｃｅ１８　Ｔｅｃｈｎｏｌｏｇｙ　Ｃｏｒｐｏｒａｔｉｏｎ）上に沈着した。本プロセスの準備として、ビーズをターミナルトランスフェラーゼ（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ）およびビオチン－１１－ｄｄＡＴＰ（Ｐｅｒｋｉｎ　Ｅｌｍｅｒ）とともにインキュベートし、ビオチン部分をＤＮＡ鋳型分子の３’端に共有結合的に付着させた。ビーズを等しい数のＭｙＯｎｅ　Ｃａｒｂｏｘｙｌｉｃ　Ａｃｉｄビーズ（Ｄｙｎａｌ）と混合し、５ｍＭのトリス塩酸塩（ｐＨ８．０）、５ｍＭのＥＤＴＡ、０．０００５％のＴｒｉｔｏｎ　Ｘ－１００、および１０％のＰＥＧ８０００（Ａｍｅｒｉｃａｎ　Ｂｉｏａｎａｌｙｔｉｃａｌ）を含有する沈着緩衝液に入れた。懸濁液をＣｏｖａｒｉｓ　Ｓ２ソニケーターで短時間超音波処理し、ストレプトアビジンでコーティングしたＯｐｔｉ－Ｃｈｅｍスライド（Ａｃｃｅｌ８　Ｔｅｃｈｎｏｌｏｇｙ　Ｃｏｒｐｏｒａｔｉｏｎ）上に沈着させた。スライドをＴＥ緩衝液で３回洗浄し、使用前に圧縮空気で乾燥させた。懸濁液をＬｉｆｔｅｒＳｌｉｐ（Ｅｒｉｅ　Ｓｃｉｅｎｔｉｆｉｃ　Ｃｏｍｐａｎｙ）で被覆し、スライド上に平坦な水層を生成し、蒸発を減少させた。これらのスライドを高湿度チャンバーに入れて室温にて４５分間インキュベートし、端部の蒸発を減少させつつ、ビーズを表面に定着させ、結合させた。ＴＥ緩衝液を充填したトレーにスライドを上下逆にして浸漬することにより、カバースリップを除去した。約１分間軽く攪拌すると、ほとんどのカルボン酸ビーズが除去された（別の実験で示された通り）。これらのスライドを即座にアセトンに浸漬し、圧縮空気を使用して乾燥させた。

【0388】

ゲル非含有スライド上でのサイクル式ライゲーション配列決定で使用した試薬は、Ｒｅｓｅｔ緩衝液を除けばアクリルアミド系ゲルの場合と同じであった。非ゲルアレイでは、１０ｍＭのＮａＯＨおよび０．１％のドデシルスルホン酸ナトリウム（Ｆｌｕｋａ）を含有するアルカリ系Ｒｅｓｅｔ緩衝液を使用した。図３８および図３９に示す通り、３００パネルゲル非含有アレイ（約１８×１８ｍｍ）に濃縮ＢＡＣ－Ｅｃｏライブラリービーズを播種し、自動小型フローセル器に入れ、アルカリ性Ｒｅｓｅｔに５０回暴露させて、ゲル非含有環境におけるビーズの安定性を検証した。５０サイクルの流動プログラムの後、ゲル非含有アレイはパネル１枚当たり２６，０００個を超えるビーズを含有した（４メガピクセルカメラ）。次いで、サイクル式ライゲーションおよび開裂を使用して、ゲル非含有アレイの配列決定を行った。各蛍光チャネルの高いＲＦＵ値によって証明されているように、サイクル１のデータの評価によって、本発明者等の２塩基４色プローブセットの効率的なライゲーションが裏付けられた。その後、ビーズ群をベースコールし、スペクトル純度プロットにプロットし、Ｓａｔａｙ分析および密度プロット評価によって優れた配列決定性能を示した。

【0389】

均等物および範囲

当該技術分野業者には、単に常套的な実験手法を用いることで、本明細書に記載した本発明の具体的な実施形態に対する多くの均等物が認識され、または確認できよう。本発明の範囲は、上記の記載に限定されることは意図されるのではなく、添付の特許請求の範囲に示されるとおりである。以下の特許請求の範囲において、「ａ」、「ａｎ」および「ｔｈｅ」などの冠詞は、そうでないと特に記載のない限り、あるいは文脈から明白にそうでない場合以外は、１つではなく、１つ以上を意味し得る。１つの群内の１つ以上のメンバー間の「または」を含む請求項または詳細説明は、そうでないと特に記載のない限り、あるいは文脈から明白にそうでない場合以外は、１つ、１つより多く、またはその群のメンバーすべてが、所与の生成物またはプロセスに存在し、使用され、あるいは、関連する場合が満たされるとみなされる。請求項における「場合により」の使用は、任意の特徴が存在する実施形態と、任意の特徴が存在しない実施形態とを本発明が包含することを示している。

【0390】

さらにまた、本発明は、記載の１つ以上の請求項からの１つ以上の限定、要素、原因、記述用語などが別の請求項に導入されたあらゆる変形例、組合せおよび置換を包含することを理解されたい。特に、別の請求項に従属する任意の請求項は、ベースとなる同じ請求項に従属する任意の他の請求項に見られる１つ以上の限定を含むように変形され得る。

【0391】

また、任意の１つ以上の実施形態は、具体的な除外が本明細書に明白に示されていない場合であっても、特許請求の範囲から明確に除外されることがあり得ることを理解されたい。また、本明細書および／または特許請求の範囲に配列決定に有用な試薬（例えば、鋳型、ミクロスフィア、プローブ、プローブファミリーなど）が開示されている場合、かかる開示はまた、該試薬を、当業者が別なふうに理解し得る場合、または本発明に特に記載のない限り、本明細書に開示された具体的な方法、または当該技術分野で知られた他の方法のいずれかに従って用いる配列決定方法も包含することを理解されたい。また、本明細書および／または特許請求の範囲に配列決定法が開示されている場合、当業者が別なふうに理解し得る場合、または本明細書においてかかる方法におけるその試薬の使用が明白に除外されている場合を除き、本明細書に開示された任意の１種類以上の試薬が該方法に使用され得る。さらに、配列決定に有用な特定の成分が本明細書および特許請求の範囲に開示されている場合、本発明は、その試薬の作製方法もまた包含することを理解されたい。用語「成分」は、配列決定に用いられる任意の要素、例えば、鋳型、自身に結合された鋳型を有する微粒子、ライブラリーなどをいうために広義で使用される。さらにまた、図面は、本明細書に組み込まれた一部分であり、本発明は、図面に示された構造、例えば、自身に結合された鋳型を有する微粒子、および図面に開示された方法を含む。

【0392】

本明細書において範囲を示す場合、両端を含む。さらにまた、ことを理解されたい。特に記載のない限り、あるいは文脈から明白にそうでない場合以外、および当業者に理解される場合、範囲として標示される値は、本発明の異なる実施形態において記載された範囲内の任意の具体的な値または下位範囲が、特に文脈においてそうでないと明白に記載されていない限り、該範囲の下限の単位の１０分の１まで想定され得る。

【図面の簡単な説明】

【0393】

【図1A】図１Ａは、初期化後、２サイクルの伸長、ライゲーションおよび同定を概略的に示す。

【図1B】図１Ｂは、伸長が鋳型の自由末端から支持体に向かって内方に進行する実施形態における初期化後、２サイクルの伸長、ライゲーションおよび同定を概略的に示す。

【図2】図２は、オリゴヌクレオチドプローブに対する色の割り当てのスキームを示し、この場合、プローブの３’塩基の正体が、フルオロフォアの色を同定することにより決定される。

【図3A】図３Ａは、鋳型の結合領域内の異なる位置における初期オリゴヌクレオチドのハイブリダイゼーション後、伸長プローブのライゲーションにより得られる伸長された二本鎖を概略的に示す。

【図3B】図３Ｂは、鋳型分子を６塩基ごとに読み出されるように設計された伸長プローブを用いて伸長、ライゲーションおよび切断方法を使用することにより、連続配列の合成を概略的に示す。

【図4A】図４Ａは、５’－Ｓ－ホスホロチオレート結合（３’－Ｏ－Ｐ－Ｓ－５’）　を示す。

【図4B】図４Ｂは、３’－Ｓ－ホスホロチオレート結合（３’－Ｓ－Ｐ－Ｏ－５’）　を示す。

【図5A】図５Ａは、３’－Ｏ－Ｐ－Ｓ－５’ホスホロチオレート結合を有する伸長プローブを用いた５’→３’方向における配列決定のための１サイクルの伸長、ライゲーションおよび切断を概略的に示す。

【図5B】図５Ｂは、３’－Ｓ－Ｐ－Ｏ－５’ホスホロチオレート結合を有する伸長プロー

ブを用いた３’→５’方向における配列決定のための１サイクルの伸長、ライゲーションおよび切断を概略的に示す。
【図6】図６Ａ～６Ｆは、単一の鋳型において行なわれた数個の配列決定反応のより詳細な概略図である。この反応では、鋳型の異なる部分に結合する初期オリゴヌクレオチドを用いている。
【図7】図７は、ｄＡおよびｄＧの３’－ホスホロアミデートの合成スキームを示す概略である。
【図8-1】図８Ａ～８Ｅは、ホスホロチオレート結合を含有する伸長プローブの成功裏のライゲーションおよび切断の２サイクルを示すゲルシフトアッセイの結果を示す。
【図8-2】図８Ｆは、ＤＮＡリガーゼによるライゲーションの機構の概略図を示す。
【図9】図９は、縮重イノシン含有オリゴヌクレオチドプローブのライゲーション効率を示すゲルシフトアッセイの結果である。
【図10】図１０は、多数の鋳型における縮重イノシン含有オリゴヌクレオチドプローブのライゲーション効率を示すゲルシフトアッセイの結果を示す。
【図11】図１１は、３’→５’伸長での２つのＤＮＡリガーゼ（Ｔ４　ＤＮＡリガーゼおよびＴａｑＤＮＡリガーゼ）の各々の忠実度を評価するために行なった解析の結果を示す。
【図12】図１２は、（Ａ）　縮重イノシン含有オリゴヌクレオチドプローブおよびライゲーション反応の直接配列決定解析のライゲーション効率を示す（Ｂ）　オリゴヌクレオチドプローブライゲーションにおけるＴ４　ＤＮＡリガーゼの忠実度を評価するために行なったゲルシフトアッセイの結果を示す。結果をパネルＣ～Ｆに表で示す。
【図13】図１３Ａ～１３Ｃは、ビーズ系鋳型をスライド上のポリアクリルアミドゲル内に包埋した場合のゲル内ライゲーションを示す実験の結果を示す。図１３Ａは、ライゲーション反応の概略を示す。ゲル内ライゲーション反応は、Ｔ４　ＤＮＡリガーゼの非存在下（Ｂ）および存在下（Ｃ）で行なった。
【図14A】図１４Ａは、第１の増幅プライマーが結合されたビーズ上で、蛍光標識された第２の増幅プライマーおよび過剰の鋳型を用いて行なったエマルジョンＰＣＲ反応の画像を示す。
【図14B】図１４Ｂ（上）は、Ｃｙ３－標識オリゴヌクレオチドがハイブリダイズする鋳型結合された結合されたビーズがポリアクリルアミドゲル固定化されたスライドの一部分の蛍光画像を示す（このスライドは、異なる実験に使用したが、ここで使用したスライドの典型である）。図１４Ｂ（下）は、ポリアクリルアミド溶液を封入するためのテフロン（登録商標）マスクを設けたスライドの概略図を示す。
【図15】図１５は、プローブの特異性および選択性の課題に取り組むために設計され３組の標識されたオリゴヌクレオチドプローブを示し、また、１組の４つのスペクトルにより分離可能な標識の励起および発光の値を示す。
【図16】図１６は、オリゴヌクレオチドプローブの４色スペクトルの正体を確認する実験の結果を示す。４つの特有の単鎖鋳型集団を含むスライド（Ａ）をハイブリダイゼーションに供し、４種類の特有のフルオロフォアプローブを含有するオリゴヌクレオチドプローブ混合物を用いたライゲーション反応物を、明るい光の下で、および（Ｂ）　４種類の帯域通過フィルターを用いた蛍光励起によりライゲーションの前と後で画像化した。個々の集団は擬似カラー表示した（Ｃ）。スペクトルの正体（これは、最小シグナル重複を示した）を（Ｄ）にプロットする。
【図17】図１７は、オリゴヌクレオチド伸長プローブのライゲーション特異性を確認する実験を示す。図１７（Ａ）は、ライゲーションの概略的概要を示す。図１７（Ｂ）は、明るい光での画像であり、図１７（Ｃ）は、ライゲーション後のポリアクリルアミドゲル内に包埋されたビーズ集団の対応する蛍光画像である。図１７（Ｄ）は、ライゲーション前（ｐｒｅ）または後（ｐｏｓｔ）の各標識から検出された蛍光を示す。
【図18】図１８は、オリゴヌクレオチド伸長プローブのライゲーションの特異性および選択性を確認する別の実験を示す。図１８（Ａ）は、ライゲーションの概略的概要を示す。

図１７（Ｂ）は、明るい光での画像であり、図１８（Ｃ）は、ライゲーション後のポリアクリルアミドゲル内に包埋されたビーズ集団の対応する蛍光画像である。図１８（Ｄ）は、ライゲーション頻度の予測対観察結果を示し、集団内の特定の伸長プローブの割合に基づいて予測した頻度と、観察された頻度との高い相関性を示す。

【図19】図１９は、オリゴヌクレオチド伸長プローブプールを含有する縮重した普遍塩基を用いて、特異的および選択的なゲル内ライゲーションがもたらされ得ることを確認する実験を示す。図１９（Ａ）は、ライゲーションの概略的概要実験を示し、ライゲーション後の４種類の差次的に標識された縮重イノシン含有プローブプールを示す。図１９（Ｂ）明るい光での画像であり、図１９（Ｃ）は、ライゲーション後のポリアクリルアミドゲル内に包埋されたビーズ集団の対応する蛍光画像である。図１９（Ｄ）は、ライゲーション頻度の予測対観察結果を示し、集団内の特定の伸長プローブの割合に基づいて予測した頻度と、観察された頻度との高い相関性を示す。図１９（Ｅ）は、生の未処理のデータおよびビーズシグナル値の上部９０％を表すフィルター処理データの散布図を示す。

【図20】図２０は、鋳型に対する初期オリゴヌクレオチド（プライマー）のハイブリダイゼーションとストリッピングの連続的サイクルにおいて検出されたシグナルを示すチャートである。図に示されるように、１０サイクルで生じたシグナル損失は最少であった。

【図21】図２１は、配列情報を、例えば、実質的に平面状の支持体内または該支持体上のアレイ状の鋳型から収集するために使用され得る自動配列決定システムの写真である。また、該システムの種々の構成部品の動作を制御するため、収集した画像データを処理および保存するため、ユーザーインターフェースを提供するためなどの専用のコンピュータを示す。図の下側部分に、重量測定気泡移動（ｇｒａｖｉｍｅｔｒｉｃ　ｂｕｂｂｌｅ　ｄｉｓｐｌａｃｅｍｅｎｔ）が達成されるように配設されたフローセルの拡大図を示す。

【図22】図２２は、実質的に平面状の支持体内または該支持体上のアレイ状の鋳型の配列を決定するために使用され得るハイスループット自動配列決定機器の概略図を示す。

【図23】図２３は、アライメントの不一致の散布図を示し、３０フレームで最少の不一致を示す。

【図24】図２４Ａ～Ｉは、さまざまな異なる方向から見た本発明フローセルまたはその一部分の概略図を示す。

【図25A】図２５Ａは、２ヌクレオチド長である拘束部分を含む部分拘束プローブを含むプローブファミリーの好ましいコレクションの例示的なコード化を示す。

【図25B】図２５Ｂは、プローブファミリーの好ましいコレクション（上側パネル）およびライゲーション、検出および切断のサイクル（下側パネル）　を示す。

【図26】図２６は、２ヌクレオチド長である拘束部分を含む拘束プローブを含むプローブファミリーの別の好ましいコレクション例示的なコード化を示す。

【図27A】図２７Ａは、表１に示すプローブファミリーの２４個の好ましいコレクションを図式的に規定するための代替法を表す。

【図27B】図２７Ｂは、表１に示すプローブファミリーの２４個の好ましいコレクションを図式的に規定するための代替法を表す。

【図27C】図２７Ｃは、表１に示すプローブファミリーの２４個の好ましいコレクションを図式的に規定するための代替法を表す。

【図28】図２８は、プローブが、２ヌクレオチド長である拘束部分を含むプローブファミリーのやや好ましいコレクションを示す。

【図29A】図２９Ａは、拘束部分３ヌクレオチド長を有するプローブを含むプローブファミリーのコレクションのための拘束部分を作製するために使用され得るダイアグラムを示す。

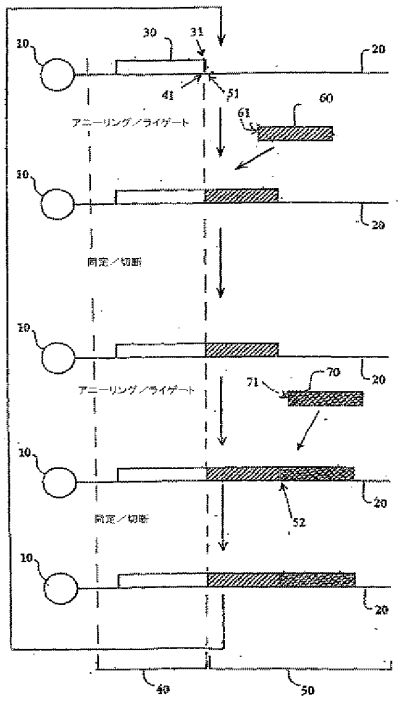
【図29B】図２９Ｂは、拘束部分３ヌクレオチド長を有するプローブを含むプローブファミリーのコレクションのための拘束部分を、プローブファミリーの２４個の好ましいコレクションから作製するために使用され得るマッピングスキームのダイアグラムを示す。

【図30】図３０は、配列決定がプローブファミリーのコレクションを用いて行なわれる方法を示す。好ましい組のプローブファミリーを用いた実施形態を示す。

【図31A】図３１Ａ～３１Ｃは、配列決定が、プローブファミリーの第１のコレクションを用いて候補配列を作製し、プローブファミリーの第２のコレクションを用いてデコード化することで行なわれる方法を示す。
【図31B】図３１Ｂは、配列決定が、プローブファミリーの第１のコレクションを用いて候補配列を作製し、プローブファミリーの第２のコレクションを用いてデコード化することで行なわれる方法を示す。
【図31C】図３１Ｃは、配列決定が、プローブファミリーの第１のコレクションを用いて候補配列を作製し、プローブファミリーの第２のコレクションを用いてデコード化することで行なわれる方法を示す。
【図32】図３２は、配列決定が、プローブファミリーのやや好ましいコレクションを用いて行なわれる方法を示す。
【図33】図３３Ａは、自身にビーズが結合されたスライドの概略図を示す。ＤＮＡ鋳型がビーズに結合されている。図３３Ｂは、スライドに結合されたビーズ集団を示す。下側パネルは、白色光下（左）および蛍光顕微鏡検査でのスライドの同じ領域を示す。上側パネルは、ビーズ密度の範囲を示す。
【図34】図３４Ａ～３４Ｃは、核酸の個々の集団としての核酸断片（鋳型）内に存在する１対のタグの両方のタグを増幅し、これを、増幅プロセスにより微粒子に捕捉するためのスキームを示す。
【図35-1】図３５Ａおよび３５Ｂは、図３５のスキームのプライマー設計および増幅の詳細を示す。明確にするために核酸断片（鋳型）の両方の鎖を示す。同じ配列を有するプライマーおよびプライマー結合領域を同じ色で示す。例えば、Ｐ１を紺色で表し、微粒子上および溶液中に存在するプライマーＰ１が、鋳型の標示した鎖と対応させて着色した部分と同じ配列を有することを示す。Ｐ１と標示した鋳型の紺色領域は、対応するプライマー（Ｐ１）が、実際には他方の鎖の相補的部分に結合し、プライマーＰ１と同じ配列を有するものであっても、プライマー結合領域とよばれ得る。
【図35-2】図３５Ｃおよび３５Ｄは、それぞれ、図３５Ａおよび３５Ｂの方法によって生成された微粒子に結合された第１および第２のタグの配列決定を示す。
【図36】図３６Ａは、対合末端ライブラリーの鋳型分子を示し、ライブラリーのメンバーに共通する、鋳型の前方アダプター部分、後方アダプター部分、および内部アダプター部分にハイブリダイズするブロッキングオリゴヌクレオチドを示している。図の下部には、アダプターとブロッキングオリゴヌクレオチドの例示的な配列が示されている。図３６Ａ～図３６Ｃの「ｄｄＢａｓｅ」とは、ジデオキシヌクレオシドを示す。「固有のＤＮＡ配列」とは、配列決定する標的領域を表す。図３６Ｂは、断片ライブラリーの鋳型分子を示し、ライブラリーのメンバーに共通する、鋳型分子の前方アダプター、後方アダプター、および内部アダプター部分にハイブリダイズするブロッカーオリゴヌクレオチドを示している。図の下部には、アダプターおよび相補性ブロッキングオリゴヌクレオチドの例示的な配列が示されている。図３６Ｃは、鋳型分子にローリングサークル増幅（ＲＣＡ）が行われたライブラリーの分子を示す。ＲＣＡは、鋳型分子の固有部分（２）、ならびにアダプター領域（１）とパドロック領域（３）の複数の複製を作成する。この図は、ライブラリーのメンバーに共通する、鋳型のアダプター部分およびパドロック部分にハイブリダイズするブロッキングオリゴヌクレオチドを示している。
【図37】いくつかのパドロックプローブ配列と、ＲＣＡを使用した鋳型分子の合成後にパドロック領域をブロックするとされるオリゴヌクレオチドの例示的な配列を示す。
【図38】半固形培地を使用せずに基材上に作成される微粒子のアレイ（ゲル非含有微粒子アレイ）を示す。
【図39】ゲル非含有微粒子アレイを使用した、ライゲーションに基づく配列決定の結果を示す。
【図40】表面上に位置する微粒子の概略図を示し、鋳型の伸長によって生じるとされる接触パッチと核酸コロニーの予想されるサイズを例示する。

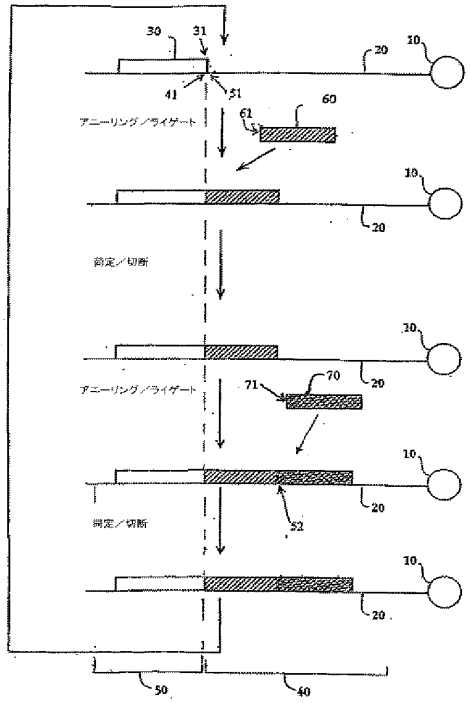
【図1A】

Fig. 1A



【図1B】

Fig. 1B



【図2】

Fig. 2

色	5'					3'
赤色	N	N	N	N	N	C
黄色	N	N	N	N	N	A
緑色	N	N	N	N	N	G
青色	N	N	N	N	N	T

【図3A】

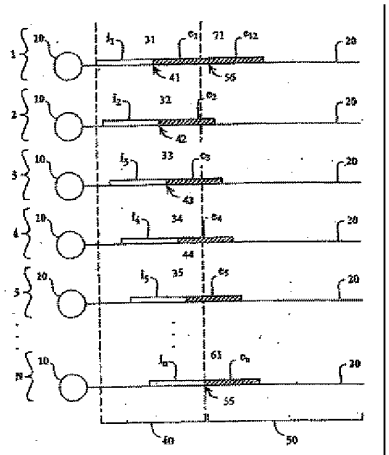


Fig. 3A

【図3B】

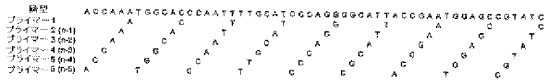


Fig. 3B

【図4A】

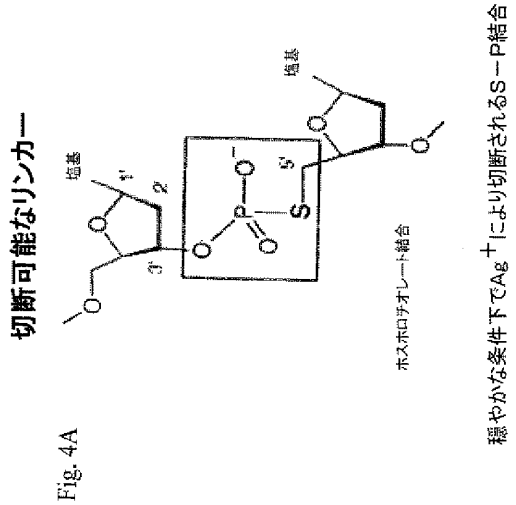


Fig. 4A

【図4B】

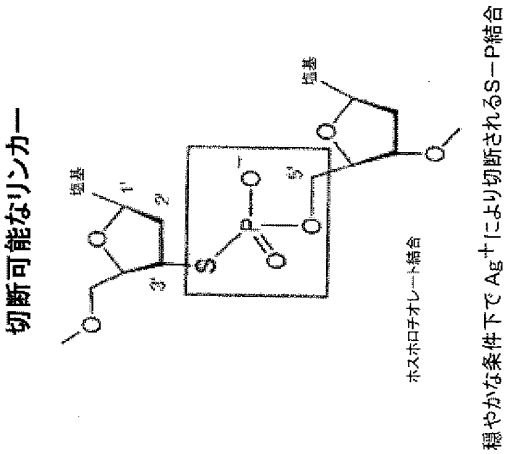
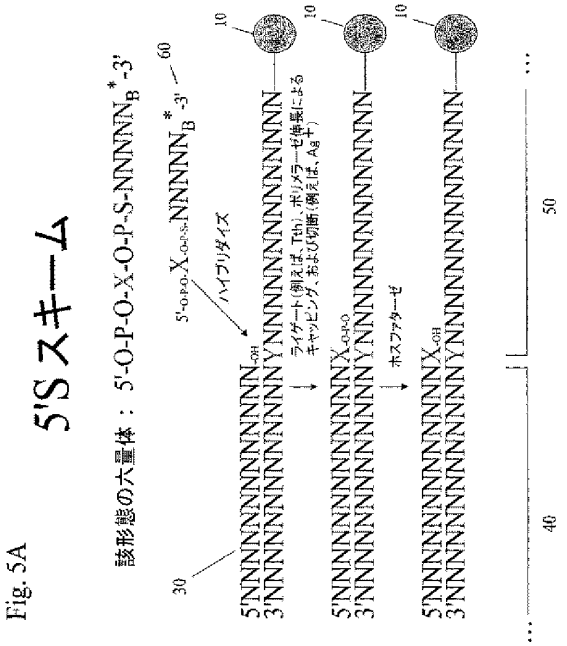


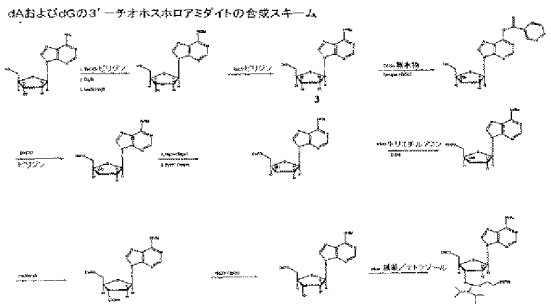
Fig. 4B

【図5A】


5'Sスキーム
該形態の六量体：　5'-O-P-O-X-O-P-S-NNNNNB*-3'
ハイブリダイズ
ライゲート(例えば、Tth)、ポリメラーゼ伸長によるキャッピング、および切断(例えば、Ag+)
ホスファターゼ
5'NNNNN

【図7】

Fig. 7



【図8-1】

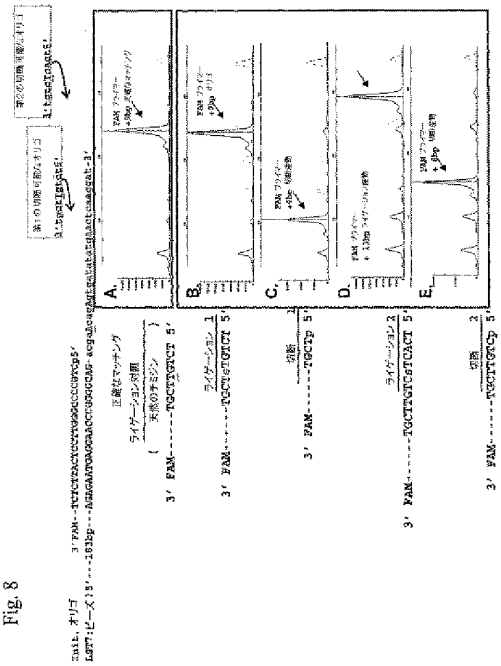
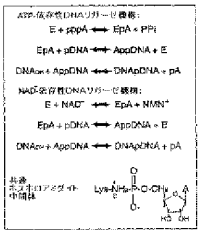


Fig. 8

【図8-2】

Fig. 8F



【図9】

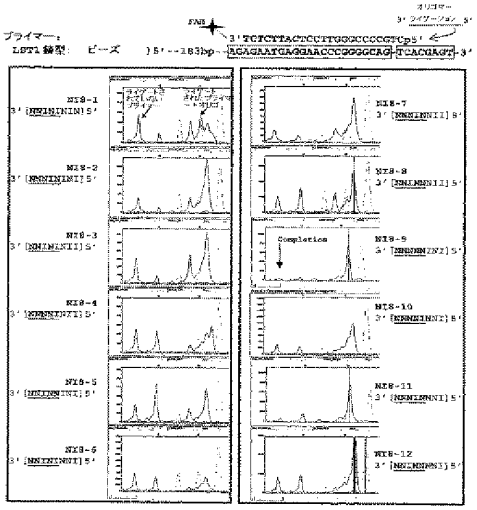


Figure 9

【図10】

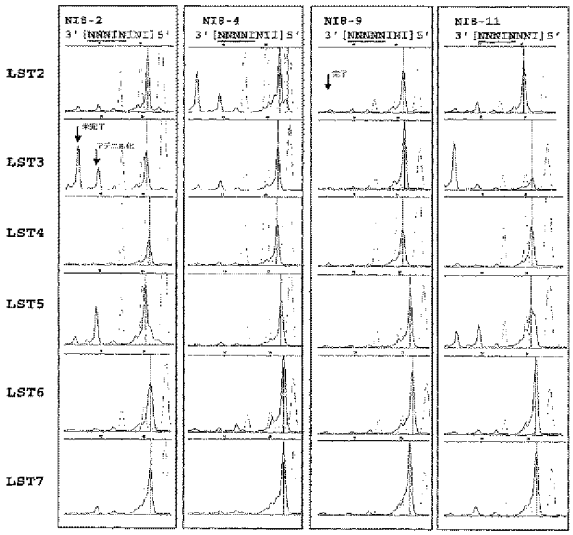


Figure 10

【図11】

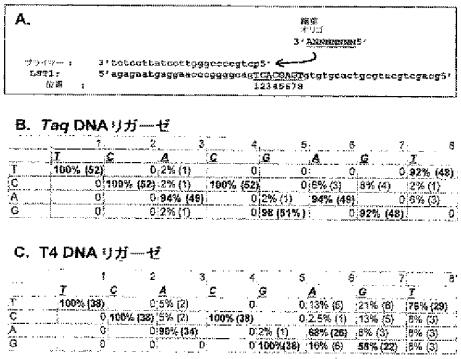


Figure 11

【図12】

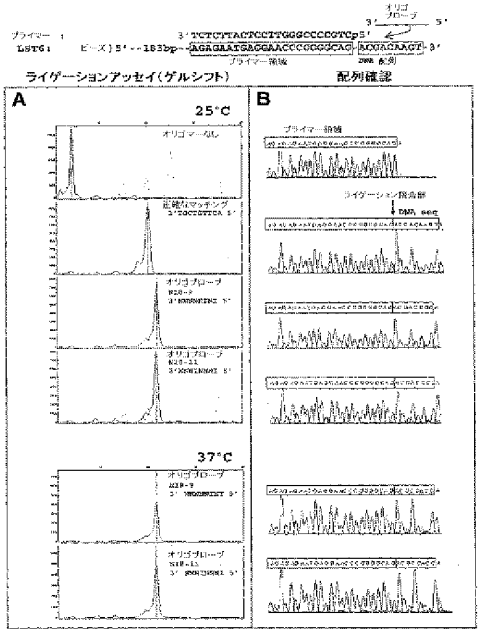


Figure 12

【図13】

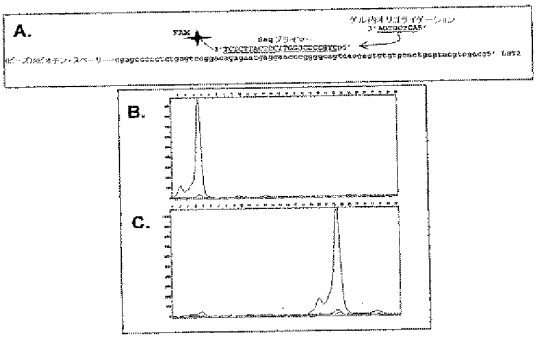


Figure 13

【図14A】

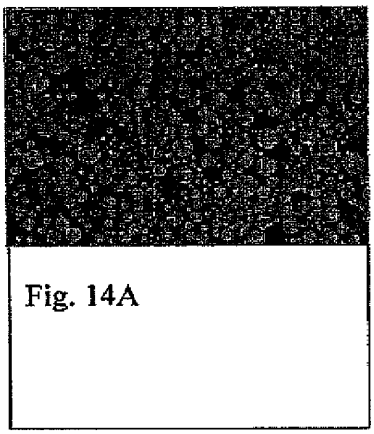


Fig. 14A

【図14B】

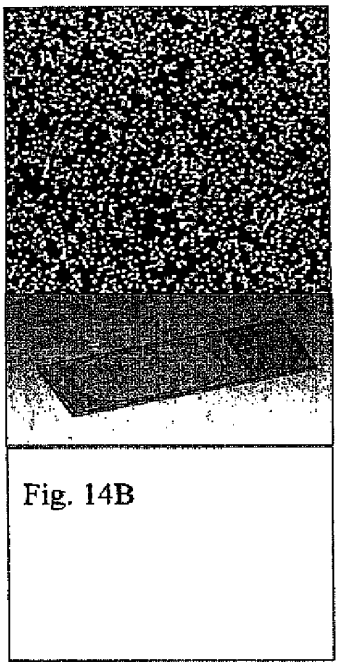
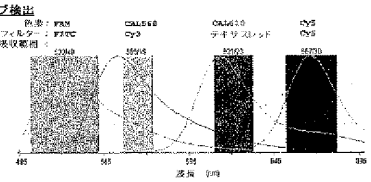


Fig. 14B

【図15】

表1．4色プローブ検出および設計

4色プローブ検出



オリゴヌクレオチドプローブ設計

蛍光色素	色素(フルネ)	Ex*	Em	特性	備考
LST1.A	FAM™ (FITC)	495	520	ASYGCTCA'-5	ANNNNNKE™
LST1.G	Cy5® (Cy5)	643	667	GGYGCTCA™	
LST1.C	CAL610® (TxRd)	590	610	CGYGCTCA™	
LST1.T	CAL560® (Cy3)	540	561	TGYGCTCA™	
LST2	Cy5	643	667	GCCACGAA™	GNBNMNNZ™
LST3	CAL610	590	610	CCACATAT™	CNNNMNNT™
LST4	CAL560	540	561	TAATAGCC™	TNNNMNNI™

* 励起および発光波長 (nm)

Figure 15

【図16】

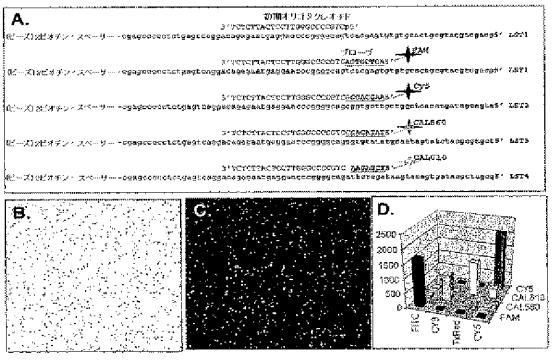


Figure 16

【図17】

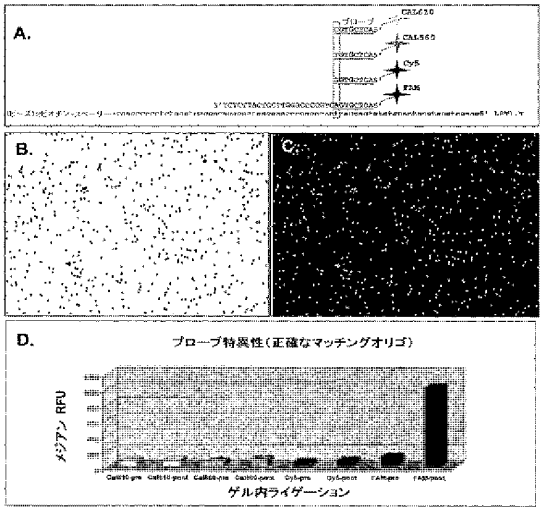
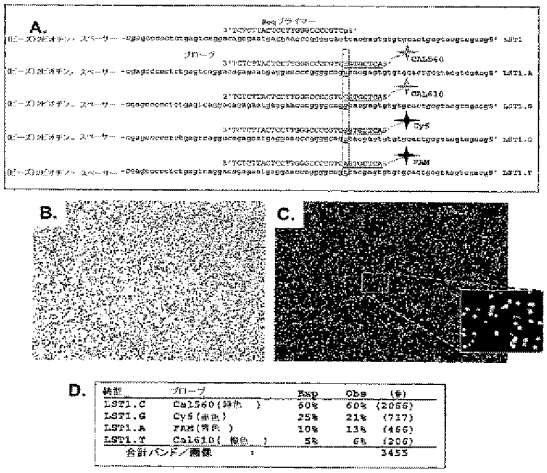


Fig. 17

【図18】

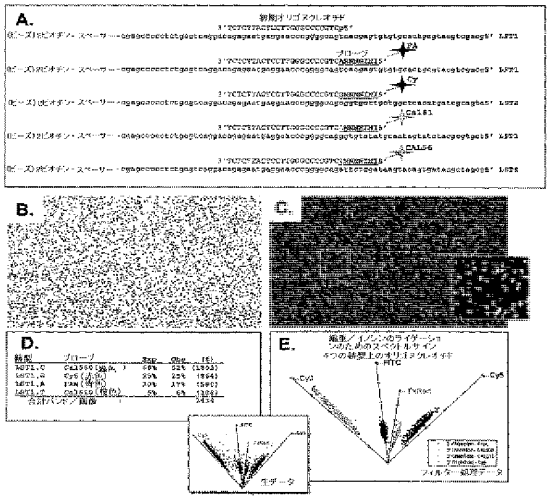
Fig. 18



検出	プローブ	Exp	Obs	(#)
LST1.C	Cal560(緑色)	50%	60%	(2056)
LST1.G	Cy5(赤色)	25%	21%	(717)
LST1.A	FAM(青色)	10%	13%	(466)
LST1.T	Cal610(橙色)	5%	6%	(206)
合計バンド／画像				3455

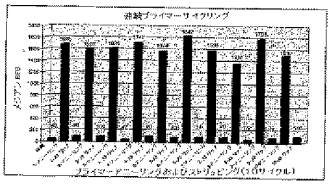
【図19】

Fig. 19



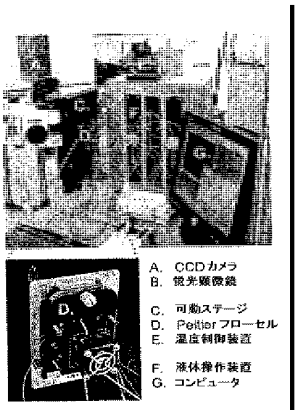
【図20】

Fig. 20



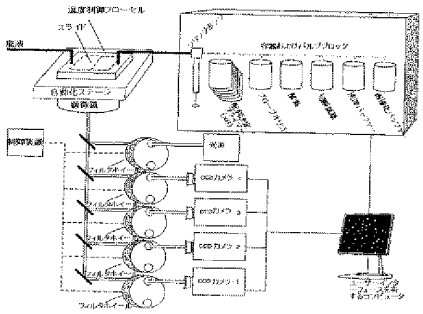
【図21】

Fig. 21



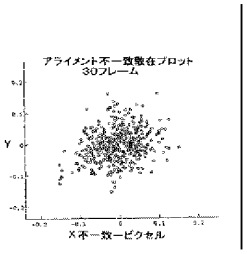
【図22】

Fig. 22



【図23】

Fig. 23



【図24A】

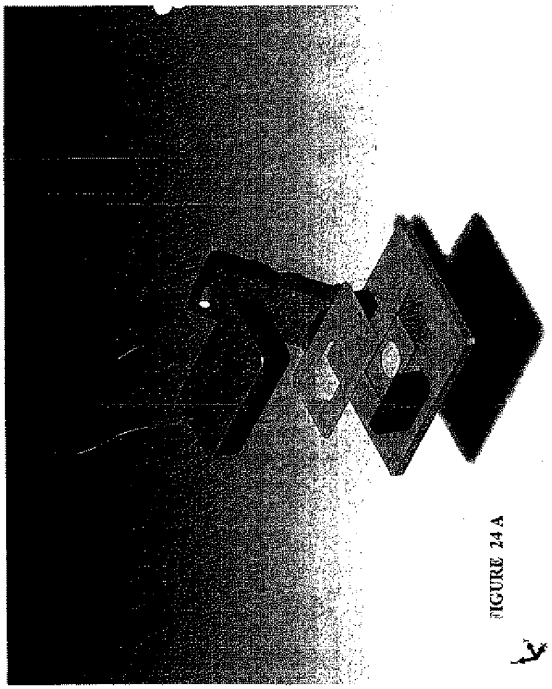


FIGURE 24 A

【図24B】

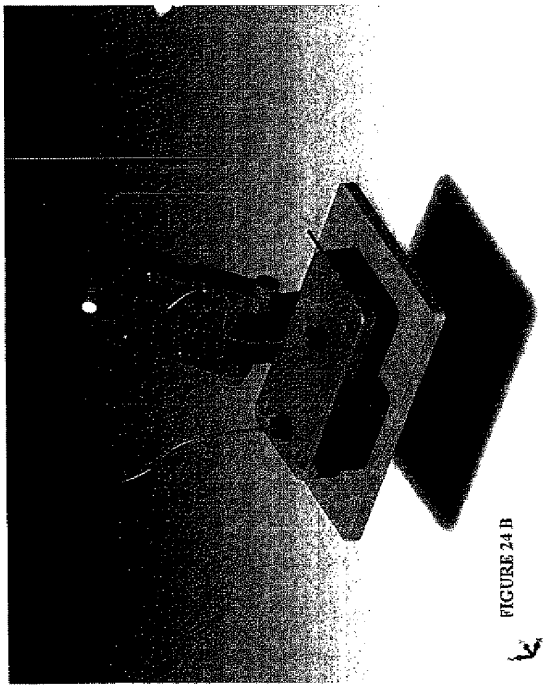


FIGURE 24 B

【図24C】

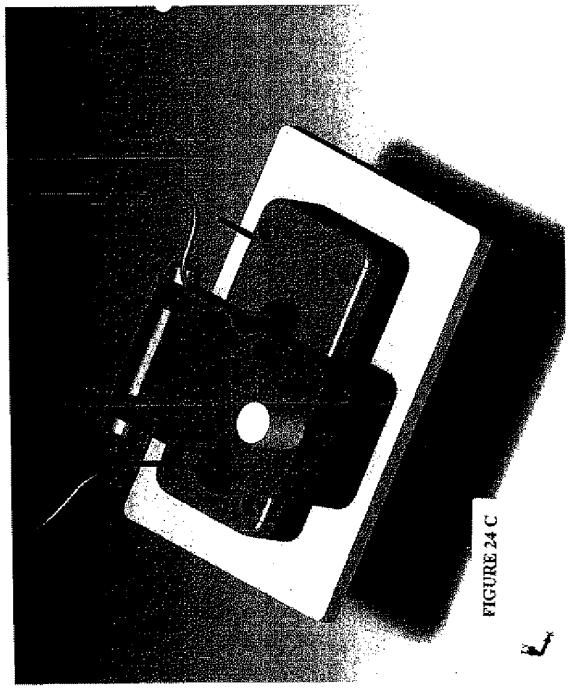


FIGURE 24 C

【図24D】

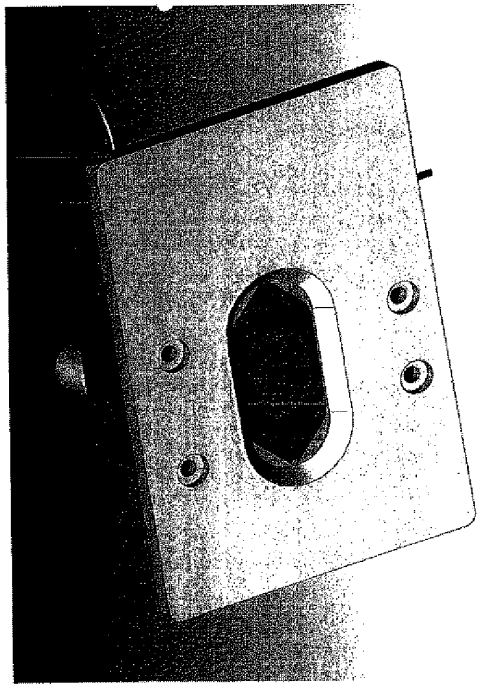


FIGURE 24D

【図24E】

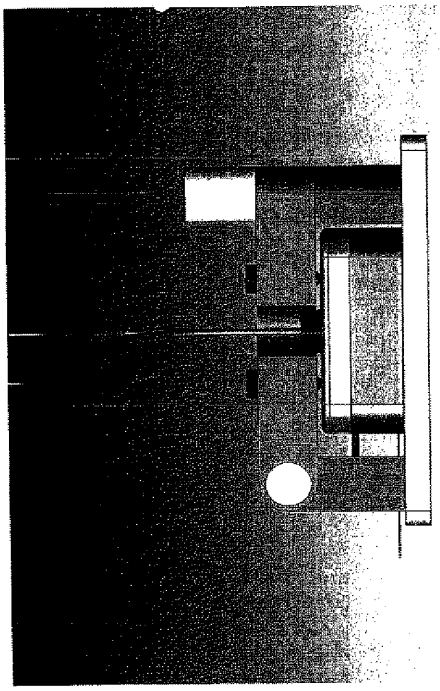


FIGURE 24E

【図24F】

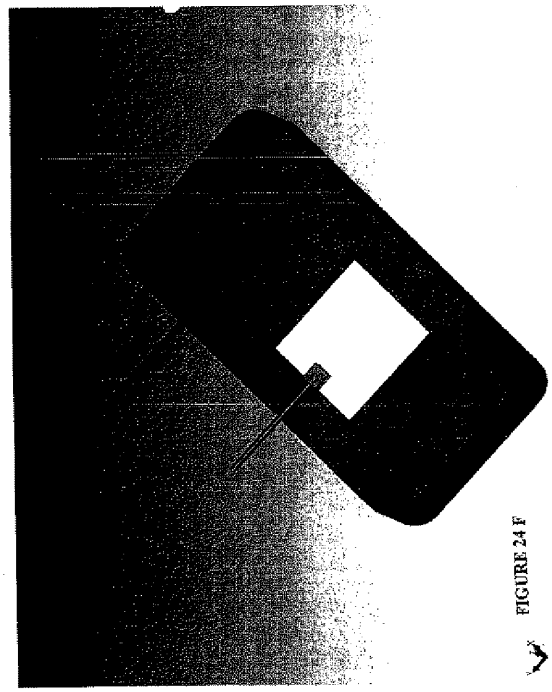


FIGURE 24 F

【図24G】

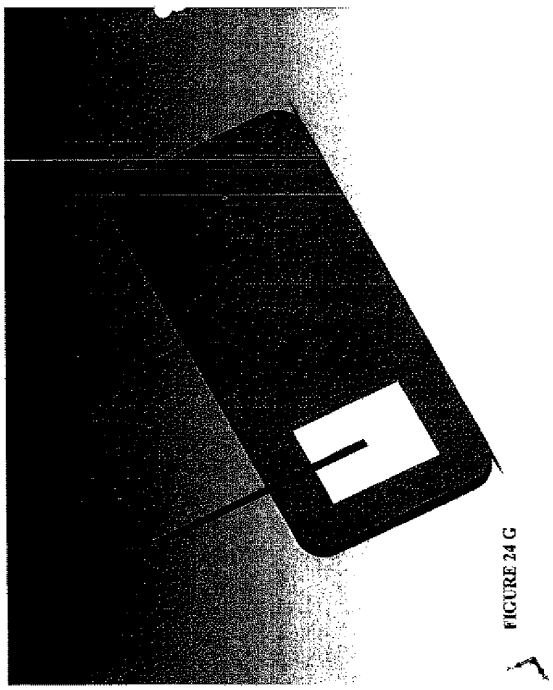
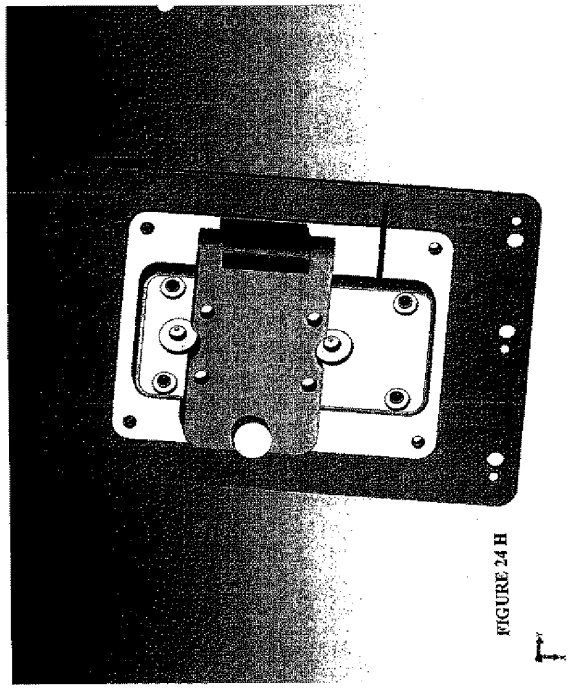
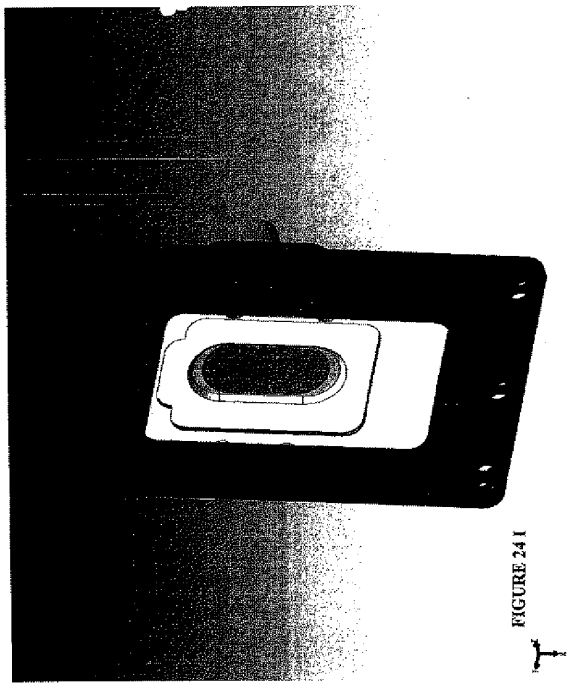


FIGURE 24 G

【図24H】



【図24I】

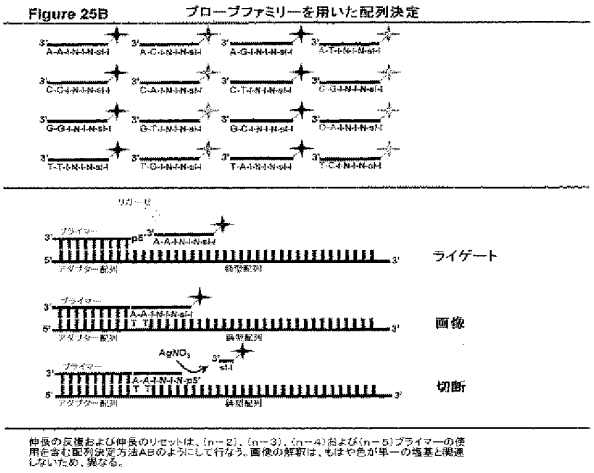


【図25A】

Figure 25A

標識	5'					3'
赤色	N	N	N	N	T	C
赤色	N	N	N	N	G	A
赤色	N	N	N	N	A	G
赤色	N	N	N	N	C	T
黄色	N	N	N	N	C	C
黄色	N	N	N	N	T	A
黄色	N	N	N	N	G	G
黄色	N	N	N	N	A	T
緑色	N	N	N	N	A	C
緑色	N	N	N	N	C	A
緑色	N	N	N	N	T	G
緑色	N	N	N	N	G	T
青色	N	N	N	N	G	C
青色	N	N	N	N	A	A
青色	N	N	N	N	C	G
青色	N	N	N	N	T	T

【図25B】



伸長の反復および伸長のリセットは、(n−2)、(n−3)、(n−4)および(n−5)プライマーの使用を含む配列決定方法ABのようにして行なう。画像の解釈は、もはや色が単一の塩基と関連しないため、異なる。

【図26】

Figure 26

標識	5'					3'
赤色	N	N	N	N	A	A
赤色	N	N	N	N	C	G
赤色	N	N	N	N	G	T
赤色	N	N	N	N	T	C
黄色	N	N	N	N	A	C
黄色	N	N	N	N	C	A
黄色	N	N	N	N	G	G
黄色	N	N	N	N	T	T
緑色	N	N	N	N	A	T
緑色	N	N	N	N	C	C
緑色	N	N	N	N	G	A
緑色	N	N	N	N	T	G
青色	N	N	N	N	A	G
青色	N	N	N	N	C	T
青色	N	N	N	N	G	C
青色	N	N	N	N	A	T

【図27A】

Figure 27A

第1塩基	第2塩基			
	標識1	標識2	標識3	標識4
A	A	C	G	T
C	C	A	T	G
G	G	T	C	A
T	T	G	A	C

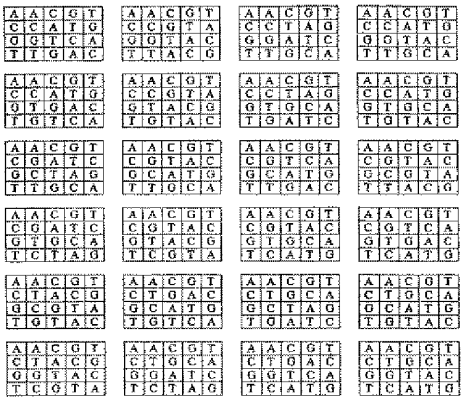
【図27B】

Figure 27B

第1塩基	第2塩基	標識
A	A	1
C	C	1
G	G	1
T	T	1
A	C	2
C	A	2
G	T	2
T	G	2
A	G	3
C	T	3
G	C	3
T	A	3
A	T	4
C	G	4
G	A	4
T	C	4

【図27C】

Figure 27C



【図28】

Figure 28

塩基 1	塩基 2	標識
A	A	1
A	C	1
A	G	3
A	T	3
C	A	2
C	C	2
C	G	4
C	T	4
G	A	1
G	C	1
G	G	3
G	T	3
T	A	2
T	C	2
T	G	4
T	T	4

【図29A】

Figure 29A

	1	2	3	4	
A	A	T	G	C	A
C	C	G	T	A	
G	G	C	A	T	
T	T	A	C	G	
A	C	A	T	G	C
C	A	C	G	T	
G	T	G	C	A	
T	G	T	A	C	
A	G	C	A	T	G
C	T	A	C	G	
G	A	T	G	C	
T	C	G	T	A	
A	T	G	C	A	T
C	G	T	A	C	
G	C	A	T	G	
T	A	C	G	T	

【図29B】

Figure 29B

A	A	C	G	T
C	C	A	T	G
G	G	T	A	C
T	T	G	C	A

	1	2	3	4	
A	A	T	G	C	A
C	C	G	T	A	
G	G	C	A	T	
T	T	A	C	G	
A	C	A	T	G	C
C	A	C	G	T	
G	T	G	C	A	
T	G	T	A	C	
A	G	C	A	T	G
C	T	A	C	G	
G	A	T	G	C	
T	C	G	T	A	
A	T	G	C	A	T
C	G	T	A	C	
G	C	A	T	G	
T	A	C	G	T	

【図30】

Figure 30

観察された標識	配列可能性				
	5	4	3	2	1
黄色				C	C
				T	A
				G	G
				A	T
緑色			A	C	
			C	A	
			T	G	
			G	T	
赤色		T	C		
		G	A		
		A	G		
		C	T		
青色	G	C			
	A	A			
	C	G			
	T	T			
4種類の可能な配列	C	G	A	C	C
	A	A	G	T	A
	G	C	T	G	G
	T	T	C	A	T

【図31A】

Figure 31A

標識	5'					3'
赤色	N	N	N	N	A	A
赤色	N	N	N	N	C	C
赤色	N	N	N	N	G	G
赤色	N	N	N	N	T	T
黄色	N	N	N	N	A	C
黄色	N	N	N	N	C	A
黄色	N	N	N	N	G	T
黄色	N	N	N	N	T	G
緑色	N	N	N	N	A	G
緑色	N	N	N	N	C	T
緑色	N	N	N	N	G	A
緑色	N	N	N	N	T	C
青色	N	N	N	N	A	T
青色	N	N	N	N	C	G
青色	N	N	N	N	G	C
青色	N	N	N	N	T	A

【図31B】

Figure 31B

観察された標識	配列可能性				
	5	4	3	2	1
黄色				**A**	**C**
				C	A
				G	T
				T	G
緑色			A	G	
			C	T	
			G	**A**	
			T	C	
赤色		A	A		
		C	C		
		G	**G**		
		T	T		
青色	A	T			
	C	**G**			
	G	C			
	T	A			
4種類の可能な配列	**C**	**G**	**G**	**A**	**C**
	A	T	T	C	A
	G	C	C	T	G
	T	A	A	G	T

【図31C】

Figure 31C

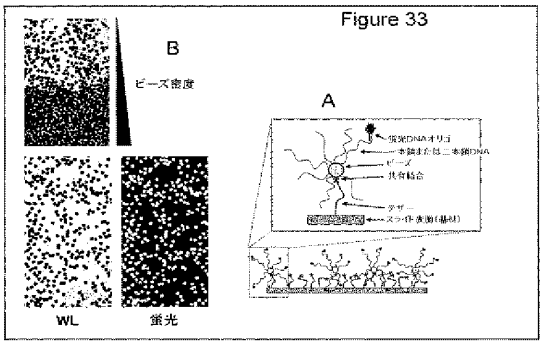
標識	5'					3'
赤色	N	N	N	N	A	A
赤色	N	N	N	N	C	G
赤色	N	N	N	N	G	T
赤色	N	N	N	N	T	C
黄色	N	N	N	N	A	C
黄色	N	N	N	N	C	T
黄色	N	N	N	N	G	G
黄色	N	N	N	N	T	A
緑色	N	N	N	N	A	G
緑色	N	N	N	N	C	A
緑色	N	N	N	N	G	C
緑色	N	N	N	N	T	T
青色	N	N	N	N	A	T
青色	N	N	N	N	C	C
青色	N	N	N	N	G	A
青色	N	N	N	N	T	G

【図32】

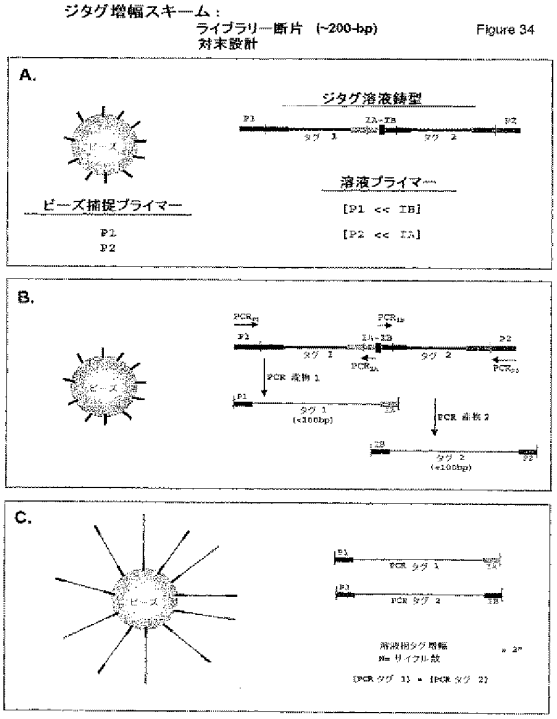
Figure 32

塩基						標識
1	2	3	4	5	6	
A	A					1
A	C					
G	A					
G	C					
	C	A				2
	C	C				
	T	A				
	T	C				
		A	G			3
		A	T			
		G	G			
		G	T			
			C	G		4
			C	T		
			T	G		
			T	T		
				A	A	1
				A	C	
				G	A	
				G	C	
A	C	A	T	G	A	4種類の可能な配列
G	C	A	T	G	A	
A	C	A	T	G	C	
G	C	A	T	G	C	

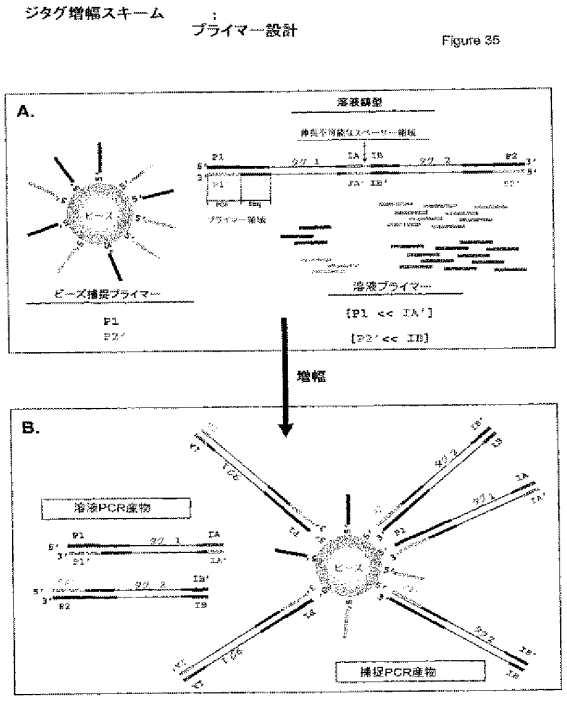
【図33】



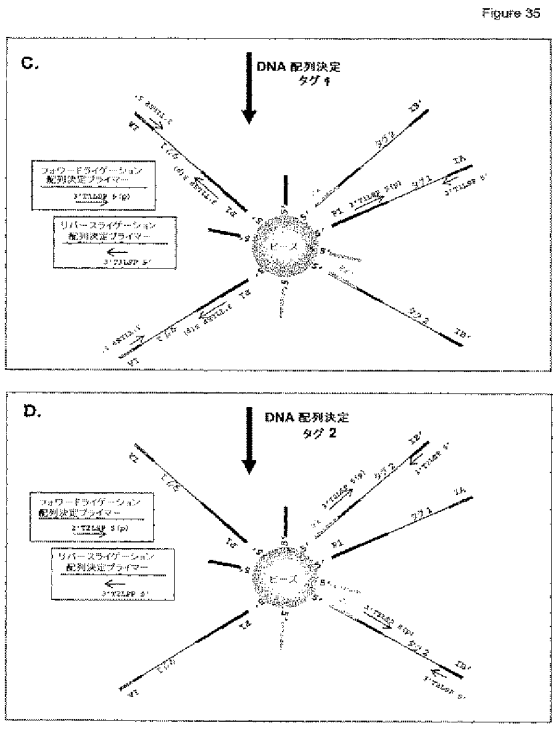
【図34】



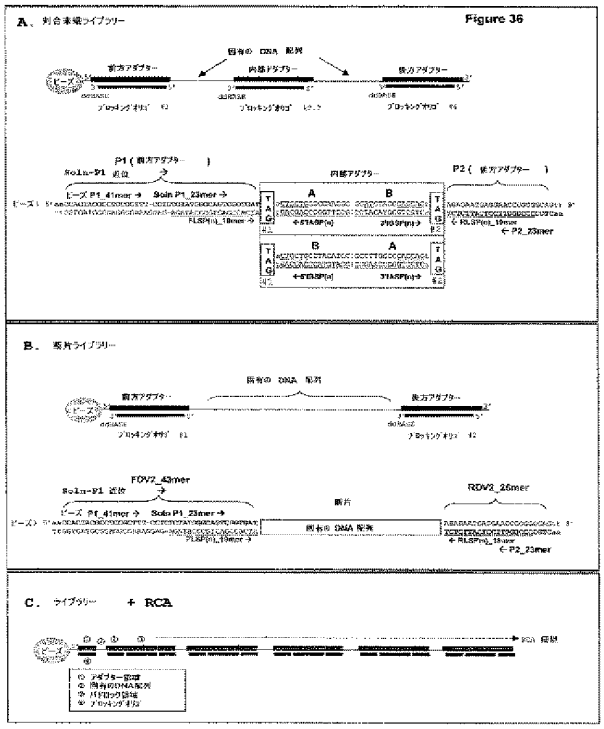
【図35-1】



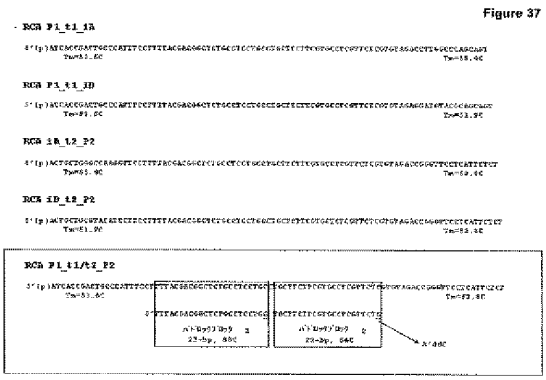
【図35-2】



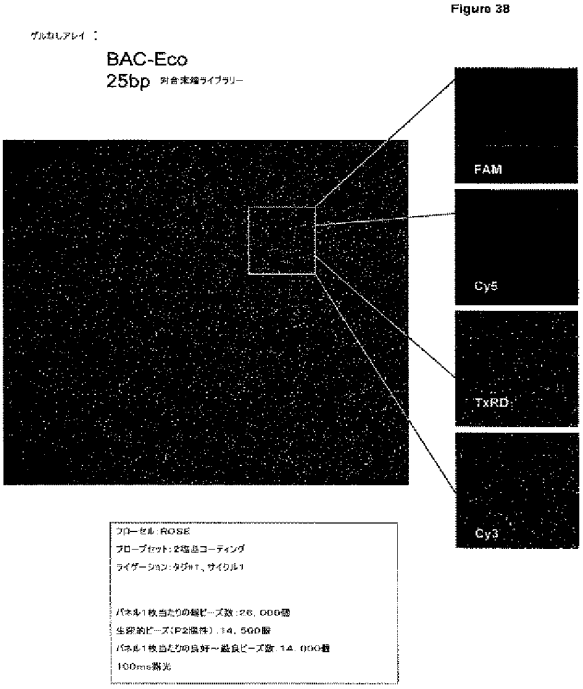
【図36】



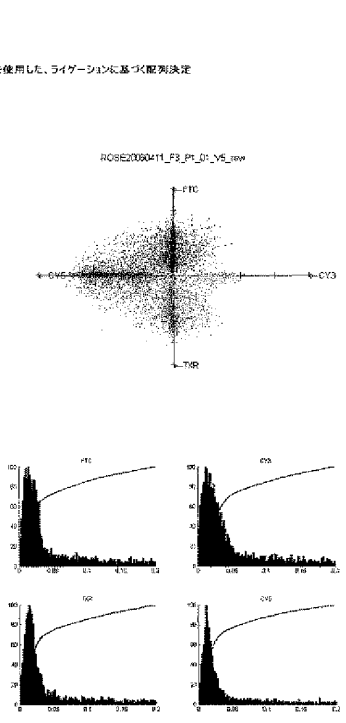
【図37】



【図38】



【図39】



【図40】

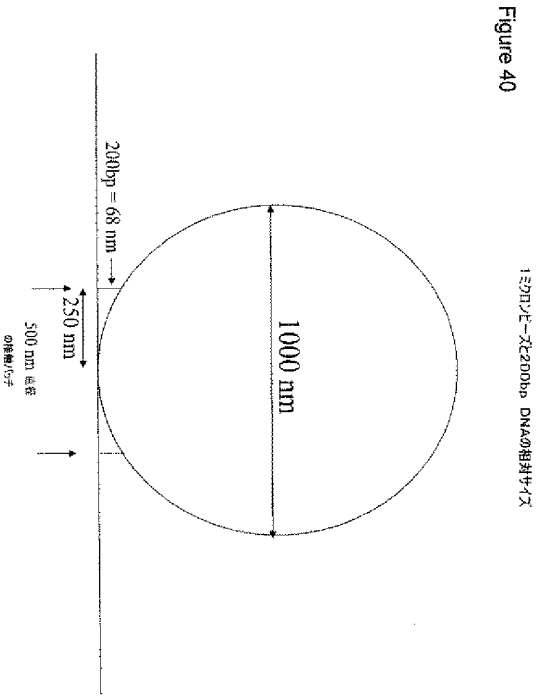


Figure 40

【手続補正書】
【提出日】平成21年1月13日(2009.1.13)
【手続補正1】
【補正対象書類名】明細書
【補正対象項目名】配列表
【補正方法】追加
【補正の内容】
【配列表】
2009538123000001.app
【手続補正書】
【提出日】平成21年1月14日(2009.1.14)
【手続補正1】
【補正対象書類名】明細書
【補正対象項目名】全文
【補正方法】変更
【補正の内容】
【発明の詳細な説明】
【技術分野】
【0001】
政府の支援
本発明は、ＮＩＨによって与えられた助成金番号Ｒ０１－ＨＧ－００３５７０の下、米国政府の支援によって為された。政府は、本発明において一定の権利を有する。
【0002】
（関連出願の引用）
本願は、２００６年４月１９日に出願された米国仮特許出願第６０／７９３，７０２号

（この全内容は、参考として本明細書に援用される）の利益、およびそれに対する優先権を主張する。本願は、２００５年２月１日に出願された米国仮特許出願第６０／６４９，２９４号；２００５年２月２５日に出願された米国仮特許出願第６０／６５６，５９９号；２００５年４月２１日に出願された米国仮特許出願第６０／６７３，７４９号；２００５年７月１５日に出願された米国仮特許出願第６０／６９９，５４１号；２００５年９月３０日に出願された米国仮特許出願第６０／７２２，５２６号；および米国特許出願第１１／３４５，９７９号に関する。これらは全て、本明細書中に参考として援用される。

【0003】

（発明の背景）

核酸配列決定手法は、基礎研究から臨床診断にわたる広範で多様な分野において非常に重要である。かかる手法から入手可能な結果としては、種々の程度の特異性の情報が挙げられ得る。例えば、有用な情報は、ある特定のポリヌクレオチドが参照ポリヌクレオチドと配列が異なるか否かの判定、試料中のある特定のポリヌクレオチド配列の存在の確認、部分配列情報、例えば、ポリヌクレオチド内の１つ以上のヌクレオチドの正体の決定、ポリヌクレオチド内のヌクレオチドの正体および順序の決定などからなるものであり得る。

【0004】

ＤＮＡ鎖は、典型的には、４種類のサブユニット、すなわち、塩基アデニン（Ａ）、シトシン（Ｃ）、グアニン（Ｇ）およびチミジン（Ｔ）を含有するデオキシリボヌクレオチドで構成されるポリマーである。これらのサブユニットは、一方のデオキシリボース基の５’炭素を次の基の３’炭素に連結するホスホジエステル共有結合によって互いに結合されている。ほとんどの天然に存在するＤＮＡは２つのかかる鎖からなり、これらは、逆平行の向きに整列され、相補的塩基間、すなわち、ＡとＴ間およびＧとＣ間で形成される水素結合によって互いに保持されている。

【0005】

ＤＮＡ配列決定は、最初、連鎖停止またはジデオキシヌクレオチド方法（非特許文献１）および化学的分解方法（非特許文献２）の開発によって大きさ規模において可能になり、このうち前者は、最も広く使用され、改良され、自動化されている。特に、蛍光標識された連鎖停止剤の使用が、自動ＤＮＡ配列決定装置の開発において主に重要であった。上記のアプローチの両方に共通するのは、大きさの異なる標識されたＤＮＡ断片の１つ以上のコレクションの作製であり、これは、次いで、該断片の３’末端のヌクレオチドの正体（連鎖停止法において）または該断片から最も新しく除去されたヌクレオチドの正体（化学的分解方法の場合）を決定するために、長さに基づいて分離されなければならない。

【0006】

現在利用可能な配列決定手法は、いくつかの完全ゲノムの配列決定などの主要な目的の達成を可能にするが、これらの手法は、いくつかの不都合点を有し、いくつかの領域において改善の相当な必要性がなお残っている。標識されたＤＮＡ断片の分離は、典型的には、ポリアクリルアミドゲル電気泳動を用いてなされている。しかしながら、この工程は、多くの状況で配列決定のスピードと精度の両方を制限する大きな障害であることが示されている。キャピラリー電気泳動（ＣＡＥ）は、ヒトゲノムプロジェクトの完成を可能にした飛躍的進歩であることが示されたが（非特許文献３）、大きな欠点がなお残っている。例えば、ＣＡＥは、依然として時間のかかる分離工程を必要とし、依然として大きさに基づく区別を伴ない、これらは、不正確となり得る。

【0007】

連鎖停止方法に対するさまざまな代替法が提案されている。多くの場合、「合成による配列決定」とよばれるアプローチの一例では、オリゴヌクレオチドプライマーを、まず標的鋳型にハイブリダイズさせる。次いで、プライマーを、種々に標識したヌクレオチドのポリメラーゼ触媒型付加の連続的サイクルによって伸長させる。成長鎖内へのその組込みを検出する。標識の正体は、鋳型内の相補的ヌクレオチドを同定する機能を果たす。あるいはまた、多数の反応が、各ヌクレオチドを用いて並行して行なわれ得、ある特定のヌクレオチドを使用する反応内への標識されたヌクレオチドの組込みにより、鋳型内の相補的

ヌクレオチドが同定される（例えば、Ｍｅｌａｍｅｄｅ，特許文献１；Ｃｈｅｅｓｅｍａｎ，特許文献２；Ｔｓｉｅｎら，特許文献３；Ｒｏｓｅｎｔｈａｌら，特許文献４；非特許文献４；非特許文献５参照のこと）。

【0008】

任意の有意な長さのポリヌクレオチドを効率的に配列決定するため、ポリメラーゼは、各サイクルにおいて正確に１つのヌクレオチドを組み込むことが望ましい。したがって、一般的に、連鎖停止剤として作用するヌクレオチド使用すること、すなわち、その組込みにより、ポリメラーゼによるさらなる伸長が抑制されることが必要である。組み込まれたヌクレオチドは、次いで、ポリメラーゼが次のヌクレオチドを組み込むことが可能になるように酵素的または化学的のいずれかで修飾されなければならない。連鎖停止剤としての機能を果たし得るが、後続の工程で伸長され得るようにその組込み後に修飾され得るさまざまなヌクレオチドアナログが提案されている。かかる「可逆的停止剤」は、例えば特許文献２；特許文献５～７に記載されている。しかしながら、高効率でポリメラーゼ組み込まれ得る可逆的停止剤を同定することは、おそらく、ヌクレオチドの大きさが小さいことを考慮すると、ヌクレオチドが停止剤としての機能を果たす能力に影響する修飾はまた、成長ポリヌクレオチド鎖内へのその組込みにも影響するという事実のため、困難であることが示されている。

【0009】

他の配列決定アプローチとしては、ピロシーケンスが挙げられ、これは、ＤＮＡ重合中に放出されるピロリン酸塩（ＰＰｉ）の検出に基づくものである（例えば、特許文献８および９参照のこと）。電気泳動による分離の必要性は回避されるが、ピロシーケンスは、今のところその広範な適用可能性を制限している多数の欠点を有する（非特許文献６）。ハイブリダイゼーションによる配列決定もまた、代替法として提案されている（特許文献１０～１２；非特許文献７）が、いくつかの不都合点（例えば、高度に類似した配列間の識別におけるエラーに可能性など）を有する。エクソヌクレアーゼによる単一分子配列決定は、１つの鎖内の塩基ごとの標識、次いで、試料流中の切断された３’末端ヌクレオチドの連続的な検出を伴うものであるが、これは、理論的には、長鎖ＤＮＡ分子の配列を速やかに決定するに非常に強力な方法である（非特許文献８）。しかしながら、この潜在性を実現する前に克服すべき種々の技術的な障害がなお残っている（非特許文献８）。

【0010】

特定の配列異型に基づく診断用試験は、さまざまな種々の疾患のために既に使用されている。ヒトゲノムの配列決定は、治療（予防的治療を含む）が患者の特定のゲノム構成にあつらえられるか、または特定の対立遺伝子もしくは変異の同定に基づいて選択される個別化医療の時代の到来を告げると広く考えられている。ＨＩＶなどの感染性因子の配列バリアントの迅速かる正確な決定の必要性が増大している。したがって、近い将来、正確かつ迅速な配列決定に対する要望が大きく拡大することは明白である。したがって、あらゆる型の改善された配列決定法が必要とされている。

【特許文献1】米国特許第４，８６３，８４９号明細書
【特許文献2】米国特許第５，３０２，５０９号明細書
【特許文献3】国際公開第９１／０６６７８号パンフレット
【特許文献4】国際公開第９３／２１３４０号パンフレット
【特許文献5】米国特許第６，２５５，４７５号明細書
【特許文献6】米国特許第６，３０９，８３６号明細書
【特許文献7】米国特許第６，６１３，５１３号明細書
【特許文献8】米国特許第６，２１０，８９１号明細書
【特許文献9】米国特許第６，２５８，５６８号明細書
【特許文献10】米国特許第５，２０２，２３１号明細書
【特許文献11】国際公開第９９／６０１７０号パンフレット
【特許文献12】国際公開第００／５６９３７号パンフレット
【非特許文献1】Ｓａｎｇｅｒら．，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．７４：５

４６３－５４６７，１９７７
【非特許文献2】Ｍａｘａｍ&Ｇｉｌｂｅｒｔ，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．７４：５６０－５６４，１９７７
【非特許文献3】Ｖｅｎｔｅｒら．，Ｓｃｉｅｎｃｅ，２９１：１３０４－１３５１，２００１；Ｌａｎｄｅｒら．，Ｎａｔｕｒｅ，４０９：８６０－９２１，２００１
【非特許文献4】Ｃａｎａｒｄら，Ｇｅｎｅ，１４８：１－６（１９９４）
【非特許文献5】Ｍｅｔｚｋｅｒら，Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓｅａｒｃｈ，２２：４２５９－４２６７（１９９４）
【非特許文献6】Ｆｒａｎｃａら．，Ｑｕａｒｔｅｒｌｙ Ｒｅｖｉｅｗｓ ｏｆ Ｂｉｏｐｈｙｓｉｃｓ，３５（２）：１６９－２００，２００２
【非特許文献7】Ｄｒｍａｎａｃら．，Ａｄｖａｎｃｅｓ ｉｎ Ｂｉｏｃｈｅｍｉｃａｌ Ｅｎｇｉｎｅｅｒｉｎｇ／Ｂｉｏｔｅｃｈｎｏｌｏｇｙ，７７：７６－１０１，２００２
【非特許文献8】Ｓｔｅｐｈａｎら．，Ｊ Ｂｉｏｔｅｃｈｎｏｌ．，８６：２５５－２６７，２００１

【発明の開示】
【課題を解決するための手段】
【0011】
（発明の要旨）
本発明は、断片分離を行なう必要性を回避する、またある特定の実施形態においては、ポリメラーゼ酵素の使用の必要性を回避する新しい改善された配列決定法を提供する。背景の項目に記載の方法の代替法は、Ｍａｃｅｖｉｃｚ（Ｍａｃｅｖｉｃｚ）の米国特許第５，７４０，３４１号および同第６，３０６，５９７号に記載されている。この方法は、単鎖鋳型に沿った二本鎖伸長の反復サイクルに基づくものである。これらの方法の好ましい実施形態において、ヌクレオチドは各サイクルで同定される。本発明は、これらの方法に対する改善を提供する。該改善により、該方法の効率的な実施が可能になり、ハイスループット配列決定に特に好適である。また、本発明は、単鎖鋳型に沿った二本鎖伸長の反復サイクルを伴うが、各サイクル中での個々のヌクレオチドの同定をなんら伴わない配列決定法を提供する。
【0012】
一態様において、本発明は、単鎖鋳型に沿った二本鎖伸長、標識された伸長プローブのライゲーションおよび標識の検出の連続的サイクルに基づく配列決定のための改善された方法を提供する。一般に、伸長は、初期化（ｉｎｉｔｉａｌｉｚｉｎｇ）オリゴヌクレオチドおよび鋳型によって形成された二本鎖から開始される。初期オリゴヌクレオチドは、オリゴヌクレオチドプローブをその末端にライゲートして伸長された二本鎖を形成させ、次いで、これを、ライゲーションの連続的サイクルによって反復的に伸長させることにより伸長される。各サイクル中、鋳型内の１つ以上のヌクレオチドの正体が、成功裏にライゲートされたオリゴヌクレオチドプローブ上またはこれと会合した標識を同定することにより決定される。新たに付加されたプローブの標識はまた、ライゲーション前、あるいはそれに加えて、ライゲーション後に検出され得る。一般的に、ライゲーション後に標識を検出することが好ましい。
【0013】
好ましい実施形態において、伸長された二本鎖のたった１回の伸長が１サイクルで起こるように、プローブは伸長不可能な部分を、末端位置に（二本鎖の成長核酸鎖にライゲートされたヌクレオチドと反対のプローブ末端に）有する。「伸長不可能な」により、該部分が、修飾のないリガーゼ基質としての機能を果たさないことが意図される。例えば、該部分は、５’リン酸基または３’ヒドロキシル基がない。該部分は、ライゲーションを抑制するブロッキング基が自身に結合されたヌクレオチドであり得る。本発明の好ましい実施形態において、伸長不可能な部分は、ライゲーション後に除去され、伸長可能な末端が生成され、その結果、二本鎖は後続のサイクルでさらに伸長され得る。

【0014】
伸長不可能な部分の除去を可能にするため、本発明のある特定の実施形態において、プローブに、ホスホジエステル結合が実質的に切断されない条件下で切断され得る少なくとも１つのヌクレオシド間結合を含有させる。かかる連結は、本明細書において「切れやすいヌクレオシド間結合」または「切れやすい連結」という。切れやすいヌクレオシド間結合の切断により、伸長不可能な部分が除去され、伸長可能なプローブ末端が再生されるか、または修飾されて伸長可能なプローブ末端を形成する末端残基が残るかのいずれかとなる。切れやすいヌクレオシド間結合は、プローブ内の任意の２つのヌクレオシド間に位置し得る。好ましくは、切れやすい連結は、新たに形成された結合から少なくとも数個のヌクレオチド離れて（すなわち、遠位に）位置する。伸長可能な末端ライゲートされた末端ヌクレオチドと切れやすい連結間の伸長プローブ内のヌクレオチドは、鋳型に完璧にハイブリダイズする必要はない。これらのヌクレオチドは、「スペーサー」としての機能を果たし、鋳型に沿った区間に位置するヌクレオチドの同定を、該区間該の各ヌクレオチドについてサイクルを行なうことなく可能にし得る。
【0015】
切れやすいヌクレオシド間結合および標識は、好ましくは、切れやすいヌクレオシド間結合の切断により、標識された部分内の伸長プローブと成長核酸鎖の一部のままである部分とが分離され、標識された部分の核酸が可能になる（例えば、温度上昇時）ように配置させる。例えば、標識は、伸長プローブの末端ヌクレオチドのライゲートされるヌクレオチドと反対の末端に結合させ得る。あるいはまた、標識は、任意のいくつかのアプローチを用いて除去してもよい。
【0016】
本発明者らは、ホスホジエステル結合内の橋絡酸素原子の１つがイオウ原子で置き換えられたホスホロチオレート結合が、特に好都合な切れやすいヌクレオシド間結合であることを見出した。ホスホロチオレート結合内のイオウ原子は、一方のヌクレオシドの３’炭素または隣接するヌクレオシドの５’炭素のいずれかに結合され得る。
【0017】
上記の方法のある特定の実施形態では、複数の配列決定反応が行なわれる。該反応では、最初のライゲーションが起こる末端が、鋳型に対して異なる位置に位置するように、鋳型の異なる配列にハイブリダイズする初期オリゴヌクレオチドが使用される。例えば、最初のライゲーションが起こる位置は、１個のヌクレオチド増加によって、互いに対してシフトまたは「位相ずれ（ｏｕｔ　ｏｆ　ｐｈａｓｅ）」させ得る。したがって、同じ長さのオリゴヌクレオチドプローブを用いた各伸長サイクル後、同じ相対位相が、異なる鋳型上の初期オリゴヌクレオチドの両末端間に存在する。該反応は、並行して、各々が同じ鋳型コピーを含む独立した区画において、または順次（すなわち、第１の初期オリゴヌクレオチドを用いて配列情報を得た後、伸長された二本鎖を鋳型から除去し、次いで鋳型の異なる配列にハイブリダイズする初期オリゴヌクレオチドを用いてさらなる反応（１つまたは複数）を行なうことにより）行なわれ得る。
【0018】
別の態様において、本発明は、さまざまな核酸操作に有用な溶液を提供する。一実施形態において、本発明は、１．０～３．０％ＳＤＳ、１００～３００ｍＭ　ＮａＣｌおよび５～１５ｍＭ重硫酸ナトリウム（$NaHSO_4$）を水中に含有するか、またはそれらから本質的になる溶液を提供する。該溶液は、約２％ＳＤＳ、約２００ｍＭ　ＮａＣｌおよび約１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を水中に含有するものであるか、または本質的にそれらからなるものであり得る。例えば、一実施形態において、該溶液は、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を水中に含有するものである。別の実施形態において、該溶液は、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含む水から本質的になるものである。ある特定の実施形態において、該溶液は、２．０～３．０、例えば２．５のｐＨを有する。該溶液は、二本鎖核酸、例えば二本鎖ＤＮＡを個々の鎖に分離、すなわち、二本

鎖核酸を変性（融解）させるのに有用である。ある特定の実施形態において、両方の鎖がＤＮＡである。他の実施形態において、両方の鎖がＲＮＡである。他の実施形態において、一方の鎖がＤＮＡであり、他方の鎖はＲＮＡである。他の実施形態において、一方または両方の鎖がＲＮＡおよびＤＮＡの両方を含有する。他の実施形態において、該鎖の一方または両方がＡ、Ｇ、ＣまたはＴ以外の少なくとも１つのヌクレオチドを含有する。ある一部の実施形態において、該鎖の一方または両方が、非天然に存在するヌクレオチドを含有する。また他の実施形態において、該残基の１つ以上がトリガー（ｔｒｉｇｇｅｒ）残基、例えば、無塩基残基または損傷塩基である。ある一部の実施形態において、１つ以上の残基が、普遍（ｕｎｉｖｅｒｓａｌ）塩基を含有する。ある一部の実施形態において、該鎖の一方または両方が切れやすい連結を含有する。

【0019】

二本鎖核酸は、完全な二本鎖または部分二本鎖であり得る。これは、溶液中で遊離状態であり得るか、または一方もしくは両方の鎖が固相もしくは半固相支持体もしくは基材と物理的に会合（例えば、共有結合もしくは非共有結合）　された状態であり得る。特に注目すべきことには、このような溶液中でインキュベートされた二本鎖核酸は、ゲル離層を引き起こし得る（例えば、核酸が、ポリアクリルアミドゲルなどの半固相支持体内に位置するか、これに結合されている場合）か、またはストレプトアビジン（ＳＡ）－ビオチン結合（例えば、核酸が支持体もしくは基材にＳＡ－ビオチン結合によって結合されている場合）などの非共有結合を破壊し得る熱または刺激の強い変性剤の非存在下で効果的に単鎖に分離される。一実施形態において、該溶液は、核酸の一方がビーズにＳＡ－ビオチン結合によって結合されている二本鎖核酸を分離するために使用される。

【0020】

本発明はまた、二本鎖核酸と任意の前述の溶液、例えば、約１．０～３．０％ＳＤＳ、約１００～３００ｍＭ　ＮａＣｌおよび約５～１５ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含有する水溶液、例えば、１．０～３．０％ＳＤＳ、１００～３００ｍＭ　ＮａＣｌおよび５～１５ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含有する水溶液と接触させる工程を含む、二本鎖核酸の鎖を分離する方法を提供する。一実施形態において、該溶液は、約２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）、例えば、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）　を含有するものである。別の実施形態において、該溶液は、２％ＳＤＳ、２００ｍＭ　ＮａＣｌおよび１０ｍＭ重硫酸ナトリウム（$NaHSO_4$）を含む水から本質的になるものである。ある特定の実施形態において、該溶液は、２．０～３．０、例えば２．５のｐＨを有する。ある一部の実施形態において、二本鎖核酸は、溶液中でインキュベートされる。他の実施形態において、二本鎖核酸（好ましくは、支持体または基材に結合されている）を該溶液で洗浄する。ある一部の実施形態において、二本鎖核酸を該溶液と、二本鎖核酸分子の少なくとも１０％が単鎖に分離されるのに充分な時間接触させる。ある一部の実施形態において、二本鎖核酸を該溶液と、二本鎖核酸少なくとも２０％、３０％、４０％、５０％、６０％、７０％、８０％、９０％、９５％、９８％、９９％またはそれ以上が単鎖に分離されるのに充分な時間接触させる。例示的な実施形態において、二本鎖核酸を該溶液と１５秒間～３時間接触させる。別の実施形態において、二本鎖核酸を該溶液と１分間～１時間接触させる。ある特定の実施形態において、二本鎖核酸を該溶液と、約１、２、３、４、５、１０、１５、２０、２５、３０、３５、４０、４５、５０、５５または６０分間接触させる。該方法は、インキュベーション期間後に、該溶液を除去または一部もしくは全部の核酸を該溶液から除去するさらなる工程を含むものであってもよい。

【0021】

該溶液には、いくつかの本明細書に記載した配列決定法の１つ以上の工程における用途が見出され、任意のこれらの方法において使用され得る。例えば、該溶液は、伸長された二本鎖を鋳型から分離するために使用され得る。該溶液は、切れやすい連結を切断して伸長プローブの伸長された二本鎖にもはや結合されていない部分を除去した後に使用され得

る。該溶液はまた、三重鎖核酸の分離、または単一の核酸鎖の互いににハイブリダイズした自己相補性部分を含有する二本鎖領域の分離に有用である。

【0022】

別の態様において、本発明は、少なくとも２つの識別可能に標識されたオリゴヌクレオチドプローブファミリーのコレクションを用いて、配列に関する情報を得るための方法を提供する。該プローブファミリー内のプローブは、非拘束部分および拘束部分を含有する。上記の方法のように、伸長は、初期オリゴヌクレオチドおよび鋳型によって形成された二本鎖から開始される。初期オリゴヌクレオチドは、オリゴヌクレオチドプローブをその末端にライゲートして伸長された二本鎖を形成させ、次いで、これを、ライゲーションの連続的サイクルによって反復的に伸長させることにより伸長される。伸長された二本鎖のたった１回の伸長が１サイクルで起こるように、プローブは伸長不可能な部分を、末端位置に（二本鎖の成長核酸鎖にライゲートされたヌクレオチドと反対のプローブ末端に）有する。各サイクル中、成功裏にライゲートされたプローブ上の、またはこれと会合させた標識が検出され、伸長不可能な部分が除去または修飾されて、伸長可能な末端が生成する。標識は、該プローブが属するプローブファミリーに対応する。

【0023】

伸長、ライゲーションおよび検出の連続的サイクルにより、連続的な成功裏にライゲートされたプローブが属するプローブファミリーの序列リスト（ｏｒｄｅｒｅｄ　ｌｉｓｔ）がもたらされる。プローブファミリーの序列リストは、配列に関する情報を得るために使用される。しかしながら、新たにライゲートされたプローブがどのプローブファミリーに属するのかがわかることは、それ自体では、鋳型内のヌクレオチドの正体を決定するのに充分でない。それどころか、新たにライゲートされたプローブがどのプローブファミリーに属するのかがわかることは、ある特定の配列がプローブの拘束部分の配列である可能性を排除し、各位置におけるヌクレオチドの正体の少なくとも２つの可能性を残す。したがって、新たにライゲートされたプローブの拘束部分内のヌクレオチド（すなわち、プローブの拘束部分内のヌクレオチドに相補的なヌクレオチド）と反対の位置に位置する鋳型内のヌクレオチドの正体には少なくとも２つの可能性がある。

【0024】

ある特定の実施形態において、所望回数サイクルを行なった後、該一連の序列のプローブファミリー正体を用いて１組の候補配列を作製する。この組の候補配列により、目的を達成するのに充分な情報が提供され得る。本発明の好ましい実施形態において、候補配列の中から正確な配列を選択するため、１つ以上のさらなる工程を行なう。例えば、該配列が既知配列のデータベースと比較され得、データベース内の該配列の１つに最も近い候補配列を正確な配列として選択する。他の実施形態において、異なるようにコード化された組のプローブファミリー用い、鋳型を、伸長、ライゲーション、検出および切断の連続的サイクルによるさらなる回の配列決定に供し、２回目で得られた情報を用いて正確な配列を選択する。他の実施形態において、少なくとも１つの情報項目を、プローブファミリー正体の序列リストから得られた情報と組み合わせ、配列を決定する。

【0025】

本発明はまた、プローブファミリーを用いて鋳型を配列決定する場合のエラーチェックを行なう方法を提供する。該方法のある特定のものは、一塩基多型（ＳＮＰ）と配列決定エラーを識別する。

【0026】

本発明はまた、目的の少なくとも２つのセグメント（例えば、少なくとも２つのタグ）および少なくとも３つのプライマー結合領域（ＰＢＲ）を、各々が目的のセグメントに対応する少なくとも２つの異なる鋳型が各断片から増幅され得るように含有する核酸断片（例えば、ＤＮＡ断片）を提供する。「プライマー結合領域」は、オリゴヌクレオチドが、該オリゴヌクレオチドが増幅プライマー、配列決定プライマー、初期オリゴヌクレオチドなどとしての機能を果たし得るようにハイブリダイズし得る核酸の一部分である。したがって、プライマー結合領域は、適当な相補的オリゴヌクレオチドの選択を可能にするため

、既知配列を有するものであるのがよい。本明細書および図面で用いる場合、本発明の方法に用いられる核酸鎖の一部分は、該方法の実施において、プライマーが実際に、該領域に結合するか、核酸鎖の相補鎖の対応する部分に結合するかに関係なく、プライマー結合領域とよばれ得る。したがって、核酸の一部分は、本発明の方法に用いられる場合、プライマーが実際に、該領域に結合するのか（この場合、プライマーの配列は、該領域のものに相補的または実質的に相補的である）、または該領域の相補的部分に結合するのか（この場合、プライマーの配列は、プライマー結合領域の配列と同一または実質的に同一である）とは無関係にプライマー結合領域とよばれ得る。目的のセグメントは、その配列情報が所望される核酸の任意のセグメントである。例えば、目的の配列は、タグであり得、本発明の開示の目的のためには、目的のセグメントがタグである（本明細書および他で「末端タグ」とも称する）ことが想定される。しかしながら、本発明は、目的のセグメントがタグであることに限定されないことを理解されたい。ある特定の実施形態において、少なくとも２つのタグは１対のタグである。該核酸断片は、１対以上のタグ、例えば、１つ以上の対のタグ、例えば、２、３、４、５対以上の対のタグを含有するものであり得る。本発明は、さらに、かかる核酸断片を含有するライブラリー、ならびに該鋳型およびライブラリーの作製方法を提供する。

【0027】

本発明は、さらに、自身に結合された核酸の少なくとも２つの異なる集団を有する微粒子（例えば、ビーズ）であって、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、該集団は、単一の核酸断片からの増幅（例えば、ＰＣＲ増幅）によって生成されたものである微粒子（例えば、ビーズ）を提供する。ある一部の実施形態において、単一の核酸断片は、１対のタグである５’タグおよび３’タグを含有する。単一の核酸断片が対である５’タグおよび３’タグを含有するある一部の実施形態において、微粒子に結合された核酸の集団の１つが５’タグの少なくとも一部分を含み、微粒子に結合された核酸の集団の１つが３’タグの少なくとも一部分を含む。好ましい実施形態において、該集団の１つが完全な５’タグを含み、該集団の１つが完全な３’タグを含む。

【0028】

該核酸断片は多数のＰＢＲを含有するものであり、その少なくとも１つがタグ間に位置し、その少なくとも２つが、該核酸断片の該タグを含有する一部分と隣接し、その結果、５’タグの少なくとも一部分を含む領域が増幅され得、３’タグの少なくとも一部分を含む領域が増幅され得、２つの異なる核酸集団がもたらされる。好ましい実施形態において、５’タグ全体および３’タグ全体が増幅され得る。例えば、該核酸断片は、５’タグと隣接する第１および第２のプライマー結合部位、さらに３’タグと隣接する第３および第４のプライマー結合部位を含有するものであり得る。第１および第２のプライマー結合部位に結合するプライマーを用いるＰＣＲ増幅により、５’タグが増幅される。第３および第４のプライマー結合部位に結合するプライマーを用いるＰＣＲ増幅により、３’タグが増幅される。プライマーは、各プライマーからの伸長により、増幅されるタグを含有するＤＮＡ断片の領域に向かって進行するように選択されるのがよいことは認識されよう。あるいはまた、第１のプライマー結合部位を一方のタグの上流に配置させ得、第２のプライマー結合部位を他方のタグの下流に配置させ得、第３のプライマー結合部位を、この２つのタグ間に配置させ得る。第３のプライマー結合部位は、一方のタグを増幅させるＰＣＲ増幅のためのフォワードプライマーの結合部位としての機能を果たし、他方のタグを増幅させるＰＣＲ増幅のためのリバースプライマー結合部位としての機能を果たす。したがって、一実施形態において、本発明は、自身に結合された核酸の少なくとも２つの異なる集団を有する微粒子（例えば、ビーズ）であって、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、第１の異なる集団が５’タグを含み、第２の異なる集団が３’タグを含む微粒子（例えば、ビーズ）を提供する。

【0029】

本発明は、さらに、個々の微粒子が自身に結合された核酸の少なくとも２つの異なる集団を有し、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、該集団

が単一のＤＮＡ断片からの増幅（例えば、ＰＣＲ増幅）によって生成されたものである微粒子（例えば、ビーズ）の集団を提供する。実質的に同一の集団は、例えば、５’タグと３’タグであり得る。本発明は、さらに、かかる微粒子のアレイ、および実質的に同一の核酸の集団の配列決定を伴う配列決定法を提供する。例えば、一実施形態において、個々の微粒子に結合された２つの実質的に同一の核酸の集団の各々が異なるプライマー結合領域（ＰＢＲ）を含み、その結果、異なる配列決定プライマーを用いることにより、一方の集団は、他方の集団から障害されることなく配列決定され得る。実質的に同一の核酸の２つより多くの実質的に同一の集団が単一の微粒子に結合されている場合、該集団の各々が特有のＰＢＲ有し得、その結果、所与のＰＢＲに結合するプライマーは、微粒子に結合された核酸の他方の実質的に同一の集団内に存在するＰＢＲに結合しない。したがって、本発明の方法は、自身に結合された核酸の少なくとも２つの異なる実質的に同一の集団（例えば、５’タグを含有する多コピーの鋳型と３’タグを含有する多コピーの鋳型）を有する微粒子であって、タグが対のタグである微粒子の作製を可能にする。本発明の方法によれば、鋳型は、配列決定プライマーの結合部位を提供する異なるＰＢＲを含むものである。したがって、５’タグを含有する鋳型内のＰＢＲに相補的な配列決定プライマーを選択することにより、配列情報は５’タグから、３’タグを含有する鋳型もまた同じ微粒子上に存在していても３’タグを含有する鋳型から障害されることなく得られ得る。３’タグを含有する鋳型内のＰＢＲに相補的な配列決定プライマーを選択することにより、配列情報は３’タグから、５’タグを含有する鋳型もまた同じ微粒子上に存在していても５’タグを含有する鋳型から障害されることなく得られ得る。対のタグの両方が同じ微粒子上に存在するという事実は、先行技術において単一の鋳型内に存在した場合に起こり得るように、５’および３’の対のタグの配列が互いに会合し得ることを意味する。

【0030】

基材に付着した微粒子のアレイも提供される。一実施形態において、微粒子は、一方の末端が微粒子に付着し、他方の末端が基材に付着した一本鎖鋳型によって基材に繋がれる。一端または両端における付着手段は、共有結合的である場合もあれば、非共有結合的である場合もある。ある実施形態において、一端または両端の付着手段は、ビオチン結合部分およびビオチンを含む。

【0031】

また、微粒子に付着した鋳型を複製し、場合によりその複製した鋳型を増幅させることによって作成される核酸コロニーを含むアレイも提供される。さらにまた、ブロッキングオリゴヌクレオチドとその使用方法、ならびにブロッキングオリゴヌクレオチドを含む組成物も提供される。

【0032】

本発明はまた、例えば、実質的に平面状の支持体内または該支持体上のアレイ状の鋳型を配列決定するために使用され得る自動配列決定システムを提供する。本発明は、さらに、コンピュータ可読媒体、例えば、ハードディスク、ＣＤ、ジップディスク、フュラッシュメモリーなど上に保存され得る画像処理方法を提供する。ある特定の実施形態において、該システムは、１秒当たり４０，０００個のヌクレオチド以上の同定を達成する。ある特定の好ましい実施形態において、該システムは、１日（２４時間）あたり、８．６ギガバイト（Ｇｂ）以上の配列データをもたらす。ある特定の好ましい実施形態において、該システムは、１日あたり、４８Ｇｂ以上の配列情報（ヌクレオチド同定）をもたらす。

【0033】

また、本発明は、本発明の配列決定法を適用することによってもたらされる情報を保存するコンピュータ可読媒体を提供する。情報は、データベースに保存してもよい。

【0034】

本出願書類において、種々の特許、特許出願、学術論文および他の刊行物に言及するが、これらはすべて、引用により本明細書に組み込まれる。また、以下の標準的な参考文献の研究：Ｃｕｒｒｅｎｔ　Ｐｒｏｔｏｃｏｌｓ　ｉｎ　Ｍｏｌｅｃｕｌａｒ　Ｂｉｏｌｏｇｙ，　Ｊｏｈｎ　Ｗｉｌｅｙ　＆　Ｓｏｎｓ，　Ｎ．Ｙ．，編集２００２年7月；Ｓａ

ｍｂｒｏｏｋ，　ＲｕｓｓｅｌｌおよびＳａｍｂｒｏｏｋ，　Ｍｏｌｅｃｕｌａｒ　Ｃｌｏｎｉｎｇ：Ａ　Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ，第３版，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ　Ｌａｂｏｒａｔｏｒｙ　Ｐｒｅｓｓ，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ，２００１は、引用により本明細書に組み込まれる。本明細書と引用により組み込まれる任意の文献とで矛盾する場合は、本明細書が支配するものとし、本発明者らの裁量に矛盾または不一致が存在するか否かの決定は、任意の時点で行なわれ得ることを理解されたい。

【0035】

図１～３５の多くは、米国特許出願第１１／３４５，９７９号ではカラーとなっており、これらの図が参考として本明細書で援用され、本明細書に示す図の代わりに使用できることに留意されたい。

【発明を実施するための最良の形態】

【0036】

定義

本発明の記載の理解を助長するために、以下に定義を示す。一般に、特に定義のない用語は、当該技術分野で一般的に認められている意味（１つまたは複数）を示すものとすることを理解されたい。

【0037】

本明細書で用いる場合、「無塩基残基」は、窒素含有塩基の除去または窒素含有塩基の充分な部分の除去後に残留し、得られた分子が、もはや、ヌクレオシドまたはヌクレオチドに特徴的な水素結合に関与しないようなヌクレオシドまたはヌクレオチドの一部分の構造を有する残基である。無塩基残基は、窒素含有塩基をヌクレオシドまたはヌクレオチドから除去することにより生成させ得る。しかしながら、用語「無塩基」は、該残基の構造的特徴をいうために使用され、残基が生成される様式とは無関係である。用語「無塩基残基」および「無塩基部位」は、本明細書において、プリンまたはピリミジン塩基がない核酸内の残基をいうために使用される。

【0038】

「無プリン（ａｐｕｒｉｎｉｃ）／無ピリミジン（ａｐｙｒｉｍｉｄｉｎｉｃ）（ＡＰ）エンドヌクレアーゼ」は、本明細書で用いる場合、ポリヌクレオチド内の無塩基残基の５’側、３’側または５’および３’側両方いずれかの結合を切断する酵素をいう。本発明のある特定の実施形態において、ＡＰエンドヌクレアーゼは、ＡＰリアーゼである。ＡＰエンドヌクレアーゼの例としては、限定されないが、大腸菌エンドヌクレアーゼＶＩＩＩおよびその類縁体ならびに大腸菌エンドヌクレアーゼＩＩＩおよびその類縁体が挙げられる。特定の酵素、例えば、大腸菌Ｅｎｄｏ　ＶＩＩＩ、Ｅｎｄｏ　Ｖなどのエンドヌクレアーゼに対する言及は、当該技術分野において類縁体であると、および損傷塩基の除去および／または無塩基残基もしくは他のトリガー残基を含有するＤＮＡの切断に関して類似した生化学的活性を有すると認められる他の種由来の類縁体を包含することが意図されることを理解されたい。

【0039】

本明細書で用いる場合、用語「アレイ」は、支持体マトリックス全体または該マトリックス内に分布された正体のコレクションをいい、好ましくは、個々の正体は、任意のさまざまな手法によってアレイの個別の特徴の同定を可能にするのに充分な距離で互いに間隔があいている。正体は、例えば、核酸分子、核酸分子のクローン性の集団、微粒子（任意選択で、自身に結合された核酸分子のクローン性の集団を有する）などであり得る。動詞で用いる場合、用語「アレイ」およびその変形体は、アレイを形成するため、例えば、正体を支持体マトリックス全体または該マトリックス内に分布させるための任意のプロセスをいう。

【0040】

「損傷塩基」は、Ａ、Ｇ、ＣまたはＴがＤＮＡグリコシラーゼによるＤＮＡからの除去のための基質となるような様式で異なるプリンまたはピリミジン塩基である。ウラシルは

、本発明の目的のために損傷塩基とみなす。本発明のある一部の実施形態において、損傷塩基はヒポキサンチンである。

【0041】

ポリヌクレオチド集団うちの１つのポリヌクレオチド内の位置に関する「縮重している」は、該位置を占めるヌクレオシドの一部を構成する塩基の正体が、該集団の種々のメンバー間で異なることを意味する。したがって、該集団は、その配列が縮重位置において異なる個々のメンバーを含む。用語「位置」は、一般的に、５’または３’末端に関してポリヌクレオチド内の各ヌクレオシドに割り当てられた数値をいう。例えば、伸長プローブの３’末端のヌクレオシドには１位が割り当てられ得る。したがって、構造３’－ＸＸＸＮＸＸＸＸ－５’の伸長プローブのプールにおいて、Ｎは４位である。４位は、該プールの異なるメンバーにおいてＮの正体が異なり得る場合、縮重しているとみなされる。また、該伸長プローブのプールはＮ位が縮重していると言える。位置は、任意のｋ個の異なる正体を有するヌクレオシドによって占められ得る場合、ｋ重に縮重していると言える。例えば、２つの異なる塩基のいずれかを含むヌクレオシドによって占められ得る位置は、２重に縮重している。

【0042】

「配列に関する情報を調べること」は「配列決定」を包含し、また、他のレベルの情報、例えば、配列に関する１つ以上の可能性の排除なども包含する。ポリヌクレオチドにおいて配列決定を行なうことにより、典型的には、完璧に相補的（１００％相補的）なポリヌクレオチドの配列に関して等価な情報がもたらされる。したがって、完璧に相補的なポリヌクレオチドにおいて直接行なわれる配列決定と等価である。

【0043】

複数の要素、例えば、オリゴヌクレオチドプローブ分子またはその部分内のヌクレオシドに関する「独立して」は、各要素の正体が、任意の他方の要素の正体を制限しないこと、およびそれらに制限されないことを意味し、例えば、各要素の正体は、任意の他方の要素（１つまたは複数）の正体とは無関係に選択される。したがって、１つ以上の要素の正体がわかることは、任意のその他の要素の正体に関する任意の情報を提供することにはならない。例えば、配列ＮＮＮＮ内のヌクレオシドは、各Ｎの正体が任意の他のＮの正体とは無関係にＡ、Ｇ、ＣまたはＴであり得る場合、独立している。

【0044】

「ライゲーション」は、鋳型駆動型反応において、２つ以上の核酸、例えば、オリゴヌクレオチドおよび／またはポリヌクレオチドの末端間に、共有結合または連結を形成することを意味する。結合または連結の性質は広く異なり得、ライゲーションは、酵素的または化学的に行なわれ得る。

【0045】

用語「微粒子」は、本明細書において、５０ミクロン以下、好ましくは１０ミクロン以下の最小の断面寸法を有する粒子をいうために用いる。ある特定の実施形態において、最小断面寸法は、およそ３ミクロン以下、およそ１ミクロン以下、およそ０．５ミクロン以下、例えば、およそ０．１、０．２、０．３または０．４ミクロンである。微粒子は、さまざまな無機または有機材料、例えば、限定されないが、ガラス（例えば、細孔性ガラス）、シリカ、ジルコニア、架橋ポリスチレン、ポリアクリレート、ポリメチルメタクリレート、二酸化チタン、ラテックス、ポリスチレンなど作製されたものであり得る。例えば、種々の適当な材料および他の考慮事項については、米国特許第６，４０６，８４８号を参照のこと。Ｄｙｎａビーズ（Ｄｙｎａｌ（オスロ、ノルウェー）から入手可能）は、本発明に有用な市販の微粒子の一例である。磁気応答性微粒子も使用され得る。ある種の好ましい微粒子磁気応答性により、増幅後の微粒子結合鋳型の容易な回収および濃縮が可能になり、さらなる工程（例えば、洗浄、試薬除去など）が容易になる。本発明のある特定の実施形態において、異なる形状（例えば、いくらか球状および他の非球状のもの）を有する微粒子の集団
が使用される。

【0046】
用語「ミクロスフィア」または「ビーズ」は、本明細書において、５０ミクロン以下、好ましくは１０ミクロン以下の直径を有する実質的に球状の微粒子をいうために用いる。ある特定の実施形態において、直径は、およそ３ミクロン以下、およそ１ミクロン以下、およそ０．５ミクロン以下、例えば、およそ０．１、０．２、０．３、または０．４ミクロンである。本発明のある特定の実施形態において、単分散性ミクロスフィアの集団が使用される、すなわち、ミクロスフィアは、実質的に均一な大きさのものである。例えば、微粒子の直径は、５％未満、例えば２％以下、１％以下などの変動係数を有し得る。しかしながら、他の実施形態において、微粒子集団の変動係数は、５％以上、例えば、５％、５％～１０％（両端を含む）、１０％～２５％（両端を含む）などである。ある特定の実施形態において、微粒子の混合集団が使用される。例えば、各々が５％未満の変動係数を有する２つの集団の混合物を用い、単分散性でない混合集団がもたらされ得る。一例として、１ミクロンおよび３ミクロンの直径を有するミクロスフィアの混合物が使用され得る。本発明のある特定の実施形態において、配列決定が、単分散性でない集団のミクロスフィアに結合された鋳型を用いて行なわれる場合、さらなる情報がミクロスフィアの大きさによって提供される。例えば、鋳型の異なるライブラリーを、異なる大きさのミクロスフィアに結合させ得る。また、より少数の鋳型分子をより小さい粒子に結合させ得るため、シグナル強度は異なり得、これにより多重配列決定が容易になり得る。
【0047】
用語「核酸配列」は、本明細書で用いる場合、核酸材料それ自体をいうことがあり得る。特定の核酸、例えば、ＤＮＡまたはＲＮＡ分子を生化学的にキャラクタライズする配列情報（すなわち５つの塩基の文字Ａ、Ｇ、Ｃ、ＴまたはＵから選択される文字の連続）に限定されない。本明細書に示す核酸は、特に記載のない限り、５’→３’方向で示す。
【0048】
「ヌクレオシド」は、糖分子に連結された窒素含有塩基を含む。本明細書で用いる場合、該用語は、その２’－デオキシおよび２’－ヒドロキシル形態において天然のヌクレオシド（ＫｏｒｎｂｅｒｇおよびＢａｋｅｒ，　ＤＮＡ　Ｒｅｐｌｉｃａｔｉｏｎ，　第２版．（Ｆｒｅｅｍａｎ，　Ｓａｎ　Ｆｒａｎｃｉｓｃｏ，１９９２）に記載のものなど）ならびにヌクレオシド類縁体を含む。例えば、天然ヌクレオシドとしては、アデノシン、チミジン、グアノシン、シチジン、ウリジン、デオキシアデノシン、デオキシチミジン、デオキシグアノシンおよびデオキシシチジンが挙げられる。ヌクレオシド「類縁体」は、修飾された塩基部分および／または修飾された糖部分を有する合成ヌクレオシドをいい、例えば、Ｓｃｈｅｉｔ，　ヌクレオチドＡｎａｌｏｇｓ（Ｊｏｈｎ　Ｗｉｌｅｙ，　Ｎｅｗ　Ｙｏｒｋ，　１９８０）に一般的に記載されている。かかる類縁体としては、結合特性の増強、縮重の低減、特異性の増大などのために設計された合成ヌクレオシドが挙げられる。ヌクレオシド類縁体としては、２－アミノアデノシン、２－チオチミジン、ピロロ－ピリミジン、３－メチルアデノシン、Ｃ５－プロピニルシチジン、Ｃ５－プロピニルウリジン、Ｃ５－ブロモウリジン、Ｃ５－フルオロウリジン、Ｃ５－ヨードウリジン、Ｃ５－メチルシチジン、７－デアザアデノシン、７－デアザグアノシン、８－オキソアデノシン、８－オキソグアノシン、Ｏ（６）－メチルグアニン、２－チオシチジンなどが挙げられる。ヌクレオシド類縁体は、本明細書に記載する任意の普遍塩基を含むものであり得る。
【0049】
用語「生物体」は、本明細書において、複製可能であり、配列決定の対象となる核酸を含む、任意の生体的（ｌｉｖｉｎｇ）または非生体的正体を示すために用いる。これには、プラスミド；ウイルス；原核生物、古細菌および真核生物の細胞、細胞株、真菌、原生動物、植物、動物などが含まれる。
【0050】
プローブおよび鋳型ポリヌクレオチドの突出した鎖に関する「完璧にマッチングした二本鎖」は、一方の突出した鎖が他方と二本鎖構造を、該二本鎖構造内の各ヌクレオシドが

、対向する鎖上のヌクレオシドとワトソン－クリック塩基対合を行なうように形成することを意味する。該用語はまた、ヌクレオシド類縁体の対合（例えば、デオキシイノシン、ヌクレオシドと２－アミノプリン塩基となど）を包含し、これは、プローブの縮重を低減させるために使用され得る（かかる対合が水素結合の形成を伴うものであってもそうでなくても）。

【0051】

用語「複数」は、１つより多いことを意味する。

【0052】

用語「多型」は、当該技術分野におけるその通常の意味で示され、同じ種の個体間でのゲノム配列における差をいう。「一塩基多型」（ＳＮＰ）は、単一の位置における多型をいう。

【0053】

「ポリヌクレオチド」、「核酸」または「オリゴヌクレオチド」は、ヌクレオシド間結合によって連結されたヌクレオシド（デオキシリボヌクレオシド、リボヌクレオシドまたはその類縁体を含む）の線状ポリマーをいう。典型的には、ポリヌクレオチドは少なくとも３個のヌクレオシドを含む。本発明のある特定の実施形態において、伸長プローブ内の１個以上のヌクレオシドは普遍塩基を含む。通常、オリゴヌクレオチドは、大きさが数個のモノマー単位（例えば、３～４個）から数百個のモノマー単位の範囲に及ぶ。オリゴヌクレオチドなどのポリヌクレオチドが「ＡＴＧＣＣＴＧ」などの一列の文字で表される場合はいつでも、特に記載のない限り、ヌクレオチドは、左から右に向かって５’→３’の順であること、および「Ａ」はデオキシアデノシンを表し、「Ｃ」はデオキシシチジンを表し、「Ｇ」はデオキシグアノシンを表し、「Ｔ」はチミジンを表すことは理解されよう。Ａ、Ｃ、ＧおよびＴの文字は、当該技術分野において標準的であるように、塩基自体、ヌクレオシドまたは該塩基を含むヌクレオチドをいうために使用され得る。

【0054】

天然に存在するポリヌクレオチドでは、ヌクレオシド間結合は、典型的にはホスホジエステル結合であり、サブユニットは「ヌクレオチド」とよばれる。しかしながら、ホスホロチオレート結合などの他のヌクレオシド間結合を含むオリゴヌクレオチドプローブが、本発明のある特定の実施形態において使用される。非ホスホジエステル結合を有するかかるオリゴヌクレオチドプローブを構成するサブユニットの１つ以上は、リン酸基を含むものであり得ることは認識されよう。ヌクレオチドのかかる類縁体は、本明細書で用いる用語「ヌクレオチド」の範囲に含まれるとみなし、ホスホジエステル結合でない１個以上のヌクレオシド間結合を含む核酸もやはり、「ポリヌクレオチド」、「オリゴヌクレオチド」などとよばれる。他の実施形態において、オリゴヌクレオチドプローブなどのポリヌクレオチドは、ＡＰエンドヌクレアーゼ感受性部位を含有する連結部を含む。例えば、オリゴヌクレオチドプローブは、無塩基残基、ＤＮＡグリコシラーゼによる除去のための基質である損傷塩基を含有する残基、またはＡＰエンドヌクレアーゼによる切断のための基質である別の残基または連結部を含有するものであり得る。別の実施形態において、オリゴヌクレオチドプローブは、二糖ヌクレオシドを含有するものである。

【0055】

用語「プライマー」は、短鎖ポリヌクレオチドをいい、典型的には、約１０～１００ヌクレオチド長であり、標的ポリヌクレオチド、すなわち「鋳型」に、該標的とハイブリダイズすることにより結合するものである。プライマーは、好ましくは、標的に相補的なポリヌクレオチドの鋳型指向型合成（これは、適切な酵素（１種類または複数種）、補因子、基質、例えば、ヌクレオチド、オリゴヌクレオチドなどの存在下で行なわれ得る）の開始点を提供するものである。プライマーは、典型的には、伸長が行なわれ得る起点となる末端を提供する。ＤＮＡポリメラーゼなどのポリメラーゼ酵素によって触媒される合成のためのプライマーの場合（例えば、「合成による配列決定」、ポリメラーゼ連鎖反応（ＰＣＲ）　増幅などにおいて）、プライマーは、典型的には、遊離３’ＯＨ基を有するものであるか、またはこれを有するように修飾されたものであり得る。典型的には、ＰＣＲ反

応では、増幅される領域の境界を定める１対のプライマー（第１および第２の増幅プライマー）、例えば、「上流」（または「フォワード」）プライマーおよび「下流」（または「リバース」）　プライマーが使用される。伸長、ライゲーション（および任意選択で、切断）の連続的サイクルによる合成のためのプライマーの場合、プライマー典型的には、ＤＮＡリガーゼの基質としての機能を果たす遊離５’リン酸基または３’ＯＨ基を有するものであるか、またはこれらを有するように修飾されたものであり得る。

【0056】

本明細書で用いる場合、「プローブファミリー」は、その各々が同じ標識を含む一群のプローブをいう。

【0057】

本明細書で用いる場合、ポリヌクレオチドに関する「配列の決定）」、「ヌクレオチド配列を決定すること」、「配列決定」などの用語は、ポリヌクレオチドの部分配列ならびに完全な配列の情報の決定を含む。すなわち、該用語は、配列の比較、フィンガープリンティングなどのレベルの標的ポリヌクレオチドに関する情報、ならびに同定という表示（ｅｘｐｒｅｓｓ）および目的の領域内の標的ポリヌクレオチド各ヌクレオシドの順序を含む。本発明のある特定の実施形態において、「配列の決定」は、単一のヌクレオチドを同定することを含み、一方、他の実施形態においては、１個より多くのヌクレオチドが同定される。本発明のある特定の実施形態において、それ自体では１サイクルにおいて任意のヌクレオチドを同定するのに不充分な配列情報が収集される。ヌクレオシド、ヌクレオチドおよび／または塩基の同定は、本明細書において等価とみなす。ポリヌクレオチドにおいて配列決定を行なうことにより、典型的には、完璧に相補的（１００％相補的）なポリヌクレオチドの配列に関して等価な情報がもたらされ、したがって、完璧に相補的なポリヌクレオチドにおいて直接行なわれる配列決定と等価であることに注意されたい。

【0058】

「配列決定反応」は、本明細書で用いる場合、伸長、ライゲーションおよび検出の１組のサイクルをいう。伸長された二本鎖が鋳型から除去され、第２の組のサイクルが該鋳型において行なわれる場合、各組みのサイクルは、独立した配列決定反応とみなすが、得られる配列情報を組み合わせて単一の配列を生成させ得る。

【0059】

「半固形」は、本明細書で用いる場合、固形および液状の両方の成分を有し、液状成分が固形マトリックス要素間の細孔、空間または他の隙間を占めている圧縮性のマトリックスをいう。例示的な半固形マトリックスとしては、ポリアクリルアミド、セルロース、ポリアミド（ナイロン）、および架橋アガロース、デキストランおよびポリエチレングリコールで構成されたマトリックスが挙げられる。半固相支持体は、該半固相支持体を支持する第２の支持体、例えば、実質的に平面状で剛性の支持体（基材ともいう）上に設けられたものであってもよい。

【0060】

「支持体」は、本明細書で用いる場合、その上面または内部に核酸分子、微粒子などが固定化され得るマトリックス、すなわち、これらが互いに対して自由に拡散または移動することが広くまたは全体的に妨げられるように、これらが共有結合または非共有結合され得るか、またはその内部もしくは上面にこれらが部分的または完全に包埋され得るマトリックスをいう。

【0061】

「トリガー残基」は、核酸内に存在する場合、それ以外は同一であるトリガー残基を含有しない核酸のおそらくの場合よりも、該核酸を、切断剤（例えば、酵素、硝酸銀など）または薬剤の組合せによる切断（例えば、核酸主鎖の切断）に対してより感受性にする残基、および／または該核酸をかかる切断に対してより感受性にする残基を生成させる修飾に対して感受性である残基である。したがって、核酸内のトリガー残基の存在は、該核酸内に切れやすい連結の存在をもたらし得る。例えば、無塩基残基は、核酸内の無塩基残基の存在によって該核酸がＡＰエンドヌクレアーゼなどの酵素による切断に対して感受性と

なるため、トリガー残基である。損傷塩基を含有するヌクレオシドは、核酸内に損傷塩基を含むヌクレオシドの存在によってもまた、例えば、ＤＮＡグリコシラーゼによる損傷塩基の除去後、該核酸がＡＰエンドヌクレアーゼなどの酵素による切断に対してより感受性となるため、トリガー残基である。切断部位は、トリガー残基と隣接残基間の結合部分であり得るか、またはトリガー残基から除去される１つ以上の残基である結合部分であり得る。例えば、デオキシイノシンは、核酸内のデオキシイノシンの存在によって、該核酸が大腸菌エンドヌクレアーゼＶおよびその類縁体による切断に対してより感受性となるため、トリガー残基である。かかる酵素は、デオキシイノシンに対して３’側の第２のホスホジエステル結合を切断する。本明細書に開示された任意のプローブは、１つ以上のトリガー残基を含有し得る。トリガー残基は、リボースまたはデオキシリボース部分を含むものであり得るが、そうである必要はない。好ましくは、切断剤は、トリガー残基の非存在下では実質的に核酸を切断しないが、同じ条件下（該条件は、該核酸が切断剤に対して感受性となるように修正する薬剤の存在を含み得る）で、トリガー残基を含有する核酸に対して有意な切断活性を示すものである。例えば、好ましくは、切断剤が一方がトリガー残基を含有し、他方がトリガー残基を含有しない以外は長さと組成が同一である核酸を含有する組成物内に存在する場合、トリガー残基を含有する核酸が切断される尤度は、少なくとも：１０；２５；５０；１００；２５０；５００；１０００；２５００；５０００；１０，０００；２５，０００；５０，０００；１００，０００；２５０，０００；５００，０００；１，０００，０００またはそれより大きく、トリガー残基を含有しない核酸が切断される尤度と同定度に大きく、例えば、トリガー残基を含有する核酸の切断の尤度の、トリガー残基を含有しないがそれ以外は同一である核酸の切断の尤度に対する比は１０～10^6または任意のその整数（ｉｎｔｅｇｒａｌ）部分的範囲である。この比は、トリガー残基の具体的な核酸および位置およびヌクレオチド状況に応じて異なり得ることは認識されよう。

【0062】

好ましくは、トリガー残基を含有する核酸が、該核酸を切断剤による切断に対して感受性とするために修飾される必要がある場合、かかる修飾は、適当な修飾剤（１種類または複数種）の存在下で、容易に行なわれ、例えば、該修飾は、適度な収率および適度な時間行なわれる。例えば、本発明のある特定の実施形態において、トリガー残基を含有する核酸の少なくとも５０％、少なくとも６０％、少なくとも７０％、好ましくは少なくとも８０％、少なくとも９０％またはより好ましくは少なくとも９５％が、例えば、２４時間以内に、好ましくは１２時間以内、より好ましくは１分未満～４時間以内に修飾される。

【0063】

さまざまな適当なトリガー残基および対応する切断試薬を本明細書において例示する。本明細書に記載のものと類似した活性を有する任意のトリガー残基および切断試薬が使用され得る。当業者なら、ある特定のトリガー残基および切断試薬組合せが本発明における使用に適しているか否か、例えば、切断の効率および速度、トリガー残基を含有する核酸の切断剤の選択性などが本発明における使用に適しているか否かを判定することができよう。「トリガー残基」は、単に制限酵素部位の一部を構成するヌクレオチドとは、トリガー残基が切断に対する感受性の増大を与える能力は、一般的に、トリガー残基が見出される特定の配列状況に有意に依存しないが、上記のように、該状況は修飾および／または切断に対する感受性に対し、ある程度の影響を及ぼし得るという点において区別されることに注意されたい。もちろん、その周囲のヌクレオチドに応じて、トリガー残基は、制限部位の一部を構成し得る。したがって、ほとんどの場合、切断剤は制限酵素ではないが、制限酵素であることと、非配列特異的切断可能性を有することの両方である酵素の使用は除外されない。

【0064】

「普遍塩基」は、本明細書で用いる場合、天然に存在する核酸内に典型的に見られる塩基の１つより多くと「対合し」得、したがって、二本鎖内のかかる天然に存在する塩基に代用され得る塩基である。該塩基は、天然に存在する塩基の各々と対合できるものである

必要はない。例えば、ある特定の塩基は、プリンのみと、もしくはこれと選択的に、またはピリミジンのみと、もしくはこれと選択的に対合する。ある特定の好ましい普遍塩基（完全普遍塩基）は、天然に存在する核酸内に典型的に見られる任意の塩基と対合し得るものであり、したがって、二本鎖内の任意のこれらの塩基に代用され得る塩基である。該塩基は、天然に存在する塩基の各々と等しく対合できるものである必要はない。プローブミックスが、天然に存在するヌクレオチドのすべてとは対合しない普遍塩基を（１つ以上の位置に）含むプローブを含有する場合、該普遍塩基の少なくとも１つがＡと対合するか、該普遍塩基の少なくとも１つがＧと対合するか、該普遍塩基の少なくとも１つがＣと対合するか、該普遍塩基の少なくとも１つがＴと対合するように、その特定のプローブ内の該位置に２つ以上の普遍塩基を用いることが望ましいことがあり得る。

【0065】

いくつかの普遍塩基が、当該技術分野で知られており、限定されないが、ヒポキサンチン、３－ニトロピロール、４－ニトロインドール、５－ニトロインドール、４－ニトロベンズイミダゾール、５－ニトロインダゾール、８－アザ－７－デアザアデニン、６Ｈ，８Ｈ－３，４－ジヒドロピリミド［４，５－ｃ］［１，２］オキサジン－７－オン（Ｐ．Ｋｏｎｇ　Ｔｈｏｏ　Ｌｉｎ．およびＤ．Ｍ．Ｂｒｏｗｎ，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，１９８９，　１７，１０３７３－１０３８３）、２－アミノ－６－メトキシアミノプリン（Ｄ．Ｍ．ＢｒｏｗｎおよびＰ．Ｋｏｎｇ　Ｔｈｏｏ　Ｌｉｎ，　Ｃａｒｂｏｈｙｄｒａｔｅ　Ｒｅｓｅａｒｃｈ，１９９１，２１６，１２９－１３９）などが挙げられる。ヒポキサンチンは、好ましい完全普遍塩基の一例である。ヒポキサンチンを含むヌクレオシドとしては、限定されないが、イノシン、イソイノシン、２’－デオキシイノシンおよび７－デアザ－２’－デオキシイノシン、２－アザ－２’デオキシイノシンが挙げられる。

【0066】

さらなる普遍塩基が当該技術分野で知られており、例えば、Ｌｏａｋｅｓ，　Ｄ．およびＢｒｏｗｎ，　Ｄ．Ｍ．，　Ｎｕｃｌ．Ａｃｉｄｓ　Ｒｅｓ．２２：４０３９－４０４３，　１９９４；Ｏｈｔｓｕｋａ，　Ｅ．ら、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６０（５）：２６０５－２６０８，１９８５；Ｌｉｎ，　Ｐ．Ｋ．Ｔ．およびＢｒｏｗｎ，　Ｄ．Ｍ．，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．２０（１９）：５１４９－５１５２，１９９２；Ｎｉｃｈｏｌｓ，　Ｒ．ら、Ｎａｔｕｒｅ　３６９（６４８０）：４９２－４９３，１９９４；Ｒａｈｍｏｎ，　Ｍ．Ｓ．およびＨｕｍａｙｕｎ，　Ｎ．Ｚ．，　Ｍｕｔａｔｉｏｎ　Ｒｅｓｅａｒｃｈ　３７７（２）：２６３－８，１９９７；Ｂｅｒｇｅｒ，　Ｍ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓｅａｒｃｈ，　２８（１５）：２９１１－２９１４，２０００；Ａｍｏｓｏｖａ，　Ｏ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．２５（１０）：１９３０－１９３４，　１９９７；ならびにＬｏａｋｅｓ，　Ｄ．，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．２９（１２）：２４３７－４７，２００１の関連部分に記載されている。普遍塩基は、対向する位置にある塩基と水素結合を形成し得るが、そうである必要はない。普遍塩基は、ワトソン－クリックまたは非ワトソン－クリック相互作用（例えば、フーグスティーン相互作用）により水素結合を形成し得る。

【0067】

本発明のある特定の実施形態において、普遍塩基を含むオリゴヌクレオチドプローブを使用するのではなく、無塩基残基を含むオリゴヌクレオチドプローブが使用される。無塩基残基は、任意の４つの天然に存在するヌクレオチドと対向する位置を占めることができ、したがって、普遍塩基を含むヌクレオチドと同じ機能を果たし得る。本発明のある一部の実施形態において、無塩基残基に隣接する連結部は、ＡＰエンドヌクレアーゼによって切断されるが、無塩基残基はまた、他の切れやすい連結（例えば、ホスホロチオレート）が存在し、他の切断試薬が使用される実施形態においても、本明細書に記載のように有用である（すなわち、普遍塩基の機能を果たすため）。

【0068】

本発明のある特定の実施形態の詳細な説明

Ａ．伸長、ライゲーションおよび切断の連続的サイクルによる配列決定

本発明の一態様の全体的なスキームを、図１Ａに図式的に示すが、これは、一般的に、米国特許第５，７４０，３４１号および同第６，３０６，５９７号（ともにＭａｃｅｖｉｃｚに対して発行）に教示された方法と類似する。便宜上の目的のため、これらの特許を本明細書において集合的に「Ｍａｃｅｖｉｃｚ」という。特に、Ｍａｃｅｖｉｃｚには、ポリヌクレオチド内のヌクレオチドの配列を同定するための方法が教示されており、該方法は、（ａ）　プローブを自身にライゲートさせ、伸長された二本鎖を形成することにより、初期オリゴヌクレオチドを、ポリヌクレオチドオリゴヌクレオチドに沿って伸長させる工程；（ｂ）　該ポリヌクレオチドの１つ以上のヌクレオチドを同定する工程；および（ｃ）　該ヌクレオチドの配列が決定されるまで工程（ａ）および（ｂ）を反復する工程を含む。

【0069】

Ｍａｃｅｖｉｃｚには、さらに、鋳型ポリヌクレオチド内のヌクレオチドの配列決定するための方法が教示されており、該方法は、（ａ）鋳型ポリヌクレオチドにハイブリダイズさせた初期オリゴヌクレオチドプローブを含むプローブ－鋳型二本鎖提供する工程（ここで、前記プローブは伸長可能なプローブ末端を有する）；（ｂ）伸長オリゴヌクレオチドプローブを前記伸長可能なプローブ末端にライゲートさせ、伸長されたオリゴヌクレオチドプローブを含む伸長された二本鎖を形成する工程；（ｃ）伸長された二本鎖において、（１）　ライゲートされたばかりの伸長プローブに相補的であるか、もしくは（２）伸長されたオリゴヌクレオチドプローブのすぐ下流にある鋳型ポリヌクレオチド内のヌクレオチド残基であるかのいずれかである鋳型ポリヌクレオチド内の少なくとも１つのヌクレオチドを同定する工程；（ｄ）もし伸長可能なプローブ末端が既に存在しない場合は、生成される末端が最後の伸長プローブがライゲートされた末端と異なるように、伸長可能なプローブ末端を伸長されたプローブ上に生成させる工程；ならびに（ｅ）標的ポリヌクレオチド内のヌクレオチドの配列が決定されるまで工程（ｂ）、（ｃ）および（ｄ）を反復する工程を含む。これらの方法のある特定の実施形態において、各伸長プローブは、連鎖停止部分を、初期オリゴヌクレオチドプローブに対して遠位の末端に有する。ある特定の実施形態において、再生させる工程は、伸長されたオリゴヌクレオチドプローブ内の化学的に切れやすいヌクレオシド間結合を切断することを含む。

【0070】

図１Ａを参照すると、未知配列のポリヌクレオチド領域５０と結合領域４０とを含むポリヌクレオチド鋳型２０は、支持体１０に結合されている。結合領域４０の遠位端のヌクレオチド４１およびポリヌクレオチド領域５０の近位端のヌクレオチド５１は、互いに隣接している。結合領域４０とハイブリダイズし、結合領域４０内のある位置に二本鎖を形成する初期オリゴヌクレオチド３０が提供される。初期オリゴヌクレオチド３０はまた、本明細書において「プライマー」ともいい、結合領域４０は、「プライマー結合領域」ということがある。二本鎖は、完璧にマッチングした二本鎖であり得るが、そうである必要はない。初期オリゴヌクレオチドは伸長可能な末端３１を有する。図１Ａにおいて、初期オリゴヌクレオチドは結合領域に、伸長可能な末端３１がヌクレオチド４１と反対の位置に位置するように結合する。しかしながら、初期オリゴヌクレオチドは、結合領域内の別の場所に結合し得、これは以下にさらに論考する。長さＮの伸長オリゴヌクレオチドプローブ６０は、初期オリゴヌクレオチドに隣接する鋳型にハイブリダイズされる。伸長オリゴヌクレオチドプローブの末端ヌクレオチド６１は、伸長可能な末端３１にライゲートされる。

【0071】

末端ヌクレオチド６１は、ポリヌクレオチド領域５０内の第１の未知ヌクレオチドに相補的である。したがって、末端ヌクレオチド６１の正体によりヌクレオチド５１の正体が特定される。好ましくは、ヌクレオチド５１は、末端ヌクレオチド６１としてＡ、Ｇ、ＣまたはＴを有することがわかっている伸長プローブと会合した標識（図示せず）検出する

ことをにより同定される。標識は、検出後に除去される。図２は、異なる標識（例えば、異なる色のフルオロフォア）を、異なる３’末端ヌクレオチドを有する伸長プローブに割り当てるためのスキームを示す。

【0072】

ライゲーションおよび検出の後、もしプローブ６０が既にかかる末端を有しない場合は、伸長可能なプローブ末端を伸長プローブ６０上に生成させる。第２の伸長プローブ７０（好ましくは、これも長さＮである）を伸長プローブ６０に隣接する鋳型にアニーリングさせ、プローブ６０の伸長可能な末端にライゲートさせる。伸長プローブ７０の末端ヌクレオチド７１の正体により、対向する位置にあるポリヌクレオチド５０内のヌクレオチド５２の正体が特定される。したがって、末端ヌクレオチド７１は伸長プローブの「配列決定部分」を構成し、これは、プローブの該部分は、そのハイブリダイゼーション特異性が鋳型内の１つ以上のヌクレオチドの正体を決定する基準として利用されることを意味することは認識されよう。典型的には、伸長プローブ内のさらなるヌクレオチドが鋳型とハイブリダイズするが、プローブ内のその正体がある特定の標識と会合しているヌクレオチドのみが、鋳型内のヌクレオチドを同定するために利用される。

【0073】

本発明の好ましい実施形態において、伸長可能な末端の生成は、ヌクレオシド間結合の切断を伴い、これは以下にさらに記載する。好ましくは、切断により標識もまた除去される。切断により、いくつかのヌクレオチドＭが伸長プローブから除去される（図示せず）。したがって、二本鎖は、各サイクルにおいてＮ－Ｍヌクレオチドによって伸長され、鋳型内のＮ－Ｍの区間内に位置するヌクレオチドが同定される。多コピーの所与の鋳型は、典型的には単一の支持体に結合され、配列決定反応が、これらの鋳型において同時に行なわれることを理解されたい。

【0074】

Ｍａｃｅｖｉｃｚには、オリゴヌクレオチドプローブは、一般的に、初期オリゴヌクレオチドまたは伸長された二本鎖にライゲートされ、次の伸長サイクルの伸長された二本鎖を生成させ得るものであるべきであること；ライゲーションは、プローブがライゲーション前に鋳型と二本鎖を形成すべきであるという点で、鋳型駆動型べきであること；プローブは、単一の伸長サイクルにおける同じ鋳型での多重プローブライゲーションを抑制するためのブロッキング部分を有するものであるべきこと；プローブは、ライゲーション後に伸長可能な末端を再生させるために処理または修飾され得るものであるべきこと；およびプローブは、成功裏のライゲーション後に、鋳型に関する配列情報の取得を可能にするシグナル伝達部分（すなわち、検出可能な部分）　を有するものであるべきことが教示されている。

【0075】

Ｍａｃｅｖｉｃｚには、一部の特定の好適な初期オリゴヌクレオチド、伸長オリゴヌクレオチドプローブ、鋳型、結合部位の特性、およびかかる成分を剛性、設計、作製または入手するための種々の方法が教示されている。Ｍａｃｅｖｉｃｚには、さらに、一部の特定の好適なリガーゼ、ライゲーション条件およびさまざまな適当な標識が教示されている。Ｍａｃｅｖｉｃｚにはまた、標識された連鎖停止ヌクレオチドを新たにライゲートされた伸長プローブに付加させるためにポリメラーゼ伸長を用いる同定の代替方法が教示されている。付加されたヌクレオチドの正体により、反対の位置に位置する鋳型内のヌクレオチドが特定される。

【0076】

当業者には認識されるように、鋳型、初期オリゴヌクレオチド、伸長プローブ、プライマーなどに対する言及は、一般的に、単一の分子ではなく、関連する領域内で実質的に同一である核酸分子の集団またはプールを意味する。したがって、例えば、「鋳型」は、一般的に、複数の実質的に同一の鋳型分子を意味し、「プローブ」は、一般的に、複数の実質的に同一のプローブ分子を意味するなどである。１つ以上の位置で縮重しているプローブの場合、ある特定のプローブを含むプローブ分子の配列は、縮重位置において異なる、

すなわち、ある特定のプローブを構成するプローブ分子の配列は、非縮重位置（１つまたは複数）においてのみ、実質的に同一であり得ることは認識されよう。記載の目的のため、単数形は、単一の分子群および実質的に同一の分子の集団を含むと理解されたい。単一の核酸分子（すなわち、１つの分子）に言及することが意図される場合、用語「鋳型分子」、「プローブ分子」、「プライマー分子」などを用いる。ある特定の状況において、実質的に同一の核酸分子の一集団の複数の性質は、明白に表示する。

【0077】

実質的に同一の核酸分子の集団は、任意のさまざまな既知の方法、例えば、化学合成、細胞内での生物学的合成、１種類以上の出発核酸分子からの酵素的インビトロ増幅などを用いて入手および作製され得る。例えば、当該技術分野でよく知られた方法を用い、目的の核酸を、適当な発現ベクター（例えば、ＤＮＡまたはＲＮＡプラスミド）内に挿入し、次いで、細胞（例えば、細菌細胞）内に導入して複製させることによりクローニングされ得る。次いで、目的の核酸コピーを含有するプラスミドＤＮＡまたはＲＮＡを該細胞から単離する。ウイルス、細胞などから単離されたゲノムＤＮＡ、またはｍＲＮＡ）の逆転写により作製されたｃＤＮＡもまた、クローニングまたはインビトロ増幅の中間工程なしで、実質的に同一の核酸分子の集団（例えば、その配列が決定される鋳型ポリヌクレオチド）の供給源となり得るが、一般的には、かかる中間工程を行なうことが好ましい。

【0078】

集団のメンバーは、１００％同一である必要はなく、例えば、ある特定数の「エラー」が、合成過程中に起こり得ることは理解されよう。好ましくは、集団のメンバーの少なくとも５０％が、参照核酸分子（すなわち、配列比較のための基準として用いられる規定配列の分子）と少なくとも９０％、またはより好ましくは少なくとも９５％同一である。より好ましくは、集団のメンバーの少なくとも６０％、少なくとも７０％、少なくとも８０％、少なくとも９０％、少なくとも９５％、少なくとも９９％またはそれ以上が、参照核酸分子と少なくとも９０％、またはより好ましくは少なくとも９５％同一であるか、さらにより好ましくは少なくとも９９％同一である。好ましくは、参照核酸分子に対して少なくとも９５％またはより好ましくは少なくとも９９％の同一性割合の集団のメンバーは、少なくとも９８％、９９％、９９．９％またはそれ以上である。同一性割合は、２つの最適にアライメントされた配列を比較し、同一の核酸塩基（例えば、Ａ、Ｔ、Ｃ、Ｇ、ＵまたはＩ）が両方の配列に存在する位置の数を測定してマッチングした位置の数を得、マッチングした位置の数を位置の合計数で除算し、結果に１００を積算して配列の同一性割合を得ることによりコンピュータ計算され得る。ある特定の状況において、鋳型、プローブ、プライマーなどの核酸分子は、より大きな核酸分子（これはまた、鋳型、プローブまたはプライマーの機能を果たさない部分も含有する）の一部分であり得ることは認識されよう。その場合、個々の集団のメンバーは、該部分に関して実質的に同一である必要はない。

【0079】

Ｍａｃｅｖｉｃｚには、図１Ａに示すような、鋳型がビーズなどの支持体に結合され、伸長が、支持体の遠位に位置する鋳型の末端に向かって進行する方法が教示されている。したがって、結合領域は未知配列よりも支持体の近くに位置し、伸長された二本鎖は、支持体から離れる方法に成長する。しかしながら、本発明者らは、予期に反して、該方法は、結合領域が支持体の遠位に鋳型の末端に位置し、伸長が支持体に向かって内方に進行する別のアプローチを用いて好都合に行なわれ得ることを見出した。この実施形態を図１Ｂに示す。図において、種々の要素は図１Ａと同様に番号付けしている。本発明者らは、鋳型の遠位端から支持体に向かう「内方への」配列決定により、優れた結果が得られることを調べた。特に、鋳型の遠位端からビーズなどの支持体に向かう配列決定により、支持体から外方への配列決定よりも高いライゲーション効率がもたらされる。

【0080】

Ｍａｃｅｖｉｃｚによってさらに教示されているように、好ましくは、オリゴヌクレオチドプローブは鋳型に、所定の長さの可能な全ての配列のオリゴヌクレオチドを含む混合

物として適用する。例えば、構造ＮＮＮＮＮＮ（これは、（Ｎ）$_{k}$（式中、ｋ＝６）とも表示され得る）の６ヌクレオチド長（六量体）の可能な全ての配列を含有するプローブの混合物は、4^{6}（４０９６）のプローブ種を含有し得る。一般的に、プローブは、構造Ｘ（Ｎ）$_{k}$Ｎ*（式中、Ｎは任意のヌクレオチドを表し、ｋは１～１００であり、*は標識を表し、Ｘは、その正体が標識に対応するヌクレオチドを表す）のものである。ある特定の実施形態において、ｋは、１～１００、１～５０、１～３０、１～２０、例えば、４～１０である。該ヌクレオチドの１つ以上は普遍塩基を含むものであり得る。一般的に、プローブは、Ｎで表される位置において４重に縮重しているか、またはＮで表される１つ以上の位置に縮重低減性ヌクレオチドを含む。所望の場合は、混合物を、プローブ（「ストリンジェンシー類型」）の部分集団に分けてもよい。それらの相補配列完璧にマッチングした二本鎖は、類似した安定性または結合自由エネルギーを有する。部分集団は、Ｍａｃｅｖｉｃｚに教示されているように、独立したハイブリダイゼーション反応に使用され得る。

【0081】

プローブ混合物の複雑度（ｃｏｍｐｌｅｘｉｔｙ）（すなわち、異なる配列の数）は、いくつかの方法によって、例えば、いわゆる縮重低減性のヌクレオチドまたはヌクレオチドアナログを用いることにより低減させ得る。例えば、８ヌクレオチドの可能な全ての配列を含有するプローブのライブラリーは、4^{8}のプローブを含み得る。プローブの数は、２つの位置に普遍塩基を用いることにより、例えば、長さなどの八量体ライブラリーの種々の望ましい特徴を維持したまま4^{6}に低減させ得る。本発明は、任意の上記または上記参考文献に記載の普遍塩基の使用を包含する。

【0082】

実施形態に応じて、伸長された二本鎖または初期オリゴヌクレオチドは、オリゴヌクレオチドプローブによって５’→３’方向または３’→５’方向のいずれかに伸長され得、これは以下にさらに記載する。一般的に、オリゴヌクレオチドプローブは、鋳型と完璧にマッチングした二本鎖を形成する必要はないが、かかる結合が好ましいことがあり得る。鋳型内の単一のヌクレオチドが各伸長サイクルで同定される実施形態では、完璧な塩基対合だけが、その特定のヌクレオチドを同定するために必要とされる。例えば、オリゴヌクレオチドプローブが伸長された二本鎖に酵素的にライゲートされる実施形態では、完璧な塩基対合、すなわち適正なワトソン－クリック塩基対合が、ライゲートされるプローブの末端ヌクレオチドと、鋳型内のその相補配列との間に必要とされる。一般的に、かかる実施形態では、プローブの残余ヌクレオチドは、次のライゲーションが鋳型に沿った所定の部位または塩基数で起こることを確実にする「スペーサー」としての機能を果たす。すなわち、その対合またはその欠如は、さらなる配列情報を提供しない。同様に、塩基同定をポリメラーゼ伸長に依存する実施形態では、プローブは、主に、スペーサーとしての機能を果たすため、鋳型への特異的ハイブリダイゼーションは不可欠ではない。

【0083】

上記の方法により、配列の部分決定、すなわち、鋳型内で互いに間隔があいている個々のヌクレオチドの同定が可能になる、本発明の好ましい実施形態において、より完全な情報を取得するため、各反応で異なる初期オリゴヌクレオチドｉが使用される複数の反応が行なわれる。初期オリゴヌクレオチドｉは、結合領域の異なる部分に結合する。好ましくは、初期オリゴヌクレオチドは、結合領域にハイブリダイズされるとき、異なる初期オリゴヌクレオチドのかかる伸長可能な末端が互いに１ヌクレオチドずれた位置に結合する。例えば、図３に示すように、配列決定反応１．．．Ｎが行なわれる。初期オリゴヌクレオチドｉ$_{i}$．．．ｉ$_{n}$は同じ長さを有し、その末端ヌクレオチド３１、３２、３３などが、結合領域４０内の連続的な隣接する位置４１、４２、４３などにハイブリダイズするように結合する。したがって、伸長プローブｅ$_{1}$．．．ｅ$_{n}$は、鋳型の連続的な隣接する領域に結合し、初期オリゴヌクレオチドの伸長可能な末端にライゲートされる。ｉ$_{\pi}$にライゲートされたプローブｅ$_{n}$の末端ヌクレオチド６１は、ポリヌクレオチド領域５０のヌクレオチド５５、すなわち、鋳型内の第１の未知ポリヌクレオチドに相補的である。伸長、ラ

イゲーションおよび検出の第２のサイクルでは、プローブｅ$_{12}$の末端ヌクレオチド７１は、ポリヌクレオチド領域５０のヌクレオチド５６、すなわち、未知配列の第２のヌクレオチドに相補的である。同様に、初期オリゴヌクレオチドｉ$_{2}$、ｉ$_{3}$、ｉ$_{4}$などにより初期化された二本鎖にライゲートされた伸長プローブの末端ヌクレオチドは、未知配列５０の第３、第４および第５のヌクレオチドに相補的である。初期オリゴヌクレオチドは、ポリヌクレオチド領域５０に対し、進行的に近づくのではなく進行的にさらに離れる領域に結合され得ることは認識されよう。

【0084】

非伸長プローブの末端ヌクレオチドのスペーサー機能により、初期オリゴヌクレオチドが結合する位置から相当除去される鋳型内の位置の配列情報の取得が、相応する多数回のサイクルが任意の所与の鋳型において行なわれることを要することなく可能になる。例えば、長さＮのプローブのライゲーションの連続的サイクル後、単一の末端ヌクレオチドを伸長プローブから除去するための切断によって、Ｎ－１ヌクレオチド区間のヌクレオチドが連続回数で同定され得る。例えば、鋳型内の１、Ｎ、２Ｎ－１、３Ｎ－２、４Ｎ－３および５Ｎ－４位のヌクレオチドが、６サイクルで同定され得、この場合、鋳型内の１位のヌクレオチドは、鋳型への初期オリゴヌクレオチドの結合によって形成される二本鎖内の伸長可能なプローブ末端にライゲートされるヌクレオチドと反対のヌクレオチドである。同様に、切断により２つのヌクレオチドが長さＮの伸長プローブから除去される場合、互いにＮ－２ヌクレオチド離れた位置のヌクレオチドが連続回数で同定され得る。例えば、鋳型内の１、Ｎ－１、２Ｎ－３、３Ｎ－５、４Ｎ－７位のヌクレオチドが６サイクルで同定され得る。したがって、プローブが８ヌクレオチド長であり、２個のヌクレオチドが各サイクルで除去される場合、１位、７、１３、１９および２５位のヌクレオチドが同定される。したがって、鋳型内の第１のヌクレオチドからＸ離れたヌクレオチドを同定するのに必要とされるサイクル数は、およそＸではなく、およそＸ／Ｍ（式中、Ｍは、切断後に残存する伸長プローブの長さである）である。

【0085】

例えば、図３Ｂに示す概略は、鋳型を６塩基ごとに読み出されるように設計された伸長プローブを用いた伸長、ライゲーションおよび切断方法の使用の最終結果を示す。結合領域内でずれる位置に結合する初期化６ヌクレオチドを用いて鋳型を逐次ストリッピングおよび配列決定し、結果を組み合わせることにより、すべての鋳型塩基が、規定された長さにわたって解明される。例えば、１０回の逐次ライゲーションが、６回の反応の各々で行なわれる場合、得られる読出し長さは６０個の連続塩基対であるが、１５回の逐次ライゲーションが各反応で行なわれる場合、得られる読出し長さは９０個の連続塩基対である。

【0086】

なんら理論に拘束されることを望まないが、本発明者らは、このアプローチとは対照的に、合成方法によるほとんどの逐次配列決定では、長い読出し長さに対する可能性を最終的に制限するエラー蓄積に取り組んでいることを提案する。ある特定の本明細書に記載の方法の好都合な特徴は、所与のサイクル数（ｙ）後、ｎ＊ｙ－（ｎ－１）番目の塩基に達する（例えば、１５サイクル後に前述の例において７１番目の塩基、または切断部位の３’側で６塩基のプローブを用いて２０サイクル後に１１５番目の塩基）ように、第ｎ塩基ごと（プローブ内の切断可能な部分の位置による）の同定を可能にすることである。ｎ－１、ｎ－２などの位置の初期オリゴヌクレオチドを「リセット」できることは、鋳型からの伸長鎖のストリッピングおよび新たな初期オリゴヌクレオチドのハイブリダイズのプロセスにより、バックグラウンドシグナルがゼロに有効にリセットされるため、所与の読出し長さのための逐次エラー蓄積（位相の散逸または自然減（ａｔｔｒｉｔｉｏｎ）により）が大いに最小化される。例えば、合成によるポリメラーゼ系配列決定と、本明細書に記載のライゲーション系アプローチを比較すると、各伸長サイクルでの信号対雑音比が９９：１である場合、１００サイクル後の比は、ポリメラーゼ系アプローチで３７：６３であり、リガーゼ系の方法では８５：１５である。リガーゼ系の方法での最終結果は、ポリメラーゼ系の方法よりも読出し長さにおいて大きく増大する。

【0087】
鋳型内の先行する各ヌクレオチドのためのサイクルを行なう必要がある場合に必要とされ得るよりも少ないサイクルを用いてヌクレオチドを同定できることは、いくつかの理由で重要である。特に、該方法において各工程が１００％効率で行なわれることはあり得ない。例えば、一部の鋳型は、伸長プローブに成功裏にライゲートされないことがあり得、一部の伸長プローブは切断されないことがあり得るなどである。したがって、各サイクルにおいて、異なる鋳型コピーにおいて行なわれる反応は、進行的に位相の散逸となり、有用で正確な情報が取得され得る鋳型の数が低減される。したがって、初期オリゴヌクレオチドの伸長可能な末端から数個より多く離れた位置に位置するヌクレオチドの読み出しに必要とされサイクル数を最小限にすることは特に望ましい。しかしながら、伸長プローブの長さを増大させることは、潜在的にプローブ混合物のより大きな複雑度をもたらし、これは、個々の各プローブ配列の有効濃度を低減させる。本明細書に記載のように、縮重低減性ヌクレオチドは、複雑度を低減させるために使用され得るが、ハイブリダイゼーション強度の減少および／またはライゲーション効率の減少をもたらし得る。本発明者らは、結果を最適化するために、これらの競合する要素を均衡させる必要性を認識した。したがって、本発明の好ましい実施形態において、８ヌクレオチド長の伸長プローブを使用し、選択した位置に縮重低減性ヌクレオチドを用いる。また、本発明者らは、適切な切れやすい連結ならびに切断条件ならびに切断工程の効率（すなわち、各切断工程において成功裏に切断された連結の割合）およびその適切な連結に対する特異性を最適化するタイミングを選択することの重要性を認識した。

【0088】
Ｂ．オリゴヌクレオチド伸長プローブ設計
Ｍａｃｅｖｉｃｚには、縮重低減性ヌクレオシド類縁体が、オリゴヌクレオチド伸長プローブに使用され得ることは記載されているが、かかる残基を含む残基を伸長プローブ内に含めるのに特に望ましい具体的な位置は教示されておらず、縮重低減性ヌクレオシドが組み込まれた具体的なプローブ構造（すなわち、配列）は教示されていない。本発明者らは、縮重低減性ヌクレオシド（例えば、普遍塩基を含むヌクレオシド）を、オリゴヌクレオチド伸長プローブ内の特定の位置に特定の数で利用することが特に好都合であり得ると認識した。例えば、本発明のある特定の実施形態において、６位以降の（Ｘから数えて）のヌクレオチドの大部分または全部が普遍塩基を含む。例えば、６位以降のヌクレオチド少なくとも５０％、少なくとも６０％、少なくとも７０％、少なくとも８０％、少なくとも９０％または少なくとも１００％が、普遍塩基を含有するものであり得る。ヌクレオチドは、すべてが同じ普遍塩基を含有するものである必要はない。本発明のある特定の実施形態において、ヒポキサンチンおよび／またはニトロ－インドールが普遍塩基として使用される。例えば、イノシンなどのヌクレオシドが使用され得る。

【0089】
本発明者らは、優れた結果は、６ヌクレオチド長より大きく、プローブの近位末端から（ライゲートされるヌクレオチドから数えて）伸長可能なプローブ末端までの６位以降のヌクレオチドの１つ以上が縮重低減性ヌクレオチドである、例えば、普遍塩基を含む（すなわち、最も近位ヌクレオチドを１位とみなした場合、６位以降のヌクレオチドの１つ以上が普遍塩基を含む）、例えば、八量体プローブの場合は、６位以降のヌクレオチドの１、２または３個が普遍塩基を含む伸長プローブを用いて達成され得ると認識した。例えば、３’→５’方向における配列決定では、構造３’－ＸＮＮＮＮｓＩＮＩ－５’　を有するプローブが使用され得、式中、ＸおよびＮは任意のヌクレオチドを表し、「ｓ」は、切れやすい連結を表し、切断は３’末端から数えて第５と第６の間の残基で起こるようになっており、切れやすい連結と５’末端間の残基の少なくとも１つは、好ましくは、Ｘの正体に対応する標識を有する。別の設計は、３’－ＸＮＮＮＮｓＮＩＩ－５’　である。また別のプローブ設計は、３’－ＸＮＮＮＮｓＩＩＩ－５’　である。この設計により、中程度の複雑度を有する１０２４個の異なる種プローブ混合物がもたらされ、有意なアデニル化産物の形成を抑制するのに充分長く（実施例１参照）、切断後に残留する得られる伸

長産物が非修飾ＤＮＡからなるものであり得るという利点を有する。欠点の１つは、このプローブがプライマーを一度に５塩基しか伸長させないことである。読出し長さは、伸長長さ×サイクル数の関数であるため、伸長長さにおける各追加の塩基は、読出し長さが１×サイクル数分増加する可能性を有する（例えば、２０　サイクルを用いる場合は２０塩基）。別のプローブ設計は、切断後に１つ以上イノシン（または他の普遍塩基）を伸長プローブの末端に残し、６塩基以上の長さの伸長された二本鎖を創製させるものである。例えば、プローブ３’－ＸＮＮＮＮＩｓＩＩ－５’により、二本鎖は一度に６塩基伸長され得、接合に５’イノシンが残る。これらの設計の各々において、切れやすい連結と５’末端間の残基の少なくとも１つは、好ましくは、Ｘの正体に対応する標識を有する。本発明のある特定の実施形態において、プローブの遠位末端から（ライゲートされるヌクレオチドと反対の末端から数えて）伸長可能なプローブ末端までの第３のヌクレオチドが普遍塩基を含む（すなわち、遠位末端を位置Ｋとみなした場合、位置Ｋ－２のヌクレオチドが普遍塩基を含む）。

【0090】

本発明のある特定の実施形態において、ロックド核酸（ＬＮＡ）塩基が、初期オリゴヌクレオチドプローブ、伸長プローブまたは両方内の１つ以上の位置に使用される。ロックド核酸は、例えば、米国特許第６，２６８，４９０号；Ｋｏｓｈｋｉｎ，　ＡＡら、Ｔｅｔｒａｈｅｄｒｏｎ，　５４：３６０７－３６３０，１９９８；Ｓｉｎｇｈ，　ＳＫら、Ｃｈｅｍ．Ｃｏｍｍ．，４：４５５－４５６，１９９８に記載されている。ＬＮＡは、自動ＤＮＡ合成装置によって標準的なホスホロアミダイト化学反応を用いて合成され得、天然に存在するヌクレオチドおよび／またはヌクレオチドアナログもまた含有するオリゴヌクレオチド内に組み込まれ得る。これらもまた、以下に記載のものなどの標識を用いて合成され得る。

【0091】

Ｃ．鋳型、ライブラリー、担体、ブロッカー、およびそれらの調製および使用方法

本発明は、核酸鋳型および担体を調製する種々の方法を提供する。本発明はまた、ライゲーションに基づく配列決定での使用、または他の目的のためのライブラリーも提供する。本発明はまた、ブロッカーオリゴヌクレオチド、ならびに他の目的でオリゴヌクレオチドのライゲーション、検出および開裂のサイクルを連続して行うことにより、配列決定においてそれらを使用する方法も提供する。

【0092】

Ｍａｃｅｖｉｃｚには、複数の実質的に同一の鋳型分子を含む鋳型を最初に、例えば、慣用的なポリメラーゼ連鎖反応（ＰＣＲ）法のようなチューブまたは他の容器内で増幅によって合成する方法が教示されている。Ｍａｃｅｖｉｃｚには、増幅させた鋳型分子を、好ましくは、合成後に磁気微粒子（例えば、ビーズ）などの支持体に結合させることが教示されている。

【0093】

本発明者らは、配列決定される鋳型は、望ましくは、支持体自体の上またはその内部で、例えば、１対の増幅プライマーの一方を、ＰＣＲ反応を行なう前に結合させる微粒子などの支持体または種々の半固相支持体材料ゲルマトリックスを用いることにより合成させるのがよいと認識した。このアプローチにより、合成後に、鋳型分子を支持体に結合させる別途の工程の必要性が回避される。したがって、異なる配列の複数の鋳型種が、並行して簡便に増幅され得る。例えば、以下に記載する方法によれば、微粒子上での合成により、各々が自身に結合された多コピーのある特定の鋳型分子（またはその相補体）を有する個々の微粒子の集団がもたらされ、各微粒子に結合された鋳型分子は、他の微粒子に結合された鋳型分子と配列が異なる。したがって、支持体の各々は、自身に結合された鋳型のクローン性の集団を有する、例えば、支持体Ａは、自身に結合された多コピーの鋳型Ｘを有し、支持体Ｂは、自身に結合された多コピーの鋳型Ｙを有し、支持体Ｃは、自身に結合された多コピーの鋳型Ｚを有するなどである。「鋳型のクローン性の集団」、「核酸のクローン性の集団」などにより、好ましくは、目的の単一の鋳型分子（出発鋳型）から開始

される連続回数の増幅によって生成される、実質的に同一の鋳型分子の集団が意図される。実質的に同一の鋳型分子は、出発鋳型またはその相補体と実質的に同一であり得る。

【0094】

増幅は、典型的には、ＰＣＲを用いて行なわれるが、他の増幅方法もまた使用され得る（下記参照）。クローン性の集団のメンバーは１００％同一である必要はなく、例えば、ある特定数の「エラー」が、合成過程中（例えば、増幅中）に起こり得ることは理解されよう。クローン性の集団のメンバーの好ましくは少なくとも５０％が、出発鋳型分子（またはその相補体）と少なくとも９０％、またはより好ましくは少なくとも９５％同一である。より好ましくは、集団のメンバーの少なくとも６０％、少なくとも７０％、少なくとも８０％、少なくとも９０％、少なくとも９５％、少なくとも９９％またはそれ以上が、出発鋳型分子（またはその相補体）と少なくとも９０％、またはより好ましくは少なくとも９５％同一であるか、またはさらにより好ましくは少なくとも９９％同一である。好ましくは、出発鋳型分子（またはその相補体）に対して少なくとも９５％またはより好ましくは少なくとも９９％の同一性割合の集団のメンバーは、少なくとも９８％、９９％、９９．９％またはそれ以上である。

【0095】

増幅プライマーは、任意のさまざまな手法を用いて支持体に結合させ得る。例えば、プライマー（５’末端）の一方の末端を結合ペアの一メンバー（例えば、ビオチン）で官能性付与させ、支持体を、結合ペア他方のメンバー（例えば、ストレプトアビジン）で官能性付与させ得る。任意の類似した結合ペアが使用され得る。例えば、規定配列の核酸タグを、相補的核酸タグを有する支持体およびプライマーに結合させ得、支持体に結合された核酸タグにハイブリダイズさせ得る。種々のリンカーおよび架橋剤もまた使用され得る。

【0096】

ＰＣＲを行なうための方法は、当該技術分野でよく知られており、例えば、米国特許第４，６８３，１９５号、同第４，６８３，２０２号および同第４，９６５，１８８号、ならびにＤｉｅｆｆｅｎｂａｃｈ，　Ｃ．およびＤｖｅｋｓｌｅｒ，　ＧＳ，　ＰＣＲ　Ｐｒｉｍｅｒ：Ａ　Ｌａｂｏｒａｔｏｒｙ　Ｍａｎｕａｌ，第２版，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ　Ｌａｂｏｒａｔｏｒｙ　Ｐｒｅｓｓ，　Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ，２００３に記載されている。微粒子上で核酸を増幅させるための方法は、当該技術分野でよく知られ、報告されており、例えば、標準的なＰＣＲは、マイクロタイタープレート（ｄｉｓｈ）のウェルまたはチューブ内で、自身に結合されたプライマーを有するビーズ（例えば、実施例１２のようにして調製されるビーズ）上で行なわれ得る。ＰＣＲは簡便な増幅方法であるが、当該技術分野で知られた任意の数多くの他の方法もまた使用され得る。例えば、多重鎖置換型増幅、ヘリカーゼ置換型増幅（ＨＤＡ）、ニックトランスレーション、Ｑβレプリカーゼ増幅、ローリングサークル型増幅、および他の等温増幅方法などが使用され得る。

【0097】

鋳型分子は、任意のさまざまな供給源から得られ得る。例えば、ＤＮＡは、被験体から入手され得るか、またはこれに由来であり得る試料から単離され得る。用語「試料」は、配列決定が行なわれる鋳型の任意の供給源を表すために広義で用いる。語句「に由来する」は、被験体から直接得られた試料および／または試料中の核酸は、鋳型分子を得るためにさらに加工処理されたものであり得ることを示すために用いる。試料の供給源は、任意のウイルス、原核生物、古細菌または真核生物種であり得る。本発明のある特定の実施形態において、供給源はヒトである。試料は、例えば、細胞を含有する血液または別の体液；精子；生検試料などであり得る。目的の任意の生物体のゲノムまたはミトコンドリアのＤＮＡが配列決定されてもよい。ｃＤＮＡが配列決定されてもよい。また、例えば、ＲＴ－ＰＣＲなどの当該技術分野で知られた方法を用いて最初に逆転写することによってｃＤＮＡを得ることによりＲＮＡが配列決定されてもよい。異なる試料および／または被験体由来のＤＮＡの混合物を合わせてもよい。試料は、任意のさまざまな様式で加工処理され得る。核酸は試料から、既知の方法を用いて単離、精製および／または増幅され得る。も

ちろん、生物体に由来しない完全に人工的な合成核酸、組換え核酸もまた配列決定され得る。

【0098】

鋳型は、二本鎖または単鎖形態で提供され得る。典型的には、まず、鋳型を二本鎖形態で提供し、続いて、この二本鎖を分離する（例えば、ＤＮＡを変性させる）場合、該二本鎖の一方だけを増幅して鋳型の局在クローン性の集団分子（例えば、微粒子に結合、半固相支持体内または該支持体上にに固定化など）を生成させる。

【0099】

鋳型は、さまざまなさらなる様式で選択または加工処理され得る。例えば、メチル感受性制限酵素（例えば、Ｍｓｐ１）による処理に供されたＤＮＡから得られる鋳型が使用され得る。かかる処理は、ＤＮＡ断片をもたらすものであり、増幅前に行なわれ得る。メチル化塩基を含有する断片は増幅されない。低メチル化（ｈｙｐｏｍｅｔｈｙｌａｔｅｄ）鋳型から得られる配列情報を、同じ供給源に由来する低メチル化の選択に供しなかった鋳型から得られる配列情報と比較し得る。

【0100】

鋳型は、ライブラリー内に挿入されるか、これで提供されるか、またはこれに由来するものであり得る。例えば、低メチル化ライブラリーは当該技術分野で知られている。ライブラリー内への鋳型の挿入により、鋳型の末端へのさらなるヌクレオチド配列の簡便な連鎖（例えば、タグ、プライマーまたは初期オリゴヌクレオチド結合部位など）が可能となり得る。例えば、ある特定のストラテジーにより、複数の結合部位（例えば、増幅プライマーの結合部位、初期オリゴヌクレオチドの結合部位、捕捉剤の結合部位など）を有するタグの付加が可能になる。

【0101】

さまざまな適当なライブラリーが、当該技術分野で知られている。例えば、特定の目的のライブラリー、およびその構築方法は、ＵＳＳＮ１０／９７８，２２４、ＰＣＴ公開公報ＷＯ２００５０４２７８１およびＷＯ２００５０８２０９８ならびにＳｈｅｎｄｕｒｅ，　Ｊ．ら、Ｓｃｉｅｎｃｅ，３０９（５７４１）：１７２８－３２，２００５，　Ｓｃｉｅｎｃｅｘｐｒｅｓｓ（２００５年８月４日）（ｗｗｗ．ｓｃｉｅｎｃｅｘｐｒｅｓｓ．ｏｒｇ）に記載されている。もちろん、かかるライブラリーの他の作製方法もまた使用され得ることは理解されよう。特定の目的のある特定のライブラリーは、複数の核酸断片（典型的には、ＤＮＡ）を含み、その各々は、配列決定工程で使用される増幅および／または配列決定用プライマーに相補的な配列によって分断された目的の２つの核酸セグメントを含有する、すなわち、これらの配列は、プライマー結合領域（ＰＢＲ）としての機能を果たす。特定の目的の実施形態において、核酸セグメントは、天然に存在するＤＮＡの連続片の一部分である。例えば、セグメントは、前述の参考文献に記載のようなゲノムＤＮＡの連続片の５’および３’末端由来のものであり得る。かかる核酸セグメントを本明細書において、前述の参考文献と一致する様式で、「タグ」または「末端タグ」という。単一の連続核酸に由来する（例えば、その５’および３’末端に由来する）２つのタグを、「１対のタグ」、「対のタグ」または「ジタグ（ｄｉｔａｇ）」とよぶ。「対のタグ」は、単数形で使用されていても２つのタグを含むことは認識されよう。１対のタグであるタグが由来するＤＮＡの連続片を所定の大きさの範囲内で選択することにより、該２つのタグが離れる間隔が限定される。

【0102】

配列決定および／または増幅用プライマーに相補的な配列によって分断されることに加え、ライブラリーの核酸断片はまた、典型的には、タグと隣接する配列決定および／または増幅用プライマーに相補的な配列を含む。すなわち、第１のかかる配列は、該断片の５’末端により近いタグの５’側に位置し得、第２のかかる配列は、該断片の３’末端により近いタグの３’側に位置し得る。タグが由来する連続核酸内に存在する２つのタグの位置は、種々の実施形態において、ライブラリーのＤＮＡ断片内のタグの位置に対応するものであり得るが、そうである必要はないことに注意されたい。

【0103】
核酸断片およびタグは、異なる範囲の大きさを有するものであり得る。典型的には、核酸断片は、例えば、８０～３００ヌクレオチド長、例えば１００～２００、１００～１５０、およそ１５０ヌクレオチド長、およそ２００ヌクレオチド長などであり得る。タグは、例えば、１５～２５ヌクレオチド長、例えばおよそ１７～１８ヌクレオチド長などであり得る。これらの長さは、例示であって、限定を意図しないことに注意されたい。より短鎖またはより長鎖の断片および／またはタグが使用され得る。
【0104】
また、対のタグを単一の連続核酸から得ることによりライブラリー構築の簡便な方法が提供されるが、対のタグの重要な点は、これらが、自身が元々由来する核酸内である間隔（「分断間隔」）だけ互いに離れており、この分断間隔が所定範囲内に含まれる間隔であることに注意されたい。両タグが所定範囲内に含まれる分断間隔だけ離れていることにより、タグの配列を参照配列（例えば、参照ゲノム配列）に対してアライメントさせることが可能になる。なんら理論に拘束されることを望まないが、このことは、ゲノム再配列決定などのある特定の適用用途に好都合であり得、この場合、参照ゲノムに対する配列の正確な配置が依然として可能なまま、より短い読出し長さの使用が可能になる。５’および３’タグの１対のタグは、より大きな核酸片（例えば、ゲノムＤＮＡ）のセグメントとなり（すなわち、その配列を有する）、該セグメントは、天然に存在するＤＮＡ片内（例えば、ゲノムＤＮＡ片内）において互いから予め規定された間隔内に位置する。例えば、本発明のある特定の実施形態において、５’および３’タグの１対のタグは、天然に存在するＤＮＡ片内で、互いに５００ヌクレオチドまで以内、互いに１ｋＢまで以内、互いに２ｋＢまで以内、互いに５ｋＢまで以内、互いに１０ｋＢまで以内、互いに２０ｋＢまで以内に位置するＤＮＡセグメントである。ある特定の実施形態において、５’および３’タグの１対のタグは、天然に存在するＤＮＡ片内で、５００ヌクレオチド～２ｋＢ離れて、例えば、７００ヌクレオチド～１．２ｋＢ離れて（およそ１ｋＢ離れてなど）位置する。１対のタグの２つのタグが離れている正確な間隔はあまり重要性ではなく、典型的には未知であることに注意されたい。また、タグは、元々は、より大きな核酸片から得られたものであるが、用語「タグ」は、タグの配列を有する任意の核酸セグメント、元々の配列状況に存在したもの、ライブラリー断片で存在したもの、ライブラリー断片からの増幅産物、配列決定対象の鋳型など、いずれも適用される。
【0105】
核酸断片（例えば、ライブラリー分子）は、下記の構造を有するものであり得る。
【0106】
リンカー１－タグ１－リンカー３－タグ１－リンカー２
タグ１およびタグ２は、は、５’および３’タグの１対のタグであり得る。タグのいずれかが５’タグまたは３’タグであり得る。リンカー１およびリンカー２は、１つ以上のプライマーのプライマー結合領域を含む。ある特定の実施形態において、リンカー１および２は、各々が増幅プライマーのＰＢＲおよび配列決定プライマーのＰＢＲを含む。各リンカー内のプライマーは、配列決定プライマーＰＢＲが増幅プライマーＰＢＲに対して内部に位置するようにネスト化（ｎｅｓｔｅｄ）されたものであり得る。リンカー３は、タグ１およびタグ２の配列決定を可能にするため、１つ以上配列決定プライマーのＰＢＲを含むものであり得る。核酸断片のライブラリーに関して使用される用語「リンカー」は、ライブラリーの多数の核酸断片（例えば、ライブラリーの実質的にすべての断片）内に存在する核酸配列をいう。リンカーは、ライブラリーの構築中に実際に連結機能を果たしたものであってもそうでなくてもよく、単に、所与のライブラリーの大部分または全部のメンバーに共通する規定配列とみなされるものであってもよい。かかる配列はまた、「普遍配列」ともいう。したがって、リンカーまたはその一部分に相補的な核酸は、ライブラリーの多数のメンバーにハイブリダイズされ得、ライブラリー内の大部分または全部の分子の増幅プライマーまたは配列決定プライマーとして使用され得る。
【0107】

本発明のある特定の実施形態において、核酸断片は下記の構造を有する。

【0108】

リンカー１－タグ１－内部アダプター－タグ２－リンカー２

タグ１およびタグ２ならびにリンカー１およびリンカー２は、上記のようなＰＢＲを含む。内部アダプターは、ＩＡおよびＩＢ（以下にさらに論考する）ということがある２つのプライマー結合領域を含む。これらのＰＢＲは、一方の集団の核酸がタグ１を含み、他方の集団の核酸がタグ２を含む自身に結合された核酸の２つの異なる実質的に同一の集団を有する微粒子生成させるのに有用である。核酸の２つの異なる集団は少なくとも部分的に異なる配列を有し、例えば、これらはタグの配列領域が異なる。内部アダプターは、スペーサー領域を２つのプライマー結合領域間に含むものであり得る。スペーサー領域は無塩基残基を含み得、これにより、該スペーサーを介するポリメラーゼの伸長が抑制される。もちろん、スペーサーを介するポリメラーゼ伸長が抑制され得る任意の他のブロッキング基を含有するスペーサー領域が使用され得る。

【0109】

他の実施形態において、核酸断片は、１つ以上（例えば、２、４、６など）のさらなるタグおよび１つ以上のさらなる内部アダプターを含む。例えば、核酸断片は下記の構造を有する。

【0110】

リンカー１－タグ１－内部アダプター１－タグ２－リンカー２－タグ３－内部アダプター２－タグ４－リンカー３

本発明の核酸断片およびかかる断片のライブラリー、核酸の２つ以上の実質的に同一の集団を含有する微粒子、ならびにかかる微粒子のアレイは、本明細書に記載のライゲーション系配列決定法以外の広範なさまざまな配列決定法に使用され得ることに注意されたい。例えば、ＦＩＳＳＥＱ、ピロシーケンスなどの配列決定法が使用され得る。例えば、ＷＯ２００５０８２０９８を参照のこと。もちろん、ライゲーション系の方法もまた好都合に使用され得る。本明細書に記載のライゲーション系の方法の状況では、用語「配列決定プライマー」は、「初期オリゴヌクレオチド」を意味すると理解され得ることは認識されよう。

【0111】

本発明のある特定の実施形態において、配列決定対象の鋳型は、ＰＣＲによって、エマルジョンの個々の水性区画（「反応器」ともいう）内で合成される。好ましくは、該区画は、各々が適当な自身に結合された第１の増幅プライマー、第１の鋳型コピー、第２の増幅プライマーおよびＰＣＲ反応に必要な成分（例えば、ヌクレオチド、ポリメラーゼ、補因子など）を有するビーズなどの微粒状の支持体を含む。エマルジョンの調製方法は、例えば、米国特許第６，４８９，１０３号（Ｇｒｉｆｆｉｔｈｓ）；同第５，８３０，６６３号（Ｅｍｂｌｅｔｏｎ）；および米国特許公開公報第２００４０２５３７３１号（Ｇｈａｄｅｓｓｙ）に記載されている。ＰＣＲをエマルジョンの個々の区画内で行ない、微粒子に結合された鋳型のクローン性の集団を生成させる方法（「エマルジョンＰＣＲ」）は、例えば、Ｄｒｅｓｓｍａｎ，　Ｄ．ら，　Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ，１００（１５）：８８１７－８８２２，２００３およびＰＣＴ公開公報ＷＯ２００５０１０１４５に記載されている。

【0112】

前述の参考文献に記載の方法またはその変形法は、配列決定のために微粒子に結合された鋳型のクローン性の集団を生成させるために使用され得る。好ましい非限定的な実施形態において、ＰＣＲに適した短鎖（＜５００ヌクレオチド）鋳型が、普遍アダプター配列を異なる標的配列（鋳型）の集団の各末端に結合させることにより（例えば、ライゲーションによって）創製される（この状況における普遍は、同じアダプター配列が各鋳型に結合され、単一の１対のＰＣＲ増幅プライマーを用いて増幅され得る「アダプター付加」鋳型創製されることを意味する）。バルクＰＣＲ反応液は、アダプター付加鋳型、１種類の遊離増幅プライマー、自身に結合された第２の増幅プライマーを有する微粒子、および他

のＰＣＲ試薬（例えば、ポリメラーゼ、補因子、ヌクレオチドなど）を用いて調製される。水性ＰＣＲ反応液を油相（低分子（１ｉｇｈｔ）鉱油および界面活性剤を含有）と１：２の比で混合する。この混合物をボルテックスして油中水型エマルジョンを創製する。４×１０^{9}より多い水性区画（各々が潜在的ＰＣＲ反応器）をエマルジョン内に創製するのに１ミリリットルの混合物で充分である。エマルジョン試料のアリコートを、マイクロタイタープレート（例えば、９６ウェルプレート、３８４ウェルプレートなど）のウェル内に分配し、サーマルサイクルに供し、微粒子上での固相ＰＣＲ増幅を行なう。クローン性を確保するため、微粒子および鋳型の濃度は、反応器が１つより多くのビーズまたは鋳型分子を含むことは稀であるように注意深く制御する。例えば、本発明のある特定の実施形態において、反応器の少なくとも１０％、２０％、３０％、４０％、５０％、６０％、７０％、８０％、９０％、９５％またはそれ以上が単一のビーズおよび単一の鋳型を含む。したがって、各鋳型のクローン性の集団のメンバーは、その微粒子への結合の結果、空間的に互いに近接して局在化される。一般に、鋳型の結合点は、該粒子の表面上に実質的に均一に分布され得る。増幅手順後の微粒子に付着したクローン性の集団（代表的に数千から数百万コピーの鋳型）の鋳型を有する微粒子は、鋳型増幅を受けたという。

【0113】

個々の微粒子が、自身に結合された５’タグおよび３’タグの１対のタグを含む増幅された核酸断片の異なる集団を有する微粒子の集団を生成させるためにＰＣＲエマルジョン法を用いることは、特に重要である。換言すると、個々の粒子がライブラリー由来の異なる核酸断片（例えば、増幅され、自身に結合された上記のものなど）を有する微粒子の集団生成させることは、特に重要である。

【0114】

ＤＮＡをエマルジョン中で増幅させるための当該技術分野で知られた方法（例えば、上記の参考文献に記載のもの）は、大きな核酸分子の増幅およびこれらの分子の微粒子への結合を達成するその能力に関して制限がある。例えば、より長いアンプリコンを用いると、ＰＣＲ効率は指数関数的に減少することが示されている。このＰＣＲ効率の減少により、対のタグおよびプライマー結合部位を含有する核酸断片（例えば、上記のものなど）が、ＰＣＲエマルジョンで増幅され得、かかる増幅によって微粒子に結合される効率が低下する。したがって、第１および第２のタグの１対のタグを含有する実質的に同一の核酸断片の単一の集団がＰＣＲエマルジョンで増幅され、かかる増幅によってビーズに結合される方法は、いくつかの制限があるという欠点を有する。

【0115】

本発明は、５’および３’タグの１対のタグを含有する単一の核酸断片を増幅によって微粒子に結合させた場合にもたらされる対のタグ情報は依然として保存されたまま、より小さいアンプリコンの使用を可能にするアプローチを提供する。本発明は、自身に結合された核酸の少なくとも２つの異なる集団を有する微粒子（例えば、ビーズ）を提供し、ここで、該少なくとも２つの集団の各々は複数の実質的に同一の核酸からなり、第１の実質的に同一の核酸の集団は、第１の核酸の目的のセグメント（例えば、５’タグ）を含み、核酸の第２の集団は、第２の核酸の目的のセグメント（例えば、３’タグ）を含む。第１および核酸の第２の集団は、該２つのタグを含み、かつ該タグと隣接し分断する適切に配置されたプライマー結合部位も含む単一の大きな核酸断片から増幅され、その結果、２つの増幅反応は、連続的または（好ましくは）同時のいずれかで、ＰＣＲエマルジョン単一の反応器内にて微粒子および増幅試薬の存在下で行なわれ得る。微粒子は自身に結合されたプライマーの２つの異なる集団を有し、該集団の一方は、該核酸断片内の一方のタグの外部のプライマー結合領域を有する配列に対応し、他方は、該核酸断片内の他方のタグの外部のプライマー結合領域を有する配列に対応する。すなわち、プライマー結合領域は該２つのタグと隣接している。

【0116】

また、２つの独立したＰＣＲ反応が行なわれ、各々が、核酸断片の一方のタグを含有する部分を増幅し得るように、該２つのタグ間に位置するプライマー結合領域に結合するプ

ライマーが提供される。増幅された核酸セグメントは、互いに異なるさらなるプライマー結合領域を含み、これらのさらなるプライマー結合領域は、核酸断片内に存在し、増幅プライマーのＰＢＲに対して内部に位置する、すなわち、これらはネスト化されている。これらのさらなるＰＢＲは、２つの異なる配列決定プライマーの結合領域としての機能を果たす。したがって、２つの異なる配列決定プライマーの一方または他方を自身に結合された実質的に同一の核酸セグメントの２つの集団を有する微粒子に適用することにより、２つの核酸セグメントの一方または他方のいずれかが、他方の核酸セグメントの存在による障害なく配列決定され得る。核酸セグメントの各々は、増幅された核酸断片よりも有意に短く、したがって、対のタグを含有する断片のライブラリーを用いてエマルジョン系ＰＣＲが行なわれ得る効率は改善されるが、１対のタグのタグ間の会合は依然として保持されたままである。

【0117】

上記の方法は、図３４および３５の種々のパネルを参照することによって、より良好に理解され得る。図において、同じ配列を有する核酸の部分には同じ色を割り当てている。上記の記載は、図３４および３５と一致するよう解釈されたい。図３４Ａおよび３５Ａは図３５Ａと同じ工程を示すが、さらなる詳細を提供する。図３４Ａおよび３５Ａに示すように、２つのタグ（タグ１およびタグ２）を含有する対末端ライブラリー断片は、内部アダプターカセット（ＩＡ－ＩＢ）および特有の隣接リンカー配列（Ｐ１およびＰ２）を有するように構築される（すなわち、Ｐ１およびＰ２は互いに異なる）。内部アダプターカセットおよび隣接リンカー配列はともに、ＰＣＲ増幅およびＤＮＡ配列決定の両方をもたらすヌクレオチド配列を含む。ＰＣＲプライマー領域は、ネスト化されたＤＮＡ配列決定プライマーの使用が可能になるように設計される。ＤＮＡ捕捉微粒子（ビーズ）は、同一である２つのオリゴヌクレオチド配列を特有の隣接リンカー配列に結合させることにより生成させる。ＰＣＲ増幅のため、Ｐ１およびＰ２　配列を有するオリゴヌクレオチドに結合させたＤＮＡ捕捉微粒子を、単一のジタグライブラリー断片（すなわち、５’タグおよび３’タグの１対のタグを含有するライブラリー断片）および溶液系ＰＣＲプライマーを含有する反応液にシード（ｓｅｅｄ）する。

【0118】

溶液系隣接リンカープライマー（Ｐ１およびＰ２）は、内部アダプタープライマー（ＩＡおよびＩＢ）と比べて限定的な量で添加され、ＰＣＲ生成タグ産物の効率的なビーズ駆動型（ｄｒｉｖｅ－ｔｏ－ｂｅａｄ）増幅を促進する機能を果たす（すなわち、［Ｐ１＜＜ＩＢ］，　［Ｐ２＜＜ＩＡ］）。所望の場合は、プライマーの量を適切に制御することによってもまた、核酸の集団が実質的に同じ数の核酸を含むことが確保され得る（例えば、個々の微粒子上のおよそ半数の核酸が第１の集団に属し、個々の微粒子上のおよそ半数の核酸が第２の集団に属する）。したがって、所望により、種々の集団の比を制御するため、非対称形態のＰＣＲが使用され得る。

【0119】

増幅中、図３４Ｂおよび３５Ｂに示すように（この場合も、図３５Ｂは、図３４Ｂに関するさらなる詳細を提供する）、単一の対末端ライブラリー断片が、４種類のオリゴヌクレオチドプライマー（Ｐ１、Ｐ２、ＩＡおよびＩＢ）の存在下で、２種類の特有のＰＣＲ産物を生成させる。一方の集団は、Ｐ１とＩＡに隣接したタグ１を含み、第２の集団は、Ｐ２とＩＢに隣接したタグ２を含む。

【0120】

増幅微後、粒子には、最初のライブラリー断片から生成されたタグ１およびタグ２に対応する２つの特有のＰＣＲ集団が負荷される。したがって、各タグは、図３４Ｃ、３５Ｃおよび３５Ｄに示すような、各タグの逐次配列決定を可能にする特有の組のプライマー（ｐｒｉｍｉｎｇ）領域を含む。図３５Ｃおよび３５Ｄは、異なる配列決定プライマーを用いたタグ１および２の連続的配列決定を示す。任意のさまざまな配列決定法が使用され得る。

【0121】

上記の方法は、（例えば、４、６、８、１２、１６、２０の集団であって、例えば、該集団は、２、３、４、６、８、１０の対のタグを含む）自身に結合された２つより多くの異なる核酸配列の集団を有する微粒子を生成させるために使用され得る。各集団は、２つのタグの場合で記載のようにして、特有のプライマー結合領域を各配列内に提供することにより個々に配列決定され得る。

【0122】

本発明は、図３４および３５に示す上記の構造を有する核酸断片、かかる断片のライブラリー、自身に結合されたかかる断片由来の核酸セグメントを有する微粒子、かかる微粒子の集団（個々の微粒子は、他の微粒子のものと異なる核酸の集団が自身に結合されている）、微粒子のアレイ、核酸断片からの核酸セグメント（タグ）の増幅のための増幅プライマー、微粒子に結合された核酸セグメントの配列決定のための配列決定プライマー、該断片、ライブラリーおよび微粒子の作製方法、ならびに微粒子に結合された核酸の配列決定法を包含する。本発明は、前述の成分の任意の組合せを含み、任意選択で１種類以上の酵素、バッファーまたは増幅、配列決定などに有用な他の試薬もまた含むキットを包含する。

【0123】

所望の場合は、さまざまな方法が、自身に結合された鋳型を有する微粒子の富化のために使用され得る。例えば、増幅産物（鋳型）の微粒子に結合された部分に相補的なオリゴヌクレオチド（捕捉剤）を、別の（好ましくは、より大きな）微粒子、マイクロタイターウェルまたは他の表面などの捕捉実体に結合させるハイブリダイゼーション系の方法が使用され得る。増幅産物の該部分は標的領域とよばれ得る。標的領域は、増幅中、鋳型内に、例えば、鋳型の未知配列を有する部分の末端に組み込まれ得る。例えば、標的領域は、微粒子に結合されない増幅プライマー内に存在し得、その結果、相補的部分が増幅された鋳型内に存在する。したがって、多数の異なる鋳型が、同じ標的領域を含み得るため、単一の捕捉剤が、多数の異なる鋳型にハイブリダイズし、１つだけのオリゴヌクレオチド配列を捕捉剤として用い、多数の微粒子の捕捉が可能になる。増幅に供された微粒子は、ハイブリダイゼーションが起こり得る条件下で捕捉剤に曝露され、その結果、自身に結合された増幅された鋳型を有する微粒子が、捕捉剤により捕捉実体に結合される。次いで、未結合微粒子を除去し、保持された微粒子を放出させる（例えば、昇温により）。微粒状の捕捉実体が使用されるある特定の実施形態において、自身に結合された微粒子を有する捕捉実体からなる凝集体を、ハイブリダイゼーション後に、結合された微粒子のない微粒状の捕捉実体から、および捕捉実体に結合されていない微粒子から、例えば、粘性溶液（例えば、グリセロール）中での遠心分離によって分離する。大きさ、密度などに基づく他の分離方法もまた使用され得る。しかし、ハイブリダイゼーションは、富化に使用され得るいくつかの方法のうちの１つである。例えば、鋳型内に組み込まれ得る（例えば、合成中）任意のいくつかの異なるリガンドに対して親和性を有する捕捉剤が使用され得る。多数回の富化が使用され得る。

【0124】

図１４Ａは、ＰＣＲ反応が、自身に結合された第１の増幅プライマーを有するビーズ上で、蛍光標識された第２の増幅プライマーおよび過剰の鋳型を用いて行なわれた油中水型エマルジョンの区画の画像を示す。水性反応器は、拡散した遊離プライマーから弱い蛍光を発するが、ビーズは、固相増幅の結果としてビーズ上に蓄積されるプライマーから強い蛍光を発する（すなわち、蛍光プライマーが、ビーズに結合された増幅された鋳型内に第１の増幅プライマーによって組み込まれている）。ビーズシグナルは、種々の大きさの反応器において不均一である。

【0125】

増幅後、微粒子を回収し（例えば、磁気粒子の場合は磁石の使用によって）、本明細書に記載の伸長、ライゲーションおよび切断の反復サイクルによる配列決定に使用する。本発明のある特定の実施形態において、微粒子は、配列決定前に半固相支持体内または該支持体上にアレイ状にする（これは、後述する）。実施例１２、１３、１４および１５は、

（ｉ）　微粒子上での鋳型の合成のための自身に結合された増幅プライマーを有する微粒子の調製（実施例１２）；（ｉｉ）ＰＣＲを行なうための複数の反応器を含むエマルジョンの調製（実施例１３）；（ｉｉｉ）エマルジョンの区画内でのＰＣＲ増幅（実施例１３）；（ｉｖ）エマルジョンの破壊および微粒子の回収（実施例１３）；（ｖ）　自身に結合されたクローン鋳型集団を有する微粒子の富化（実施例１４）；（ｖｉ）　半固形ポリアクリルアミド支持体の基材として供するガラススライドの調製（実施例１５）；ならびに（ｖｉｉ）　微粒子と非重合アクリルアミドとの混合による自身に結合された鋳型を有する微粒子のアレイの形成、基材上のアクリルアミド内への包埋（実施例１５）ために使用され得る代表的で非限定的な方法のさらなる詳細を提供する。また、実施例１５には、ポリメラーゼトラッピング（ｔｒａｐｐｉｎｇ）のプロトコルを記載しており、これは、半固相支持体内でＰＣＲ行なう場合のある特定の方法で使用される。当業者には、これらの方法に対する数多くの変形法が使用され得ることが認識されよう。

【0126】

本発明の他の実施形態において、鋳型は、内部に固定化された適当な増幅プライマーを有するゲルなどの半固相支持体内でのＰＣＲによって増幅される。鋳型、さらなる増幅プライマー、およびＰＣＲ反応に必要な試薬を半固相支持体内に存在させる。１対の増幅プライマーの一方または両方を半固相支持体に、適当な連結部分、例えば、アクリダイト基によって結合させる。結合は、重合中に行なわれ得る。さらなる試薬（例えば、鋳型、第２の増幅プライマー、ポリメラーゼ、ヌクレオチド、補因子など）を、半固相支持体の形成の前に存在させてもよく（例えば、ゲル形成前に液体中）、または試薬の１種類以上をその形成後に半固相支持体中に拡散させてもよい。半固相支持体の細孔径は、かかる拡散を可能にするように選択される。当該技術分野でよく知られているように、ポリアクリルアミドゲルの場合は、細孔径は、主にアクリルアミドモノマー濃度によって、および程度は低いが架橋剤によって決定される。同様の考慮事項が、他の半固相支持体材料の場合に適用される。所望の細孔径を得るために適切な架橋剤および濃度が選択され得る。本発明のある特定の実施形態において、カチオン脂質、ポリアミン、ポリカチオンなどの添加剤を、重合前に該溶液中に含め、ゲル内ミセルを形成させるか、または微粒子周囲に凝集させる。米国特許第５，７０５，６２８号、同第５，８９８，０７１号、および同第６，５３４，２６２号に記載の方法もまた使用され得る。例えば、種々の「クラウディング（ｃｒｏｗｄｉｎｇ）試薬」が、クローンＰＣＲ用のビーズ付近にＤＮＡをクラウディングさせるために使用され得るＳＰＲＩ（登録商標）磁気ビーズ手法および／または条件もまた使用され得る。例えば、１０％ポリエチレングリコール（ＰＥＧ）の存在下での有効なＰＣＲ増幅を示した米国特許第５，６６５，５７２号を参照のこと。本発明の方法のある特定の実施形態において、増幅（例えば、ＰＣＲ）、ライゲーションまたは両方が、ベタイン、ポリエチレングリコール、ＰＶＰ－４０などの存在下で行なわれる。これらの試薬は、溶液に添加しても、エマルジョン中に存在させても、および／または半固相支持体中に拡散させてもよい。

【0127】

半固相支持体は、実質的に平面状の剛性の基材に配置するか、またはその上面に配設する。ある特定の好ましい実施形態において、該基材は、典型的な標識（例えば、蛍光標識、量子ドット、プラスモン共鳴粒子、ナノクラスター）の励起および検出に使用される励起および発光波長（例えば、およそ４００～９００ｎｍ）の放射線に対して透過性である。ガラス、プラスチック、石英などの材料が適当である。半固相支持体を基材に接着してもよく、任意選択で、任意のさまざまな方法を用いて基材に固定させ得る。

基質は、接着または結合を増強する物質（例えば、シラン、ポリリシンなど）でコーティングされたものであってもそうでなくてもよい。米国特許第６，５１１，８０３号には、半固相支持体内でのＰＣＲを用いて鋳型のクローン性の集団合成するための方法、実質的に平面状の基材上に半固相支持体を作製するための方法などが記載されている。同様の方法が本発明に使用され得る。基材は、半固形基材の形成前の液体を入れるためのウェルまたは凹部を有するものであってもよい。あるいはまた、高くした障壁またはマスクがこの

目的のために使用され得る。

【0128】

上記のアプローチは、空間的に局在化されるクローン鋳型の集団を生成させるためのエマルジョン中の反応器（ｒｅａｃｔｏｒ）の使用の代替法を提供する。クローン性の集団は半固相支持体内の別個の位置に、新たにライゲートされた伸長プローブを検出する（例えば、画像化によって）目的のために、シグナルが、配列決定中に各集団から取得され得るように存在する。本発明のある一部の実施形態において、２つ以上の異なるクローン性の集団が単一の核酸断片から増幅され、半固相支持体内の別個の位置に混合物として存在する。混合物中のクローン性の集団の各々は、例えば、別個の位置に５’タグを含有する断片および３’タグを含有する断片が含まれるようにタグを含み得る。５’タグおよび３’タグを含むクローン鋳型は異なる配列決定プライマーを含み、その結果、これらは、互いに独立して配列決定され得る。このアプローチは、多数の実質的に同一の核酸の集団を微粒子上に生成させ、単一の微粒子から１対のタグの両方のメンバーの配列決定情報得るための上記のアプローチ同一である。

【0129】

一般に、任意の本発明の方法における使用のための半固相支持体は、約１００ミクロン以下の厚さ、例えば約５０ミクロン厚以下、例えば約２０～４０ミクロン厚（両端を含む）の層を形成したものである。カバースリップまたは実質的に平面状の表面を有する他の類似した物体を、半固相支持体材料の上に、好ましくは重合前に配設し得、均一なゲル層の作製、例えば、実質的に平面状および／または実質的に均一な厚さのゲル層の作製が補助される。

【0130】

また本発明の他の実施形態において、上記の方法に対し、鋳型が、自身に結合された適当な増幅プライマーを有する微粒子上でのＰＣＲによって合成され、該微粒子が鋳型合成前に半固相支持体内または該支持体上に固定化される、すなわち、これらは、完全または部分的に半固相支持体内に包埋される変形法が使用される。一般的に、微粒子は半固相支持体材料に完全に囲まれるが、基材上に載っていてもその下方にあってもよい。したがって、微粒子は、半固相支持体が破壊されな限り、互いに対して実質的に固定された位置に維持される。このアプローチは、空間的に局在化されるクローン鋳型の集団を生成させるためのエマルジョンの使用別の代替法を提供する。微粒子は、半固相支持体の形成前に液体と混合してもよい。あるいはまた、微粒子を、実質的に平面状の基材上でアレイ状にし、重合、架橋などの前に液体を微粒子アレイに添加してもよい。微粒子は、自身に結合された第１の増幅プライマーを有する。第２の増幅プライマーは、半固相支持体に結合されたものであり得るが、そうである必要はない。さらなる試薬（例えば、鋳型、第２の増幅プライマー、ポリメラーゼ、ヌクレオチド、補因子など）を、半固相支持体の形成前に存在させてもよく（例えば、ゲル形成前に液体中）、またはこれらの試薬の１種類以上をゲル形成後に半固相支持体中に拡散させてもよい。半固形基材は、一般的に、上記のようにして例えばガラススライド上に形成される。

【0131】

本発明のある特定の実施形態において、ゲルは、鋳型合成後に、クローン鋳型集団が結合された微粒子が簡便に回収され得るように（例えば、磁気粒子の場合は磁石の使用によって）、可溶化（例えば、消化または解重合または溶解）させ得る。可溶化、消化、解重合、溶解させなどさせ得るゲルを、本明細書において「可逆的」という。慣用的なポリアクリルアミド重合は、Ｎ－Ｎ’メチレンビスアクリルアミド（ＢＩＳ）を架橋剤と、重合を開始させる適当な触媒（例えば、Ｎ，Ｎ，Ｎ’，Ｎ’－テトラメチルエチレンジアミン（ＴＥＭＥＤ））と一緒に使用する。可逆的ゲルを作製するため、代替的な架橋剤、例えば、Ｎ－Ｎ’ジアリル酒石酸ジアミド（ＤＡＴＤ）などが使用され得る。この化合物は、ＢＩＳに構造的に類似しているが、過ヨウ素酸によって（例えば、過ヨウ素酸ナトリウムを含有する溶液中）切断され得るシス－ジオール基を有する（Ａｎｋｅｒ，　Ｈ．Ｓ．：Ｆ．Ｅ．Ｂ．Ｓ．Ｌｅｔｔ．，　７：２９３，１９７０）。したがって、ＤＡＴＤゲルは

、容易に可溶化され得る。架橋剤としてＤＡＴＤを用いて作製されたゲルは、高度に透明であり、ガラスに良好に結合する。可逆的ゲルを形成するＤＡＴＤ様特性を有する別の架橋剤は、エチレンジアクリレートである（Ｃｈｏｕｌｅｓ，　Ｇ．Ｌ．およびＺｉｍｍ，　Ｂ．Ｓ．：Ａｎａｌ．Ｂｉｏｃｈｅｍ．，１３：３３６－３３９，１９６５）。Ｎ，Ｎ’－ビスアクリリルシスタミン（ＢＡＣ）は、可逆的ポリアクリルアミドゲルを形成するために使用され得る別の架橋剤である。過ヨウ素酸塩に溶解されるゲルを形成するために使用され得る別の架橋剤はＮ，Ｎ’－（１，２－ジヒドロキシエチレン）ビス－アクリルアミド（ＤＨＥＢＡ）である。可逆的半固相支持体を形成する任意のさまざまな他の材料もまた使用され得る。例えば、熱可逆的ポリマー、例えば、Ｐｌｕｒｏｎｉｃｓ（ＢＡＳＦから入手可能）　などが使用され得る。Ｐｌｕｒｏｎｉｃｓは、ポリ（エチレンオキシド）－ポリ（プロピレンオキシド）－ポリ（エチレンオキシド）（ＰＥＯ－ＰＰＯ－ＰＥＯ）トリブロックコポリマーのファミリーの１つである（Ｎａｃｅ，　Ｖ．Ｍ．ら、Ｎｏｎｉｏｎｉｃ　Ｓｕｒｆａｃｔａｎｔｓ，　Ｍａｒｃｅｌ－Ｄｅｋｋｅｒ，　ＮＹ，１９９６）。これらの材料は、高温（例えば、室温より高い温度）で半固形（ゲル）になり、冷却すると液化する。種々の方法が、Ｐｌｕｒｏｎｉｃｓを化学的に誘導体化し、例えば、プライマーとの結合を助長するために使用され得る（例えば、Ｎｅｆｆ，　Ｊ．Ａ．ら、Ｊ．Ｂｉｏｍｅｄ．Ｍａｔｅｒ．Ｒｅｓ．，４０：５１１，１９９８；Ｐｒｕｄ’ｈｏｍｍｅ，　ＲＫら、Ｌａｎｇｍｕｉｒ，１２：４６５１，１９９６を参照）。

【0132】

可溶化後、微粒子を回収し、伸長、ライゲーションおよび切断の反復サイクルを用いた配列決定に供し得る。配列決定前に、微粒子を第２の半固相支持体内または該支持体上に、例えば、第１の半固相支持体内または該支持体上に存在したときより高い密度でアレイ状にしてもよい。半固相支持体は、典型的には、それ自体が、実質的に平面状で剛性の基材、例えばガラススライドによって支持されている。

【0133】

したがって、２つの一般的なアプローチは、半固相支持体内または該支持体上に包埋されたクローン鋳型集団を担持する微粒子のアレイを有する半固相支持体を作製するために使用され得る。第１のアプローチは、半固相支持体内に存在しない微粒子上で増幅を行ない（例えば、エマルジョン系ＰＣＲによって）、次いで、微粒子を半固相支持体内または該支持体上に固定化することを伴う。第２の一般的なアプローチは、微粒子を半固相支持体内または該支持体上に固定化し、次いで、増幅を行なうことを伴う。いずれの場合も、微粒子の凝集を低減させるため、および／または微粒子を実質的に単一の焦点面に整列させるための手順を用いることが望ましいことがあり得る。例えば、粒子をポリアクリルアミドゲル内に固定化させる場合、モノマーおよび架橋剤の濃度は、重合が完了する前に粒子が溶液の底面に沈降し、その結果、下方の平面状の基材上に沈殿し、したがって、単一の平面状に整列するように選択される。本発明のある特定の実施形態において、カバースリップなどの実質的に平面状の表面を有する物体を、微粒子を含有する液状アクリルアミド（または半固相支持体を形成できる他の材料）の上面に配置し、アクリルアミドが２つの層間に捕捉され「サンドイッチ」構造となるようにする。次いで、該サンドイッチを裏返し、重力の作用によって、微粒子が下方に沈降し、カバースリップ（実質的に平面状の表面を有する他の物体）上に堆積するようにする。重合後、カバースリップを取り除く。したがって、微粒子は、実質的に単一の平面状に半固相支持体の表面に近接して（例えば、表面に接して（ｔａｎｇｅｎｔ））包埋される。

【0134】

上記のように微粒子などの支持体を半固形マトリックス内に固定化するのではなく、本発明のある特定の実施形態では、微粒子を、固定化のための半固相支持体を使用することなく、共有結合または非共有結合のいずれかで実質的に平面状で剛性の基材に結合させ、それによって「ゲル非含有」または「ゲル無し」微粒子アレイをもたらす。微粒子を基材、例えば、ガラス、プラスチック、石英、シリコンなどなどに結合させるためのさまざまな方法が、当該技術分野で知られている。基材は、コーティングされたものであってもそ

うでなくてもよく（例えば、スピンコーティング）、または結合を助長する材料（例えば、任意のさまざまなポリマー）もしくは薬剤により官能性付与させたものであってもよい。コーティングは、薄膜、自己組織型単層などであり得る。微粒子、微粒子に付着した部分、または微粒子に付着したオリゴヌクレオチド（例えば、鋳型）のいずれかを基材に付着させることができる。本発明のある実施形態において、基材はシラン化剤で処理されず、あるいはそのように処理されたとしても、その処理は有効なシラン化をもたらさず、すなわち例えば、本明細書に記載のライゲーションに基づく配列決定を複数回繰り返す時に行われるような、その後の操作および／または流体との接触に対して安定した形で、平坦なガラス表面上のポリアクリルアミド層により固定された微粒子のアレイを形成するように、シラン化が有効に作用しない。この文脈において「安定」とは、一般的に、操作および／または流体との接触時にゲルが基材に固着したまま維持され、著しい屈曲や、脱離、離層を生じないことを意味する。本発明者等は、微粒子アレイの作成にゲルなどの半固形培地の使用を避けることにより、多くの利点が得られることを認識した。例えば、（ｉ）半固形培地の非存在下では、試薬の拡散がより早くなり、かつライゲーションされていないプローブや酵素などの望ましくない種の除去もより早くなり、（ｉｉ）有効なシラン化が行われなければ、アクリルアミドなどのゲルが基材に安定して固着される状態が維持されず、（ｉｉｉ）重合は酸素などの環境特性に敏感であることから、重合手順を排除することで、アレイ生成プロセスにおける不均一性の潜在的な原因が除去され、（ｉｖ）半固形培地を使用しないことで、一つの焦点面により多くの微粒子が収まるようになり、（ｖ）微粒子は、基材に付着した場合の方が、半固形培地（特に重合が損なわれるもの）に埋め込まれた場合よりも、適切な位置により安定して固着される。

【0135】

一般的に、当該技術分野で既知の多様な方法のいずれを使用しても、このような核酸が微粒子あるいはその他の担体または基材に付着しやすくなるように、オリゴヌクレオチドプライマー、プローブ、鋳型などの核酸を修飾することができる。さらに、当該技術分野で既知の多様な方法のいずれを使用しても、核酸が付着しやすくなるように微粒子またはその他の担体を修飾して、担体や基材などに微粒子が付着しやすくなるようにすることができる。所望の官能基が付着しやすくなるような界面化学物質を有するミクロスフェアが使用可能である。これらの界面化学物質のいくつかの例には、アミノ基（例えば、脂肪族および芳香族アミン）、カルボン酸、アルデヒド、アミド、クロロメチル基、ヒドラジド、ヒドロキシル基、スルホン酸塩、および硫酸塩が含まれるが、これらに限定されない。これらの基は核酸中に存在する基と反応する場合があり、あるいは核酸が反応基の付着によって修飾される場合もある。さらに、ホモ二官能性およびヘテロ二官能性リンカーを含めた多くの安定した二官能基も当該技術分野で周知である。例えば、ＵＲＬ：ｗｗｗ．ｐｉｅｒｃｅｎｅｔ．ｃｏｍのウェブサイトで閲覧可能なＰｉｅｒｃｅ　Ｃｈｅｍｉｃａｌ　Ｔｅｃｈｎｉｃａｌ　Ｌｉｂｒａｒｙ（１９９４－９５　Ｐｉｅｒｃｅ　Ｃａｔａｌｏｇに最初に公開）、およびＧ．Ｔ．Ｈｅｒｍａｎｓｏｎ，Ｂｉｏｃｏｎｊｕｇａｔｅ　Ｔｅｃｈｎｉｑｕｅｓ，Ａｃａｄｅｍｉｃ　Ｐｒｅｓｓ，Ｉｎｃ．，１９９６を参照されたい。また、米国特許第６，６３２，６５５号も参照されたい。

【0136】

一般に、結合ペアを形成するように互いに対して親和性を示す任意の対の分子が、微粒子または鋳型を基材に結合させるために使用され得る。結合ペアの第１のメンバーを共有結合または非共有結合により基材に結合させ、結合ペアの第２のメンバーを共有結合または非共有結合により微粒子または鋳型に結合させる。説明上、結合対の第１のメンバー、すなわち基材に付着した結合パートナーは、本明細書でＢＰ１と呼び、結合対の第２のメンバー、すなわち微粒子または鋳型に付着した結合パートナーは、（ＢＰ２）と呼ぶ。第１の結合パートナー（ＢＰ１）をリンカーによって基材に結合させてもよい。第２の結合パートナー（ＢＰ２）をリンカーによって微粒子または鋳型に結合させてもよい。例えば、アプローチの一例によれば、スライドまたは他の適当な基質をアミン反応性基（例えば、アミン反応性基を含有するＰＥＧリンカーを使用）で修飾する。アミン反応性基は、水

性条件で（例えば、ｐＨ８．０で）任意のタンパク質（例えば、ストレプトアビジン）中のアミン（例えば、リシン）と反応する。したがって、アミンを担持する部分により官能性付与させた微粒子は、基材上に固定化された状態になる。アミン担持する部分は、タンパク質または適当に官能性付与された核酸、例えば、ＤＮＡ鋳型であり得る。多数の部分がビーズに結合され得る。例えば、ビーズは、ＮＨＳエステルと反応してビーズを基材に結合させる自身に結合されたタンパク質を有し得、また、ＤＮＡ自身に結合された鋳型を有し得、これは、ビーズが基材に結合された後、配列決定され得る。アミン反応性ＮＨＳ部分を一方の末端に有するポリマーテザー（ｔｅｔｈｅｒ）　を担持する適当にコーティングされスライドは、例えば、Ｓｃｈｏｔｔ　Ｎｅｘｔｅｒｉｏｎ，　Ｓｃｈｏｔｔ　Ｎｏｒｔｈ　Ａｍｅｒｉｃａ，　Ｉｎｃ．，　Ｅｌｍｓｆｏｒｄ，　ＮＹ　１０５２３から市販されている。あるいはまた、コーティングされたスライド（例えば、ビオチン－コートスライド）が、Ａｃｃｅｌｒ８　Ｔｅｃｈｎｏｌｏｇｙ　Ｃｏｒｐｏｒａｔｉｏｎ，　Ｄｅｎｖｅｒ，　ＣＯから市販されている。そのＯｐｔｉＣｈｅｍ™技術は、基材への微粒子の結合方法の代表であるが一例である。例えば、米国特許第６，８４４，０２８号を参照のこと。あるいはまた、微粒子は基材に、ビーズ上のポリヌクレオチドをビオチンにより官能性付与し（例えば、末端トランスフェラーゼとビオチン－ジデオキシＡＴＰおよび／またはビオチン－デオキシＡＴＰの使用によって）、次いで、これをストレプトアビジン－コーティングされたスライドのような基材（例えば、Ａｃｃｅｌｒ８　Ｔｅｃｈｎｏｌｏｇｙ　Ｃｏｒｐｏｒａｔｉｏｎ，　Ｄｅｎｖｅｒ，　ＣＯから入手可能）（米国特許第６，８４４，０２８号を参照のこと）と、ビオチン－ストレプトアビジン結合の形成を促進する条件下で接触させることにより結合させ得る。一実施形態において、ストレプトアビジンは、ＰＥＧリンカーを使用して基材に付着する。一実施形態において、微粒子結合ポリヌクレオチドは、合成後にビオチンで官能基化される。別の実施形態において、ビオチンは、例えばエマルジョンＰＣＲを実施する時などの増幅時に、ビオチン化プライマーを使用することにより、合成時にポリヌクレオチドに組み込まれる。例えば、第１のプライマーＰ１は、微粒子に共有結合的にまたは非共有結合的に付着する。微粒子に結合していない第２のプライマーＰ２はビオチン部分を含み、したがって、得られるＰＣＲ産物はビオチンを含む。

【0137】

そのため、本発明は、核酸鋳型が付着した微粒子を捕捉し、基材（例えば、ガラススライドなどの実質的に平面状で剛性の基材）の表面に、それらの微粒子を繋ぐ方法を提供する。特に興味深い実施形態においては、鋳型の種々のクローン群が付着した微粒子の群が作成され（例えば、エマルジョンＰＣＲを使用して）、鋳型がビオチン部分を含む。ビオチンは、増幅後に標準的な方法を使用して鋳型に付着する場合がある。次に、微粒子は、ビオチン結合部分（例えば、ストレプトアビジンなどのビオチン結合性タンパク質）が付着したガラススライドなど、実質的に平面状で剛性の基材と接触する。鋳型分子上のビオチンは、ビオチン結合部分に結合し、それによってビオチンおよびビオチン結合性タンパク質を含む連結を介して、微粒子を基材に付着させる。したがって、微粒子の基材への付着は、鋳型がテザーの役割を果たす間接的なものである場合がある。一実施形態において、鋳型分子の一端は、ビーズに付着したビオチン結合部分に付着し、鋳型分子の他端は、基材に付着したビオチン結合部分に付着する。

【0138】

ある実施形態において、一本鎖鋳型の一方の末端は微粒子に付着し、一本鎖鋳型の他方の末端は基材に付着する。したがって、一実施形態においては、一本鎖鋳型の３’および５’の両末端が、微粒子を基材に付着させる役割を果たす連結に関与し、第１の連結は微粒子と鋳型の間にあり、第２の連結は鋳型と基材との間にある。得られる構造は、ハイブリダイズした核酸が解離しやすくなる傾向があるとされる熱や他の条件に対しても安定している。

【0139】

実施例１６に記載の通り、ストレプトアビジンでコーティングされた微粒子に付着した

鋳型は、合成後のエマルジョンＰＣＲ時にビオチン化することができ、得られるビオチン化した鋳型は、ストレプトアビジンでコーティングされた基材に効率的かつ強固に結合することが発見されている。一実施形態において、ビオチンとストレプトアビジンの連結は、本方法において以下の２つの段階で使用される：（ｉ）鋳型の増幅前に（例えば、エマルジョンＰＣＲ前に）、ビオチン化プライマーをストレプトアビジンでコーティングされた微粒子に付着させる；（ｉｉ）増幅後、自由端（微粒子に付着していない端部）がビオチン化された微粒子結合鋳型をストレプトアビジンでコーティングされた基材に付着させ、それによって微粒子も基材に固定する。場合により、手順（ｉ）の後、エマルジョンＰＣＲ（またはその他の増幅方法）を行った微粒子群において、増幅された微粒子を濃縮することもできる。手順（ｉｉ）の前、そして場合により濃縮の後には、ストレプトアビジンを露出した微粒子表面の部分を被覆するために、微粒子をビオチン化オリゴヌクレオチドとともにインキュベートすることもできる。これらの方法により、半固形培地を必要とすることなく、基材の表面に安定して付着した微粒子のアレイをもたらされる。特に興味深い実施形態において、基材は、ガラススライドなどの実質的に平面状で剛性の基材である。本明細書ではビオチンとストレプトアビジンの相互作用が例示されているが、ストレプトアビジンは、ビオチンに結合するいくつかのタンパク質の内の１つにすぎず、これらのタンパク質はいずれも本発明で使用できることが理解される。例えば、アビジンは卵白タンパク質であり、細菌性ストレプトアビジンのように、高い親和性と選択性を持ってビオチンに結合する。ＮｅｕｔｒＡｖｉｄｉｎは、炭水化物を除去する処理を行ったアビジンの誘導体である。ＣａｐｔＡｖｉｄｉｎは、ｐＨ９を超えるビオチン化分子に対する親和性を減少させたアビジンの誘導体である。したがって、ビオチン化分子は、中性のｐＨで結合させることができ、約ｐＨ１０で解放することができる。ＮｅｕｔｒＡｖｉｄｉｎおよびＣａｐｔＡｖｉｄｉｎについては、オンライン版のＴｈｅ　Ｈａｎｄｂｏｏｋ　ｏｆ　Ｆｌｕｏｒｅｓｃｅｎｔ　Ｐｒｏｂｅｓ　ａｎｄ　Ｒｅｓｅａｒｃｈ　Ｐｒｏｄｕｃｔｓ（ｈｔｔｐ：／／ｐｒｏｂｅｓ．ｉｎｖｉｔｒｏｇｅｎ．ｃｏｍ／ｈａｎｄｂｏｏｋ／ｓｅｃｔｉｏｎｓ／０７０６．ｈｔｍｌ；２００６年４月１７日閲覧）に記載されており、Ｉｎｖｉｔｒｏｇｅｎ（米国カリフォルニア州カールズバッド）から入手することができる。さらに、本発明は、特異的かつ高親和性の相互作用を示すいずれかの分子対の使用を包含する。例えば、特異的結合対のメンバーは、抗体と抗原、受容体と受容体リガンド（例えば、小分子またはペプチド）、金属と金属結合剤（例えば、Ｎｉ＋と６ＸＨｉｓタグ）などであると考えられる。本発明は、上述のいずれかの方法を使用して基材に付着した微粒子を提供し、さらに、種々の鋳型が付着している、基材に付着した微粒子を含むアレイも提供する。

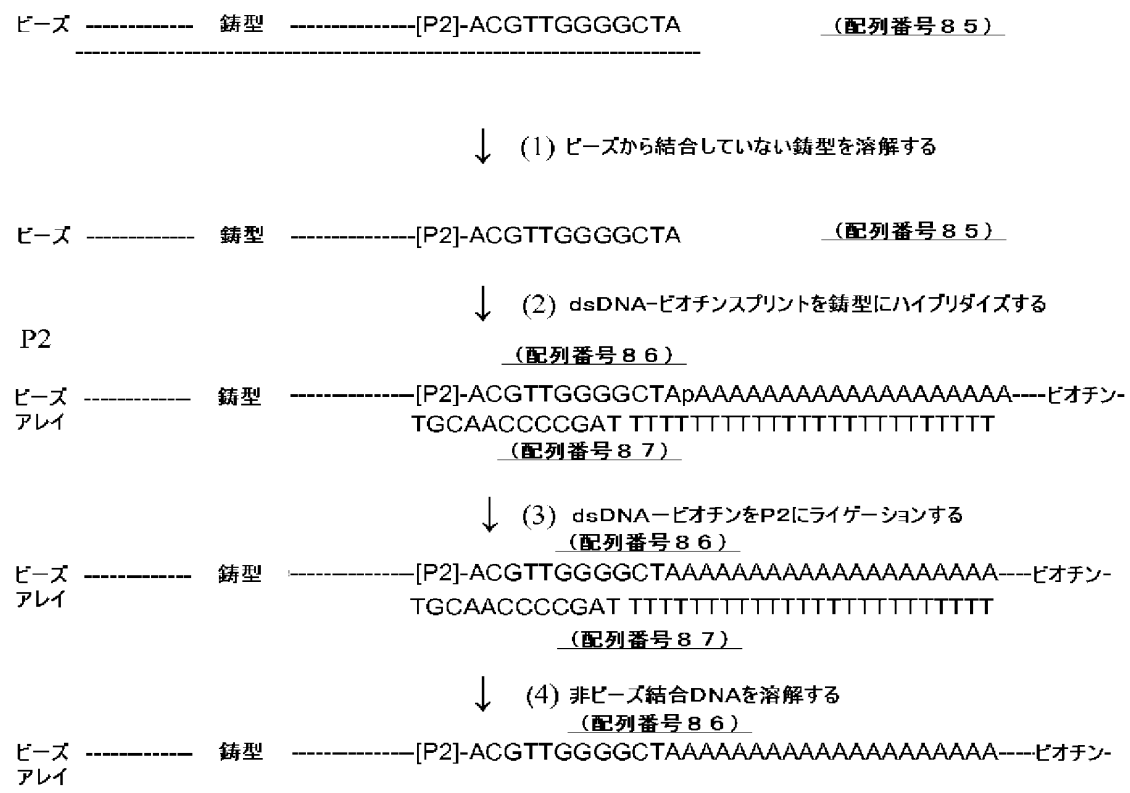
【0140】

本発明のある実施形態において、ゲル非含有微粒子アレイの形成は、鋳型の複数の複製が付着した（例えば、鋳型の少なくとも数千、典型的には数百万の複製が付着した）微粒子を、鋳型の複数の複製が付着していない微粒子から分離する役割を果たす。一実施形態において、基材には第１の結合パートナー（ＢＰ１）が付着し、微粒子に付着した鋳型分子は第２の結合パートナー（ＢＰ２）を含み、ＢＰ１とＢＰ２は互いに特異的に結合し、すなわち、これらは特異的結合対のメンバーである。上述のようにゲル非含有微粒子アレイが形成されると、ＢＰ２が付着した鋳型を有する微粒子のみが基材に付着することになる。別の実施形態において、基材には第１の反応部分（Ｒ１）が付着しており、微粒子に付着した鋳型分子は第２の反応部分（Ｒ２）を含み、Ｒ１とＲ２は互いに反応して、共有結合を形成する。上述のようにゲル非含有微粒子アレイが形成されると、ＢＰ２またはＲ２が付着した鋳型を有する微粒子のみが基材に付着することになる。結合または反応させた後、付着していない微粒子は、例えば軽い撹拌および／または洗浄によって除去することができる。本方法は、典型的には、鋳型の種々のクローン群が付着した微粒子を含み、また鋳型の複数の複製が付着していない微粒子も含む、微粒子群に適用される。例えば、本方法は、鋳型の増幅が行われた（例えば、エマルジョンＰＣＲ時に）微粒子を、実質的な鋳型増幅が行われていない微粒子から分離させるために使用される場合がある。一実施

形態において、本方法は以下の手順を含む：（ｉ）特異的結合対の第１のメンバーまたは反応部分が付着した基材を提供する（ｉｉ）（結合対のメンバー間、あるいは反応部分間で）結合を行うのに好適な条件下で、特異的結合対の第２のメンバーまたは反応部分が付着した鋳型の複数の複製を少なくともいくつか有する微粒子群と、基材を接触させる；（ｉｉｉ）非結合微粒子を除去する。強い非共有結合連結を形成する特異的結合パートナー（例えば、ストレプトアビジンとビオチン）は、濃縮を達成するのに特に興味深い。別の実施形態において、相補性オリゴヌクレオチド間のハイブリダイゼーションが使用される。例えば、一実施形態においては、エマルジョンＰＣＲ時に鋳型に組み込まれる遊離ＰＣＲプライマー（遊離ＰＣＲプライマーとは、微粒子に付着していないプライマーである）の一部と相補的であるように選択されるオリゴヌクレオチドが、基材に付着する。遊離ＰＣＲプライマーは、増幅が成功した場合にのみ微粒子上に存在するため、鋳型増幅に成功した微粒子のみが基材に付着する。リガーゼを使用して、ハイブリダイゼーション事象の品質を確認し、ビオチン化スプリントまたはプライマーをビーズ上の鋳型の３’端に共有結合的に結合させる場合もある。例えば、以下の一連の手順を実施することができるが、これらの手順において、「ビーズ」とは微粒子を表し、Ｐ２とは増幅プライマー配列の少なくとも一部を表し、「ｄｓ」とは「二本鎖」を意味し、「アレイ」とは、増幅に成功した微粒子がビオチンを介して付着することができる基材を指す。二本鎖鋳型が付着した微粒子が提供される。第１の手順では、例えば温度を上昇させることによって、非結合鋳型が除去される。第２の手順では、一本鎖伸長物を有する二本鎖核酸が、鋳型にハイブリダイズされる。二本鎖核酸は、ビオチンを鋳型に安定して結合させることができるブリッジまたはスプリントの役割を果たす。一本鎖伸長物を有さない二本鎖核酸の鎖は、一本鎖伸長物とは反対の末端にビオチン部分が付着している。第３の手順では、リガーゼが存在する。ビオチンを含む二本鎖核酸は、ハイブリダイゼーションに成功した場合に鋳型にライゲーションされ、それによってビオチンを鋳型に安定して結合させる。第４の手順では、鋳型にライゲーションされなかったスプリントの鎖が、例えば温度を上昇させることによって解放される。ビオチンと、基材または担体に結合したストレプトアビジンとの相互作用によって、微粒子のアレイが作成される。

【数1】

【図40】



本方法は、複数の鋳型が付着した微粒子を、複数の鋳型が付着していない、あるいは実質的により少ない鋳型が付着した微粒子から分離させるために使用することができ、これらの鋳型は増幅または合成後に微粒子に付着する。分離させる微粒子は、微粒子結合鋳型の増幅または合成を行うか、あるいは増幅された鋳型の複数の複製が微粒子に付着することができるいずれかの種類の条件にさらされている場合がある。増幅方法は、ＰＣＲ増幅、ローリングサークル増幅、またはその他いずれかの種類の核酸増幅である場合がある。本方法は、本発明の他の方法および組成物のいずれかと組み合わせる、および／または併用することができる。接触手順は、典型的には液体培地内で行われる。本発明のある実施形態において、接触手順では、微粒子を含有する液体を、特異的結合対または反応部分が付着した基材に流す。基材は、例えば、流体入口と流体出口とを有するフローセルなどのチャンバー内に配置される場合がある。微粒子は、基材に付着した微粒子の所望の密度または数に達するまで、基材を流す場合がある。一定期間、密度または数の変化を監視する場合もある（例えば、画像化による）。特に興味深い実施形態において、本方法は、エマルジョンＰＣＲ時に増幅が行われた微粒子を、エマルジョンＰＣＲ時に実質的な鋳型の増幅が行われなかった微粒子から分離させるために使用される。本方法では、鋳型の増幅が行われた微粒子を濃縮する。基材に結合した微粒子に付着した鋳型には、種々のさらなる反応または操作を行うことができる。これらは、例えば、本明細書に記載のようなライゲーションに基づく配列決定、またはＦＩＳＳＥＱやパイロシーケンシングなどの他の配列決定法などの配列決定であってよい。例えば、半固形培地を使用せずに、および／または半固形培地の非存在下で、基材に付着した微粒子に付着した鋳型に対して、本明細書に記載の本発明の配列法のいずれも実施することができる。

【0141】

微粒子が基材または半固形培地に付着する本発明の実施形態のいずれにおいても、微粒子はその後に解放し、場合により除去することができる（例えば、洗浄による）。微粒子を解放するのに適切な方法は、微粒子が基材または半固形培地に付着する上で介する特定の共有結合または非共有結合連結によって異なる。ＤＮＡ鋳型を実質的に損傷しない限り

、あるいは基材または半固形培地からＤＮＡ鋳型を解放しない限り、いずれの好適な方法も使用することができる。例えば、一実施形態において、微粒子は、例えばジスルフィドまたはエステル連結を含有するリンカーなどの開裂可能なリンカーによって基材または半固形培地に付着する。

【0142】

本発明のある実施形態において、微粒子は、半固形培地に安定して付着した鋳型のクローン群のアレイを作成するために使用される。本方法では、１つ以上の鋳型分子が付着した微粒子が、例えば実質的に平面状で剛性の基材上に位置するポリアクリルアミドゲルなど、基材上に位置する半固形培地の存在下でインキュベートされ、半固形培地に固定したおよび／または付着したプライマーに鋳型がハイブリダイズされる。次にプライマーが伸長され（例えば、ＤＮＡポリメラーゼを使用）、半固形培地に付着したまたは固定した相補性鋳型の合成を生じる。微粒子は、例えばインキュベーションのストリンジェンシーを上昇させることによって（例えば、温度を上昇させることによって）解放され、それによって２本の相補性鋳型鎖が分離する。例えば、微粒子に付着した鋳型を開裂させることによって、あるいは微粒子を鋳型から脱離させることによって、微粒子を解放する代替の方法を使用することもできると考えられる。

【0143】

本プロセスは、微粒子結合鋳型の複製または「インプリント」を半固形培地に転写する。本プロセスの効率は、微粒子から半固形培地に複製した鋳型分子の数を、微粒子に付着した鋳型分子の数で除算したものとして定義される場合がある。幾何学的および物理的考察に基づくと、決して本発明を限定するものではないが、２００ｂｐのサイズの鋳型分子約１５０，０００個が付着した直径１μｍの微粒子は、図４０に示す通り、直径約５００ｎｍの接触パッチを有する場合がある。接触パッチは、半固形培地または基材の中または上に位置するプライマーを伸長することによって、微粒子に付着したものと相補的な鋳型を合成できるように、培地の表面上に位置するかあるいは培地に部分的に埋め込んだ微粒子に十分に近接している、半固形培地または基材の領域を指す。具体的には、直径１ミクロンのビーズは、３．１×１０^{6} ｎｍ^{2} の面積を有し、したがってビーズ上の１５０，０００個のＤＮＡ分子は、平均面積２０．９ｎｍ^{2} または平均距離４．５７ｎｍとなる。Ｂ－ＤＮＡの直径は約１．９ｎｍであり、２００ｂｐのＢ－ＤＮＡの長さは６８ｎｍである。したがって、６８ｎｍの分離点までの１ミクロンビーズの接触パッチは、半径２５２ｎｍまたは面積１９９，０００ｎｍ^{2} である。ＤＮＡ分子１個当たり２０．９ｎｍ^{2} で、パッチは９，５００個もの分子、またはビーズの下半分上の分子の数の約１３％を含有すると予想される。

【0144】

場合により、半固形培地と連結したままの鋳型の増幅が１回以上行われる。一実施形態において、増幅はローリングサークル増幅（ＲＣＡ；米国特許第５，８５４，０３３号；第６，１４３，４９５号）である。ＲＣＡを行う前に、（ｉ）鋳型の２つの非近接領域への環状化可能プローブ（「パドロックプローブ」）のハイブリダイゼーション、（ｉｉ）ポリメラーゼを使用した、発生したギャップの充填、（ｉｉｉ）末端のライゲーション、を含む手順が行われる場合がある。ＲＣＡで使用する鋳型分子は、配列決定する部分だけでなく、環状化可能なプローブと相補的な領域も含まなければならないことが理解される。

【0145】

プライマー伸長と場合による増幅によって、半固形培地に付着または固定した、「スポット」のアレイまたは核酸「コロニー」がもたらされる。コロニーは、微粒子を沈着した位置に対応する場所に位置する。コロニーの多くまたはほとんどは、鋳型の単一のクローン群、あるいは本発明のある実施形態においては、鋳型の２つまたは数個までのクローン群（微粒子に複数種類の鋳型が付着していた場合）から構成される。類似の手法を使用して、プライマーを、基材上に位置する半固形培地ではなく、基材自体に付着させることによって、半固形培地を使用することなく、ガラススライドなどの基材上に核酸コロニーの

アレイを直接作成することもできると考えられる。

【0146】

いかなる理論にも拘束されるものではないが、上述のように微粒子を使用して核酸コロニーのアレイを形成することで、多くの利点が得られる。微粒子は、アレイを形成するために使用する前に、鋳型の増幅を行うことができ、場合により濃縮も行うことができ、それによって各核酸スポットは、単一の鋳型の増幅によってではなく、単一の微粒子に由来する鋳型の複数の複製の増幅によって生じることになる。さらに、半固形培地の表面上に互いに対して近接して配置できる微粒子を使用することで、半固形培地の表面の効果的な使用が可能になるとともに、検出時に互いに容易に区別できる離散したスポットももたらされる。スポットは、典型的には微粒子よりもサイズが小さく、互いにより明確に区別できるようになる。例えば、粒子と平坦な表面との接点の２５０ｎｍ以内に位置する直径１ミクロンの粒子上のＤＮＡが、平坦な表面に付着し、複製される場合には、粒子が解放された後に、直径５００ｎｍのＤＮＡパッチが表面にできることになる。１ミクロンのビーズ２つが接している場合には、あとにできるＤＮＡパッチの中心が１ミクロン離れており、各パッチの最も近い端部の間に５００ｎｍの空間ができる。ガラススライドなどの小さな基材の表面上に数百万の微粒子を充填する能力により、本プロセスは、近隣のコロニーに干渉されずに容易に画像化でき、また複数の配列決定サイクルにわたって簡単かつ信頼性の高い検出が可能となる十分な数の鋳型分子を含有する、鋳型コロニーの高密度アレイを実現する効率的な手段を提供する。

【0147】

基材に結合した微粒子に付着した鋳型には、種々のさらなる反応または操作を行うことができる。これは、例えば、本明細書に記載のようなライゲーションに基づく配列決定、またはＦＩＳＳＥＱやパイロシーケンシングなどの他の配列決定方法などの配列決定であってよい。例えば、上述のように微粒子を使用して形成される、半固形培地内の核酸コロニーに存在する鋳型に対して、本明細書に記載の本発明の配列法のいずれも実施することができる。

【0148】

本明細書に記載の方法に従って形成される微粒子または核酸コロニーのアレイは、一般的にランダムであり得る。本明細書で用いる場合、用語「ランダムパターンの」または「ランダム」は、不規則な非Ｃａｒｔｅｓｉａｎ分布（換言すると、格子のｘ軸およびｙ軸に沿った所定の点もしくは位置、または放射状パターンの中心からの角度もしくは半径の規定の「時計位置」で整列していない）支持体上での正体（特徴）であって、意図的な設計（もしくはかかる設計が達成され得るプログラム）または個々の正体の配置によって得られないものをいう。かかる「ランダムパターンの」または「ランダム」アレイの正体は、正体のプールを含む溶液、エマルジョン、エーロゾル、気体状のまたは乾燥した調製物を、支持体上または支持体内に滴下、噴霧、めっき、塗布、分布などさせ、支持体内または支持体上の特定の部位に指向させるなんらかの様式の介在なく支持体上または支持体内に沈降させることにより得られ得る。例えば、正体は、半固相支持体の前駆物質（例えば、アクリルアミドモノマー）を含有する溶液中に懸濁させ得る。次いで、この溶液を、第２の支持体上に分布させ、半固相支持体を第２の支持体上に形成させる。正体は、半固相支持体内または該支持体上に包埋される。もちろん、非ランダムアレイもまた使用され得る。微粒子の密接な充填は、規則正しいグリッド様のアレイの微粒子またはそれから合成される核酸コロニーをもたらし得る。一般的に、本明細書において使用されるアレイを形成するための方法は、例えば、ポリヌクレオチドの合成が、基質上の予め規定された位置の個々のヌクレオチドサブユニットの逐次適用によって行なわれる方法と異なる。

【0149】

図１４Ｂ（上）は、その上面にポリアクリルアミドゲルを有するスライド（１インチ×３インチ）の蛍光画像を示す。ビーズに結合させた鋳型にハイブリダイズされた蛍光標識されたオリゴヌクレオチドを有するビーズ（１ミクロン直径）は、ゲル内に固定化されている。この画像は、およそ２８０００００００ビーズ／スライドの画像化に充分なビーズ

表面密度（すなわち、ビーズが位置する領域内の基材の単位面積あたりのビーズの数）を示す。表面密度および画像化可能な領域は、単一のスライド上で少なくとも５００００００００ビーズの画像化に充分である。例えば、図１４Ｂ（下）は、ビーズが半固相支持体層内に包埋されるポリアクリルアミドゲルなどの透明な領域を覆うテフロン（登録商標）マスクを有するスライドの概略図を示す。このマスクの面積は８６４ｍｍ^{2}である。５００００００００個のビーズで、表面密度は５７８，０００ビーズ／ｍｍ^{2}である。１ミクロンビーズの最密六方アレイでは１，１５５，０００ビーズ／ｍｍ^{2}となるため、この実施形態は、理論最大密度の５２％を有するアレイをもたらす。この特定の実施形態より少数およびより多数のビーズ、およびより大きいかより小さいビーズ表面密度が使用され得ることは認識されよう。

【0150】

微粒子は、実質的に平面状の半固相支持体内もしくは該支持体上で、または別の支持体もしくは基材上で、いくつかの様式で規定され得るさまざまな密度でアレイ状であり得る。例えば、密度は、実質的に平面状のアレイの単位面積あたりの微粒子の数（例えば、球状微粒子）に関して表示され得る。本発明のある特定の実施形態において、実質的に平面状のアレイの単位面積あたりの微粒子の数は、六方アレイ内の微粒子の数の少なくとも８０％である。（「六方アレイ」により、米国特許第６，４０６，８４８号に記載のようなアレイ内のどの微粒子も少なくとも６個の他の隣接微粒子と等しい面積で接触している微粒子の実質的に平面状のアレイが意図される）。しかしながら、本発明の他の実施形態において、微粒子密度は低く、例えば、実質的に平面状のアレイの単位面積あたりの微粒子の数は、六方アレイ内の微粒子の数の８０％未満、７０％未満、６０％未満または５０％未満である。なんら理論に拘束されることを望まないが、例えば、酵素、プライマー、補因子などの試薬の充分な拡散を可能にするため、およびある種の試薬が微粒子に対して異なる親和性を有するか、その内部の捕捉された場合に起こり得る試薬分配効果を回避するため、これらなどより小さい密度を用いることが好ましいこと場合があり得る。かかる効果は、アレイ上の異なる位置で異なる反応条件をもたらし得、これらの試薬がアレイ上のある特定の位置に接近するのを妨げることすらあり得る。このような問題は、反応がフローセル内で行なわれる場合、試薬がフローセル内を方向的に移動するため、悪化し得る。本発明のある特定の実施形態において、混合装置、例えば、機械的または音波的手段によって液体混合を行なう装置をフローセルのチャンバ内に含める。いくつかの適当な混合装置が、当該技術分野で知られている。

【0151】

本発明の配列決定法は、あらゆる型（例えば、ランダムおよび非ランダム両方のアレイ）のアレイ形式に整列させた鋳型を用いて行なわれ、アレイは、微粒子のアレイまたは鋳型自体のアレイであり得る。例えば、その上面にアレイ状の鋳型を有する支持体は、米国特許第５，６４１，６５８号およびＰＣＴ公開公報ＷＯ００１８９５７に記載されている。アレイは、広範なさまざまな基材、例えば、フィルター、膜（例えば、ナイロン）、金属表面などの上に配置させ得る。その上面で伸長、ライゲーションおよび切断の反復サイクルによる配列決定が行なわれ得るアレイ形式のさらなる例は、ウェル内で光ファイバーの束の個々の光ファイバーの末端または遠位端に配置したビーズのアレイである。例えば、ＵＳ公開公報および特許、例えば、６，０２３，５４０；６，４２９，０２７、２００４０１８５４８３、２００２１８７５１５、ＰＣＴ出願ＵＳ９８／０５０２５およびＰＣＴ　ＵＳ９８／０９１６３ならびにＰＣＴ公開公報ＷＯ００３９５８７に記載されたビーズアレイおよび「アレイのアレイ」を参照のこと。自身に結合された鋳型を有するビーズは、本明細書に記載のようなアレイ状であり得る。増幅は、好ましくは、アレイの形成前に行なわれる。かかる基材上に形成されたアレイは、必ずしも実質的に平面状である必要はない。

【0152】

他の実施形態において、ＰＣＲは、基材または支持体に結合されたオリゴヌクレオチドを含むアレイ上で行なわれる（例えば、米国特許第５，７４４，３０５号；同第５，８０

０，９９２号；同第６，６４６，２４３号および関連特許（Ａｆｆｙｍｅｔｒｉｘ）；ＰＣＴ公開公報ＷＯ２００４０２９５８６；ＷＯ０３０６５０３８；ＷＯ０３０４０４１０（Ｎｉｍｂｌｅｇｅｎ）　を参照）。一般に、かかるオリゴヌクレオチドは、遊離３’または５’末端を有する。所望の場合は、該末端は、例えば、リン酸基またはＯＨ基を３’末端に（既に存在しない場合）付加することにより修飾され得る。支持体または基材に結合されたオリゴヌクレオチドに相補的な領域を含む鋳型分子は、該オリゴヌクレオチドにハイブリダイズされ、ＰＣＲがアレイ上でインサイチュで行なわれ、クローン鋳型集団がアレイ上の各位置にもたらされる。アレイに結合されたオリゴヌクレオチドは、増幅プライマーの１つとしての機能を果たし得る。次いで、鋳型を本明細書に記載のライゲーション系の方法を用いて配列決定する。配列決定はまた、Ｕ．Ｓ．公開公報２００３００６８６２９に記載のように、アレイ内の鋳型上で行なわれ得る。

【0153】

表面上へのＤＮＡアレイの作製のためのさらに他の方法が使用され得る。例えば、末端アルデヒド基で修飾されたアルカンチオールが、自己組織型単層（ＳＡＭ）を金表面上に作製するために使用され得る。該単層のアルデヒド基は、アミン修飾オリゴヌクレオチドまたは他のアミン担持生体分子と反応し、シッフ塩基を形成し得、次いで、これは、シアノ水素化ホウ素ナトリウムでの処理によって安定な第２級アミンに還元され得る（Ｐｅｅｌｅｎ　＆　Ｓｍｉｔｈ，　Ｌａｎｇｍｕｉｒ，　２１（１）：２６６－７１，　２００５）。次いで、鋳型のＰＣＲ増幅が行なわれ得る。あるいはまた、自身に結合された鋳型のクローン性の集団を有する微粒子を、微粒子または鋳型上のアミン基または粒子に結合されたオリゴヌクレオチドをかかる表面と反応させることによりにより表面に結合させ得る。

【0154】

自身に結合されたクローン鋳型集団を有する微粒子を得るさらに別の方法は、米国特許第５，６０４，０９７号に記載された「固相クローニング」アプローチであり、これは、同じ配列のポリヌクレオチドだけが任意の特定の微粒子に結合されるような、微粒子上のポリヌクレオチドを分取するためのオリゴヌクレオチドタグを利用する。

【0155】

本発明のある特定の実施形態において、伸長、ライゲーションおよび切断の反復サイクルによる配列決定は、配列決定試薬（例えば、伸長プローブ、リガーゼ、ホスファターゼなど）を支持体内または支持体上に固定化された鋳型のクローン性の集団を有する半固相支持体（例えば、ゲル）中に、各クローン性の集団が支持体の空間的に異なる領域に局在化されるように拡散させることによって行なわれる。ある特定の実施形態において、鋳型は、上記のように、半固相支持体に直接結合させる。しかしながら、他の実施形態では、代わりに半固相支持体内または該支持体上に固定化させた微粒子などの第２の支持体に、鋳型を固定化させる（これも上記のようのとおり）。

【0156】

実施例１に記載のように、本発明者らは、ロバスト（ｒｏｂｕｓｔ）なライゲーションおよび切断が、ポリアクリルアミドゲル内に固定化した鋳型結合ビーズ上で行なわれ得ることを示した。したがって、本発明は、（ａ）半固相支持体内または該支持体上に固定化された第１のポリヌクレオチドを提供する工程；（ｂ）第１のポリヌクレオチドを第２のポリヌクレオチドおよびリガーゼと接触させる工程；および（ｃ）第１および第２のポリヌクレオチドを、リガーゼの存在下ライゲーションに適した条件下で維持する工程を含む、第１のポリヌクレオチドを第２のポリヌクレオチドにライゲートさせる方法を提供する。好適な条件としては、使用される具体的なリガーゼに適切なバッファー、補因子、温度、時間などの提供が挙げられる。好ましい実施形態において、半固相支持体は、アクリルアミドゲルなどのゲルである。さらに好ましい実施形態において、第１のポリヌクレオチドは、それ自体が、例えば、部分的または完全に包埋させることにより支持体マトリックス内に半固相支持体内または該支持体上に固定化されたビーズなどの支持体への結合の結果、半固相支持体内または該支持体上に固定化されている。あるいはまた、第１のポリヌ

クレオチドは、アクリダイト部分などの連結部によって半固相支持体に直接結合させ得る。連結は共有結合または非共有結合であり得る（例えば、ビオチン－アビジン相互作用による）。米国特許第６，５１１，８０３号には、核酸分子を本発明の好ましい支持体、すなわち、ポリアクリルアミドゲルに（ａ）結合させるために使用され得るさまざまな方法が記載されている。

【0157】

本発明は、さらに、（ａ）半固相支持体内または該支持体上に固定化され、切れやすい連結を含むポリヌクレオチドを提供する工程；（ｂ）該ポリヌクレオチドを切断剤と接触させる工程；および（ｃ）該ポリヌクレオチドを該切断剤の存在下、切断に適した条件下で維持する工程を含む、ポリヌクレオチドを切断する方法を提供する。好適な条件としては、特定の切断剤に適切なバッファー、温度、時間などの提供が挙げられる。好ましい実施形態において、半固相支持体は、アクリルアミドゲルなどのゲルである。さらに好ましい実施形態において、ポリヌクレオチドは、それ自体が半固相支持体内に固定化されたビーズなどの支持体への結合の結果、半固相支持体に固定化されている。あるいはまた、ポリヌクレオチドは、アクリダイト部分などの連結部によって半固相支持体に直接結合させ得る。連結は共有結合または非共有結合であり得る（例えば、ビオチン－アビジン相互作用による）。当然ながら、本明細書に記載の多くの方法にしたがって調製されるＤＮＡ鋳型は、典型的には配列決定する領域を含有し、また３’端および５’端のいずれかまたは両方に保存プライミング領域も含有する（ＰＢＲ）。「保存」または「共通」領域とは、配列決定する種々の領域を含有する複数の鋳型に共通する配列を指し、すなわち、鋳型は、配列の一部は異なるものの、同一の部分も含有する。鋳型は、１つ以上の保存内部アダプター配列を含有する場合もある。さらに、ＤＮＡ鋳型のローリングサークル増幅（ＲＣＡ）は、これらの保存配列のさらなる複製を作成するだけでなく、ＲＣＡプローブからさらに別の領域の保存配列の複製も導入する。そのため、配列決定するライブラリー分子の部分（「標的領域」、「対象部分」などとも呼ばれる）は、実際の鋳型核酸の半分未満に相当する場合がある。本発明は、これらの既知の／共通する非標的領域が、一本鎖の場合に、配列決定プローブを封鎖することができ、また配列決定プライマー（例えば、開始オリゴヌクレオチド）のミスプライムの可能性がある部位であるという認識を包含する。本発明は、ポリヌクレオチド鋳型中に存在する非標的配列と相補的なブロッキングオリゴヌクレオチドを提供する。本明細書で使用される「ブロッキングオリゴヌクレオチド」とは、鋳型の非標的配列に安定してハイブリダイズするオリゴヌクレオチドであり、この非標的配列は、配列決定に好適な条件下で種々の標的領域を含む複数の鋳型に共通する。非標的領域は、開始オリゴヌクレオチドが結合するとされる領域とは異なる。本発明はさらに、１つ以上のブロッキングオリゴヌクレオチドがハイブリダイズするポリヌクレオチド鋳型も提供する。

【0158】

本発明のある実施形態において、鋳型はエマルジョンＰＣＲを使用して合成される。

【0159】

特に興味深い実施形態において、ＤＮＡ鋳型は、断片ライブラリーのメンバーであり、図３６Ｂに示す通り前方および後方アダプターを含有する。第１のブロッキングオリゴヌクレオチドは前方アダプターと相補的であり、第２のブロッキングオリゴヌクレオチドは後方アダプターと相補的である。他の実施形態において、ＤＮＡ鋳型は、対合末端ライブラリーのメンバーであり、図３６Ａに示す通り、前方および後方アダプターと、さらに内部アダプターも含有する。第１のブロッキングオリゴヌクレオチドは前方アダプターと相補的であり、第２のブロッキングオリゴヌクレオチドは後方アダプターと相補的であり、第３のブロッキングオリゴヌクレオチドは内部アダプターと相補的である。他の実施形態において、鋳型は、ＲＣＡを使用して増幅され、図３６Ｃおよび図３７に示す通りアダプター領域とパドロック領域とを含有する。ブロッキングオリゴヌクレオチドは、鋳型に存在するアダプター領域およびパドロック領域と相補的である。ＲＣＡでは、パドロックプローブがポリメラーゼによって複製され、その相補体が生成されることが理解される。し

たがって、鋳型に存在するＲＣＡ相補体をブロックするために、パドロックプローブと同じ配列が、ブロッキングオリゴヌクレオチドとして使用される。図３６および図３７に示す特異的なオリゴヌクレオチドとそれらの相補体は、本発明の態様であるが、ブロッキングオリゴヌクレオチドの配列は、鋳型中に存在し、様々に異なることがある特定の保存配列と相補的になるように選択されることが認識される。さらに、本発明には、図３６または図３７に示すものと１個、２個、３個、４個または５個のヌクレオチドのみ配列が異なるオリゴヌクレオチドが含まれる。

【0160】

決して本発明を限定するものではないが、本発明者等は、例えば、鋳型の複雑性を低減させるツールとして作用する、ミスプライムの可能性がある部位を排除する、および／または鋳型の標的領域への伸長オリゴヌクレオチドのアクセスを容易にすることにより、前述の問題、あるいはこれらの共通配列の多くの複製が存在するために生じる場合があるその他の問題に対抗するために、ブロッキングオリゴヌクレオチドが使用される場合があることを認識した。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、例えばシグナル／ノイズ比の向上など、配列決定効率の向上をもたらす。

【0161】

ブロッキングオリゴヌクレオチドは、典型的には、配列決定プライマーのアニーリング前に一本鎖鋳型ＤＮＡにハイブリダイズされ、これらの領域がその後に配列決定プライマー（例えば、ライゲーションに基づく配列決定における開始オリゴヌクレオチド）またはプローブ（例えば、ライゲーションに基づく配列決定における伸長プローブ）のいずれかとハイブリダイズするのが防止される。これらのオリゴヌクレオチドは、典型的にはライゲーション、検出、（および、伸長オリゴヌクレオチドが開裂される本発明の実施形態においては、開裂）の連続サイクルを通して存在し続けるとされる。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、ポリメラーゼまたはリガーゼの基材ではなく、例えば、これらのオリゴヌクレオチドは、典型的なポリメラーゼまたはリガーゼ酵素によって酵素的に伸長可能ではない。一実施形態において、ブロッキングオリゴヌクレオチドは、３’ヒドロキシル基および５’リン酸塩が欠損している。これらの基は、存在しない場合もあれば、合成後に除去される場合もあり、あるいはオリゴヌクレオチドの３’端および／または５’端が、伸長またはライゲーションの基材ではない部分でキャップまたはブロックされる場合もある。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、３’末端ジデオキシヌクレオシドを含む。本発明のある実施形態において、ブロッキングオリゴヌクレオチドは、３’末端ジデオキシシトシン（３‘ｄｄＣ）を含む。本発明のある実施形態において、対合タグライブラリーとともに使用するパドロックプローブは、単一タグそれぞれ（タグ１のみ、タグ２のみ）の、あるいは両タグの間（タグ１‐内部‐タグ２）のＲＣＡが可能となるように設計される（図３７）。

【0162】

ブロッキングオリゴヌクレオチドは、保存領域よりも短くてよく、すなわち、これらのオリゴヌクレオチドは、保存領域の一部のみと相補的であってよい。ブロッキングオリゴヌクレオチドは、保存領域と完全に相補的である必要はないが、そうであるのが好ましい場合がある。典型的には、ブロッキングオリゴヌクレオチドは、保存領域のすべてまたは一部と少なくとも８０％、好ましくは少なくとも９０％相補的となる。ブロッキングオリゴヌクレオチドのサイズは、ブロックする共通配列の長さによって変動する場合がある。典型的な長さは、１０～５０ヌクレオチドである。それぞれがブロックする保存領域の一部と相補的である複数のブロッキングオリゴヌクレオチドが、単一のより長いオリゴヌクレオチドの代わりに使用される場合もある。

【0163】

ブロッキングオリゴヌクレオチドは、本明細書に記載のようなライゲーションに基づく配列決定において特定の用途を見出す場合がある。したがって、本明細書に記載のいずれの方法も、鋳型を開始オリゴヌクレオチドと接触させる前に、プローブと鋳型の二本鎖を形成または提供する前に、および／または伸長した二本鎖を形成する前に、鋳型ポリヌク

レオチドを１つ以上のブロッキングオリゴヌクレオチドと接触させる手順を含む場合がある。但し、ブロッキングオリゴヌクレオチドはまた、ＦＩＳＳＥＱやパイロシーケンシングなどの他の配列決定法を実施する場合に使用される場合もある。

【0164】

Ｄ．種々の初期オリゴヌクレオチドを用いた再度初期化を伴う配列決定

本発明の好ましい実施形態において、第１の初期オリゴヌクレオチドを伸長させることによって生成された伸長鎖は、充分なサイクル数の後、鋳型から除去され、第２の初期オリゴヌクレオチドが結合領域にアニーリングされた後、伸長、ライゲーションおよび検出のサイクルが行なわれる。該プロセスは、任意の数の異なる初期オリゴヌクレオチドを用いて反復される。伸長プローブが切断される実施形態では、好ましくは、使用される異なる初期オリゴヌクレオチドの数（したがって、反応の数）は、プローブの遠位部分の放出後に鋳型にハイブリダイズされたままの伸長プローブの部分の長さに等しい。したがって、この実施形態によれば、配列情報（例えば、各ヌクレオチドの順番および正体）は、単一の支持体に結合された鋳型から得られ得るが、連続したヌクレオチドが各サイクルで同定される場合に必要とされ得るよりも実質的に少ないサイクルを用いて、さらに深く読み出しが行なわれる。

【0165】

初期オリゴヌクレオチドが同じ鋳型に連続的に結合される実施形態は、Ｍａｃｅｖｉｃｚにより教示された方法などの、鋳型を多数のアリコートに分割することを要するアプローチと比べてある種の利点を有する。例えば、初期オリゴヌクレオチドを同じ鋳型に適用することにより、多数のアリコートから取得されたデータを追跡し、後で組み合わせる必要性が回避される。個々の支持体の位置が所定でないような支持体がランダム様式のアレイ状である実施形態では、各々が自身に結合された同じ配列の鋳型を有する多数の支持体からの部分配列情報を信頼できるように組み合わせることは困難または不可能であり得る。

【0166】

Ｅ．単一の鋳型における各サイクルでの多数のヌクレオチドの同定

Ｍａｃｅｖｉｃｚには、伸長、ライゲーションおよび検出の各サイクルにおける鋳型内の単一のヌクレオチドの同定が教示されている。しかしながら、本発明者らは、該方法は、各サイクルで鋳型内の多数のヌクレオチドの同定が可能となるように変形し得ると認識した。この場合、伸長プローブは、伸長された二本鎖に接合された２つ以上の、好ましくは連続したヌクレオチドの正体が標識から決定され得るように標識される。換言すると、伸長プローブの配列決定部分は、１つより多くのヌクレオチドであり、典型的には、近位ヌクレオチド、直接隣接ヌクレオチド、およびおそらく１つ以上のさらなる、好ましくは連続したヌクレオチドを含み、これらはすべて、鋳型に特異的にハイブリダイズする。例えば、塩基Ａ、Ｇ、ＣおよびＴを特定する４つの標識を用いるのではなく、１６個の識別可能に標識されたプローブまたはプローブの組合せが、１６個の可能なジヌクレオチドＡＡ、ＡＧ、ＡＣ、ＡＴ、ＧＡ、ＧＧ、ＧＣ、ＧＴ、ＣＡ、ＣＧ、ＣＣ、ＣＴ、ＴＡ、ＴＧ、ＴＣおよびＴＴを特定するために使用される。各識別可能に標識された伸長プローブの配列決定部分は、これらのジヌクレオチドの１つに相補的である。より多くの標識を用いる同様の方法により、各サイクルで、より長鎖のヌクレオチド配列の同定が可能になる。

【0167】

Ｆ．標識

用語「標識」は、本明細書において、プローブと結合または会合した任意の検出可能な部分または複数の検出可能な部分であって、それにより異なる種のプローブ（例えば、異なる末端ヌクレオチドを有するプローブ）が互いに識別され得る部分を表すために広義で用いる。したがって、標識と特異的検出可能な部分との間に１対１の対応がある必要はない。例えば、多数の検出可能な部分が単一のプローブに結合され得、プローブが、自身に結合された異なる検出可能な部分または検出可能な部分の組を有するプローブと識別されるのを可能にする複合シグナルがもたらされ得る。例えば、検出可能な部分の組合せが、

米国特許第６，６３２，６０９号およびＳｐｅｉｃｈｅｒら、Ｎａｔｕｒｅ　Ｇｅｎｅｔｉｃｓ，１２：３６８－３７５，１９９６に記載された「コンビナトリアルマルチカラーコーディング（Ｃｏｍｂｉｎａｔｏｒｉａｌ　Ｍｕｌｔｉｃｏｌｏｒ　Ｃｏｄｉｎｇ）」とよばれる標識スキームに従って使用され得る。

【0168】

本発明のプローブは、さまざまな様式、例えば、基質と接触させると検出可能なシグナルを生成する蛍光性または化学発光性部分、比色性部分、酵素性部分などの直接または間接的結合によって標識され得る。Ｍａｃｅｖｉｃｚには、プローブを蛍光染料、例えば、Ｍｅｎｃｈｅｎら，米国特許第５，１８８，９３４号；Ｂｅｇｏｔら　ＰＣＴ出願ＰＣＴ／ＵＳ９０１０５５６５に開示されているもので標識させ得ることが教示されている。用語「蛍光染料」および「フルオロフォア」は、本明細書で用いる場合、規定の励起波長の光エネルギーを吸収し、異なる波長の光エネルギーを放出する部分をいう。好ましくは、所与のプローブ混合物での使用のために選択される標識は、スペクトルにより分離可能なものである。本明細書で用いる場合、「スペクトルにより分離可能な」は、標識が、作業条件下で、そのスペクトル特性、特に蛍光発光波長に基づいて識別され得ることを意味する。例えば、１つ以上の末端ヌクレオチドの正体は、最大光発光強度の異なる波長、またはおそらく異なる波長での強度比と相関し得る。標識を検出および同定するために用いられる標識のスペクトル特性（１つまたは複数）を、本明細書では「色」という。標識が、特異的スペクトル特性、例えば、単一の検出可能な部分からなる標識の場合は最大発光強度の周波数、または、多数の検出可能な部分からなる標識の場合は発光ピークの周波数に基づいて高頻度で同定されることは認識されよう。

【0169】

好ましくは、４種類のスペクトルにより分離可能な蛍光染料の各々と、４つの可能なプローブ末端ヌクレオチドとの１対１対応を可能にする４種類のプローブが提供される。スペクトルにより分離可能な色素の組は、米国特許第４，８５５，２２５号および同第５，１８８，９３４号；国際出願ＰＣＴ７ＵＳ９０／０５５６５；ならびにＬｅｅら，　Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓｅａｒｃｈｓ，２０：２４７１－２４８３（１９９２）に開示されている。ある特定の実施形態において、ＦＩＴＣ、ＨＥＸ™、テキサスレッドおよびＣｙ５からなる組が好ましい。数多くの好適な蛍光染料が、例えば、Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，　Ｉｎｃ．，　Ｅｕｇｅｎｅ　ＯＲから市販されている。蛍光染料の具体例としては、限定されないが、Ａｌｅｘａ　Ｆｌｕｏｒ色素（Ａｌｅｘａ　Ｆｌｕｏｒ　３５０、Ａｌｅｘａ　Ｆｌｕｏｒ　４８８、Ａｌｅｘａ　Ｆｌｕｏｒ　５３２、Ａｌｅｘａ　Ｆｌｕｏｒ　５４６、Ａｌｅｘａ　Ｆｌｕｏｒ　５６８、Ａｌｅｘａ　Ｆｌｕｏｒ　５９４、Ａｌｅｘａ　Ｆｌｕｏｒ　６３３、Ａｌｅｘａ　Ｆｌｕｏｒ　６６０　ａｎｄ　Ａｌｅｘａ　Ｆｌｕｏｒ　６８０）、ＡＭＣＡ、ＡＭＣＡ－Ｓ、ＢＯＤＩＰＹ色素（ＢＯＤＩＰＹ　ＦＬ、ＢＯＤＩＰＹ　Ｒ６Ｇ、ＢＯＤＩＰＹ　ＴＭＲ、ＢＯＤＩＰＹ　ＴＲ、ＢＯＤＩＰＹ　５３０／５５０、ＢＯＤＩＰＹ　５５８／５６８、ＢＯＤＩＰＹ　５６４／５７０、ＢＯＤＩＰＹ　５７６／５８９、ＢＯＤＩＰＹ　５８１／５９１、ＢＯＤＩＰＹ　６３０／６５０、ＢＯＤＩＰＹ　６５０／６６５）、ＣＡＬ色素、カルボキシローダミン６Ｇ、カルボキシ－Ｘ－ローダミン（ＲＯＸ）、Ｃａｓｃａｄｅ　Ｂｌｕｅ、Ｃａｓｃａｄｅ　Ｙｅｌｌｏｗ、シアニン色素（Ｃｙ３、Ｃｙ５、Ｃｙ３．５、Ｃｙ５．５）、Ｄａｎｓｙｌ、Ｄａｐｏｘｙｌ、ジアルキルアミノクマリン、４’，５’－ジクロロ－２’，７’－ジメトキシ－フルオレセイン、ＤＭ－ＮＥＲＦ、エオシン、エリスロシン、フロレセイン、ＦＡＭ、ヒドロキシクマリン、ＩＲＤｙｅｓ（ＩＲＤ４０、ＩＲＤ　７００、ＩＲＤ　８００）、ＪＯＥ、リサミンローダミンＢ、マリンブルー、メトキシクマリン、ナフトフルオレセイン、オレゴングリーン４８８、オレゴングリーン５００、オレゴングリーン５１４、Ｏｙｓｔｅｒ色素、Ｐａｃｉｆｉｃ　Ｂｌｕｅ、ＰｙＭＰＯ、ピレン、ローダミン６Ｇ、ローダミングリーン、ローダミンレッド、Ｒｈｏｄｏｌ　Ｇｒｅｅｎ、２’，４’，５’，７’－テトラ－ブロモスルホン－フルオレセイン、テトラメチル－ローダミン（ＴＭＲ）、カルボキシテトラメチルローダミン（ＴＡＭＲＡ）、テ

キサスレッド、テキサスレッド－Ｘが挙げられる。Ｔｈｅ　Ｈａｎｄｂｏｏｋ　ｏｆ　Ｆｌｕｏｒｅｓｃｅｎｔ　Ｐｒｏｂｅｓ　ａｎｄ　Ｒｅｓｅａｒｃｈ　Ｐｒｏｄｕｃｔｓ，第９版，　Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ，　Ｉｎｃ．，　ｆｏｒ　ｆｕｒｔｈｅｒ　ｄｅｓｃｒｉｐｔｉｏｎを参照のこと。

【0170】

それ自身が直接検出可能であるのではなく、一部の蛍光基は、非放射性蛍光共鳴エネルギー転移（ＦＲＥＴ）のプロセスにてエネルギーを別の基に転移させ、第２の基が、検出されるシグナルを生成する。すなわち、消光剤の使用もまた本発明の範囲内である。用語「消光剤」は、可視光を発光せずに励起された蛍光標識のエネルギーを、これに近接して配置された場合に吸収でき、消散できる部分をいう。消光剤の例としては、限定されないが、ＤＡＢＣＹＬ（４－（４’－ジメチルアミノフェニルアゾ）安息香酸）スクシンイミジルエステル、ジアリールローダミンカルボン酸、スクシンイミジルエステル（ＱＳＹ－７）および４’，５’－ジニトロフルオレセインカルボン酸、スクシンイミジルエステル（ＱＳＹ－３３）（すべて、Ｍｏｌｅｃｕｌａｒ　Ｐｒｏｂｅｓ製）、消光剤１（Ｑ１；Ｅｐｏｃｈ製）または「ブラックホール（Ｂｌａｃｋ　ｈｏｌｅ）消光剤」ＢＨＱ－１、ＢＨＱ－２およびＢＨＱ－３（ＢｉｏＳｅａｒｃｈ，　Ｉｎｃ製）が挙げられる。

【0171】

上記の種々の検出可能な部分に加え、本発明はまた、スペクトルにより分離可能な量子ドット、金属ナノ粒子またはナノクラスターなどの使用の使用を包含し、これらは、オリゴヌクレオチドプローブに直接結合されたものであり得るか、または高分子マトリックス内に包埋もしくはこれと会合させ、次いでプローブに結合させたものであり得るかのいずれかである。上記のように、検出可能な部分は、それ自体が直接検出可能である必要はない。例えば、これらは、検出される基質に対して作用するものであってもよく、検出可能となるために修飾を必要とするものであってもよい。

【0172】

上記のように、本発明のある特定の実施形態において、標識は、複数の検出可能な部分からなる。これらの検出可能な部分からの複合シグナルにより色がもたらされ、これを、プローブの同定のために使用する。例えば、ある特定の配列の「紫色」のプローブは、「青色」および「赤色」の検出可能な部分を結合することにより構築され得る。あるいはまた、同じ配列を有するが異なる検出可能な部分で標識された２種類のプローブを組み合わせて混合プローブを作製することにより異なる色が生成され得る。したがって、ある特定の配列の「紫色」のプローブは、該配列を有する２種類のプローブを構築することにより作製され得る。「赤色」の検出可能な部分を第１の種に結合させ、「青色」の検出可能な部分を第２の種に結合させる。これらの２種類のアリコートを混合する。種々の濃度（ｓｈａｄｅ）の紫色が、アリコートを種々の比率で混合することにより生成され得る。このアプローチはいくつかの利点をもたらす。第１に、より少数の検出可能な部分を用いて多数の識別可能なプローブの生成を可能にする。第２に、混合プローブの使用により、特定の検出可能な部分と特定のヌクレオチド間の相互作用に起因し得る偏重の低減を補助し得る冗長（ｒｅｄｕｎｄａｎｃｙ）度が提供され得る。

【0173】

本発明のある特定の実施形態において、検出可能な部分は、ライゲーションおよび検出後に検出可能な部分の除去を可能にする切断可能な連結によってオリゴヌクレオチド伸長プローブ内のヌクレオチドに結合させる。任意のさまざまな異なる切断可能な連結が使用され得る。本明細書で用いる場合、オリゴヌクレオチドプローブにおける検出可能な部分およびヌクレオチドの文脈において、用語「切断可能な連結」は、検出可能な部分をヌクレオチドに接合させ、所望の場合は、結合されたヌクレオチドまたは核酸分子を本質的に改変することなく、切断されて検出可能な部分をヌクレオチドから除去し得る化学的部分をいう。切断は、連結の性質に応じて、例えば、酸もしくは塩基処理、または連結の酸化もしくは還元、または光処理（光切断）によってなされ得る。切断可能な連結および切断剤の例は、Ｓｈｉｒｎｋｕｓら．，１９８５，　Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ

．ＵＳＡ　８２：２５９３－２５９７；Ｓｏｕｋｕｐら．，１９９５，　Ｂｉｏｃｏｎｊｕｇ．Ｃｈｅｍ．６：１３５－１３８；Ｓｈｉｍｉｋｕｓら．，１９８６，　ＤＮＡ　５：２４７－２５５；ならびにＨｅｒｍａｎおよびＦｅｎｎ，１９９０，　Ｍｅｔｈ．Ｅｎｚｙｍｏｌ．１８４：５８４－５８８に記載されている。より一般的には、「開裂可能な連結」とは、２つの分子または正体を連結するために使用できる部分であって、容易に開裂できることによって、例えば分子または正体の安定性と両立する条件下で、構造を実質的に変更することなく、分子または正体の分離を可能にする部分を指す。

【0174】

例えば、米国特許第６，５１１，８０３号に記載のように、ジスルフィド結合が、ジチオトレイトール（ＤＴＴ）などのチオール化合物還元剤を用いて還元され、それにより切断され得る。ヌクレオチドと反応性アリールアミノ基（例えば、ｄＣＴＰ）のように、フルオロフォアが、コンジュゲーションに利用され得るスルフヒドリル（ＳＨ）基とともに利用可能である（例えば、シアニン５またはシアニン３フルオロフォアとＳＨ基；Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｎｕｃｌｅａｒ－ＤｕＰｏｎｔ）。反応性ピリジルジチオールは、スルフヒドリル基と反応してスルフヒドリル結合をもたらし、これは、ジチオトレイトールなどの還元剤により切断可能である。ＮＨＳ－エステルヘテロ二官能性架橋剤（Ｐｉｅｒｃｅ）は、反応性アリールアミノ基を含むデオキシヌクレオチドをピリジルジチオール基に連結させるために使用され得、これは、次いでフルオロフォア上のＳＨと反応性となり、本発明の方法に有用なジスルフィド結合された切断可能なヌクレオチドフルオロフォア複合体をもたらす。あるいはまた、ヌクレオチドとフルオロフォア間のシスーグリコール連結は過ヨウ素酸塩によって切断され得る。さまざまな切断可能な連結が、米国特許第６，６６４，０７９号および同第６，６３２，６５５号、ＵＳ出願公開公報２００３０１０４４３７、ＷＯ　０４／１８４９７およびＷＯ　０３／４８３８７に記載されている。

【0175】

本発明の他の実施形態において、光などの電磁エネルギーへの曝露によって非検出可能となり得る検出可能な部分（光退色性）が使用される。

【0176】

切断可能な連結によってプローブに結合された標識を有するか、または光退色性となり得る標識を有する伸長プローブを用いる本発明の実施形態において、配列決定法は、典型的には、ライゲーションおよび標識検出が行なわれた後、１回以上のサイクルにおいて切断または光退色工程を含む。上記のように、オリゴヌクレオチド伸長プローブ内に存在する切れやすい連結の切断は、完結するまで進行し得ない（すなわち、新たにライゲートされたプローブの１００％未満が、ライゲートされたサイクルにおいて切断され得る）。かかるプローブは一般的に、伸長不可能な末端を含むか、またはキャップされているため、連続的サイクルに寄与しない。しかしながら、プローブが切断されないことは、標識が、プローブライゲートされる鋳型分子と会合したままであることを意味し、これは、後続のサイクルにおけるノイズを増大させ得るバックグラウンドシグナル（すなわち、バックグラウンド蛍光）に寄与する。標識を除去するため、または非検出可能とするための切断または光退色の工程を組み込むことは、このバックグラウンドを低減させ、信号対雑音比が改善される。切断または光退色は、各サイクルごとにできるだけ多く行なってもよく、サイクル１回おき、３回に１回、５回以上に１回などの低頻度で行なってもよい。本発明のある特定の実施形態において、切断可能なリンカーの切断を達成するための任意のさらなる工程を実際に加える必要はない。例えば、ＤＴＴなどの切断剤を、非ライゲート伸長プローブを除去するために使用され得る洗浄バッファー中に既に存在させてもよい。

【0177】

Ｇ．好ましい切れやすい連結

本発明者は、少なくとも１つのホスホロチオレート結合を有する伸長プローブが、伸長、ライゲーション、検出および切断の連続的サイクルによる配列決定のための方法の実施において特に有用であることを見出した。かかる連結では、ホスホジエステル結合の橋絡酸素原子の１つがイオウ原子で置き換えられている。ホスホロチオレート結合は、図４Ａ

に示すような５’－Ｓ－ホスホロチオレート結合（３’－Ｏ－Ｐ－Ｓ－５’）または図４Ｂに示すような３’－Ｓ－ホスホロチオレート結合（３’－Ｓ－Ｐ－Ｏ－５’）のいずれかであり得る。図４Ａおよび４Ｂに示すように、３’－Ｏ－Ｐ－Ｓ－５’または３’－Ｓ－Ｐ－Ｏ－５’で表される連結内のリン原子は、２つの非橋絡酸素原子に結合され得ることを理解されたい（典型的なホスホジエステル結合の場合と同様）。あるいはまた、リン原子は、任意のさまざまな他の原子または基、例えば、Ｓ、CH_3、BH_3などに結合され得る。したがって、本発明の一態様は、ホスホロチオレート結合を含む標識されたオリゴヌクレオチドプローブである。プローブには、本明細書に記載した配列決定法において具体的な用途が見られるが、さまざまな他の目的にもまた使用され得る。特に、本発明は、（ｉ）形態５’－Ｏ－Ｐ－Ｏ－Ｘ－Ｏ－Ｐ－Ｓ－（Ｎ）$_k$ N_B * －３’のオリゴヌクレオチド；および（ｉｉ）形態５’－N_B *（Ｎ）$_k$－Ｓ－Ｐ－Ｏ－Ｘ－３’のオリゴヌクレオチドを提供する。これらのプローブの各々において、Ｎは任意のヌクレオチドを表し、N_Bはリガーゼによって伸長可能でない部分を表し、＊は検出可能な部分を表し、Ｘはヌクレオチドを表し、ｋは１～１００である。ある特定の実施形態において、ｋは、１～５０、１～３０、１～２０、例えば４～１０である、ただし、検出可能な部分は、（Ｎ）$_k$の任意のヌクレオシド上にN_Bの代わりまたはこれに加えて存在するものとする。任意のこれらのプローブ内の末端ヌクレオチドは、リン酸基またはヒドロキシル基を含むものであってもそうでなくてもよい。さらにまた、該リン原子は、好ましい実施形態において、一般的に、２つのさらなる（非橋絡）酸素原子に結合されていることは認識されよう。

【0178】

５’－Ｓ－ホスホロチオレートまたは３’－Ｓ－ホスホロチオレート結合を含有するオリゴヌクレオチドを合成するための方法は、当該技術分野で知られており、これらの方法のある特定のものは、自動固相オリゴヌクレオチド合成に適している。合成手順は、例えば、Ｃｏｏｋ，ＡＦ，Ｊ．Ａｍ．Ｃｈｅｍ．Ｓｏｃ，９２：１９０－１９５，１９７０；Ｃｈｌａｄｅｋ，Ｓ．ら、Ｊ．Ａｍ．Ｃｈｅｍ．Ｓｏｃ，９４：２０７９－２０８４，１９７２；Ｒｙｂａｋｏｖ，ＶＮら、Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．，９：１８９－２０１，１９８１；Ｃｏｓｓｔｉｃｋ，Ｒ．およびＶｙｌｅ，ＪＳ，Ｊ．Ｃｈｅｍ．Ｓｏｃ．ＣＨｅｍ．Ｃｏｍｍｕｎ．，９９２－９９２，１９８８；Ｍａｇ，Ｍ．ら、Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．，１９（７）；１４３７－１４４１，１９９１；Ｘｕ，Ｙ，およびＫｏｏｌ，ＥＴ，Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．，２６（１３）：３１５９－３１６４，１９９８；Ｃｏｓｓｔｉｃｋ，Ｒ．およびＶｙｌｅ，ＪＳ，Ｔｅｔｒａｈｅｄｒｏｎ Ｌｅｔｔ．，３０：４６９３－４６９６，１９８９；Ｃｏｓｓｔｉｃｋ，Ｒ．およびＶｙｌｅ，ＪＳ，Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．，１８：８２９－８３５，１９９０；Ｓｕｎ，ＳＧおよびＰｉｃｃｉｒｉｌｌｉ，ＪＡ，Ｎｕｃｌ．Ｎｕｃｌ，１６：１５４３－１５４５，１９９７；Ｓｕｎ ＳＧら、ＲＮＡ，３：１３５２－１３６３，１９９７；Ｖｙｌｅ，ＪＳら、Ｔｅｔｒａｈｅｄｒｏｎ Ｌｅｔｔ．，３３：３０１７－３０２０，１９９２；Ｌｉ，Ｘ．ら、Ｊ．Ｃｈｅｍ．Ｓｏｃ．Ｐｅｒｋｉｎ Ｔｒａｎｓ．，１：２１２３－２２１２９，１９９４；Ｌｉｕ，ＸＨ ａｎｄ Ｒｅｅｓｅ，ＣＢ，Ｔｅｔｒａｈｅｄｒｏｎ Ｌｅｔｔ．，３７：９２５－９２８，１９９６；Ｗｅｉｎｓｔｅｉｎ，ＬＢら、Ｊ．Ａｍ．Ｃｈｅｍ．Ｓｏｃ，１１８：１０３４１－１０３５０，１９９６；ならびにＳａｂｂａｇｈ，Ｇ．ら、Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．，３２（２）：４９５－５０１，２００４に記載されている。また、本発明者らは、新たな合成方法を開発した。例えば、図７は、ｄＡの３’－ホスホロアミデートの合成スキームを示す。同様のスキームが、ｄＧの３’－ホスホロアミデートの合成に使用され得る。これらのホスホロアミデートは、プリンヌクレオシドと会合した３’－Ｓ－ホスホロチオレート結合を含有するオリゴヌクレオチドを、例えば、自動ＤＮＡ合成装置を用いて合成するために使用され得る。

【0179】

ホスホロチオレート連結は、さまざまな金属含有薬剤を用いて切断され得る。金属は、

例えば、Ａｇ、Ｈｇ、Ｃｕ、Ｍｎ、ＺｎまたはＣｄであり得る。好ましくは、該薬剤は、Ag^+、Hg^{++}、Cu^{++}、Mn^{++}、Zn^+またはCd^+アニオンを提供する水溶性の塩である（他の酸化状態のイオンを提供する塩もまた使用され得る）。I_2もまた使用され得る。硝酸銀（$AgNO_3$）などの銀含有塩またはAg^+イオンを提供する他の塩，は特に好ましい。好適な条件としては、例えば、５０ｍＭ　$AgNO_3$、約２２～３７℃で１０分以上（例えば、３０分間）が挙げられる。好ましくは、ｐＨは、４．０～１０．０、より好ましくは５．０～９．０、例えば約６．０～８．０、例えば約７．０である。例えば、Ｍａｇ，　Ｍ．ら、Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．，１９（７）：１４３７－１４４１，１９９１を参照のこと。例示的なプロトコルを実施例１に示す。

【0180】

５’→３’方向の配列決定は、３’－Ｏ－Ｐ－Ｓ－５’連結を含有する伸長プローブを用いて行なわれ得る。図５Ａは、形態５’－Ｏ－Ｐ－Ｏ－Ｘ－Ｏ－Ｐ－Ｓ－$NNNNN_B$*－３’（式中、Ｎは任意のヌクレオチドを表し、N_Bはリガーゼによって伸長可能でない部分を表し（例えば、N_Bは、３’ヒドロキシル基がないヌクレオチドであるか、またはブロッキング部分が結合されている）、＊は検出可能な部分を表し、Ｘは、その正体が検出可能な部分に対応するヌクレオチドを表す）の伸長プローブを用いたハイブリダイゼーション、ライゲーションおよび切断１サイクルを示す。あるいはまた、多重ライゲーションを抑制するため、任意の多数のブロッキング部分を３’末端ヌクレオチドに結合させ得る。例えば、バルキー基をヌクレオチドの糖鎖部分、例えば、２’位または３’位に結合させることにより、ライゲーションが抑制される。蛍光標識は、適切なバルキー基としての機能を果たし得る。

【0181】

結合領域４０および未知配列のポリヌクレオチド領域５０を含有する鋳型を支持体、例えば、ビーズに結合させる。好ましい実施形態において、図５Ａに示すように、結合領域は、支持体への結合点と反対の鋳型の末端に位置する。伸長可能な末端（この場合は、遊離３’ＯＨ基）を有する初期オリゴヌクレオチド３０を、結合領域４０にアニーリングさせる。伸長プローブ６０をポリヌクレオチド領域５０内の鋳型にハイブリダイズさせる。ヌクレオチドＸは、鋳型内の未知ヌクレオチドＹと相補塩基対を形成する。伸長プローブ６０を初期オリゴヌクレオチドにライゲートさせる（例えば、Ｔ４リガーゼを用いて）。ライゲーション後、伸長プローブ６０に結合された標識を検出する（図示せず）。標識は、ヌクレオチドＸの正体に対応する。したがって、ヌクレオチドＹが、ヌクレオチドＸに相補的なヌクレオチドとして同定される。次いで、伸長プローブ６０をホスホロチオレート結合で切断し（例えば、$AgNO_3$またはAg^+イオンを提供する別の塩を用いて）、伸長された二本鎖が得られる。切断によりリン酸基が伸長された二本鎖の３’末端に生じる。ホスファターゼ処理が、伸長可能なプローブ末端を伸長された二本鎖に生成させるために使用される。該プロセスを所望回数サイクル反復する。

【0182】

好ましい実施形態では、配列決定は、３’→５’方向に３’－Ｓ－Ｐ－Ｏ－５’連結を含有する伸長プローブを用いて行なわれる。図５Ｂは、形態５’－N_B*－ＮＮＮＮ－Ｓ－Ｐ－Ｏ－Ｘ－３’（式中、Ｎは任意のヌクレオチドを表し、N_Bはリガーゼによって伸長可能でない部分を表し（例えば、ＮＢは、５’リン酸基がないヌクレオチドであるか、またはブロッキング部分が結合されている）、＊は検出可能な部分を表し、Ｘは、その正体が検出可能な部分に対応するヌクレオチドを表す）の伸長プローブを用いたハイブリダイゼーション、ライゲーションおよび切断の１サイクルを示す。

【0183】

結合領域４０および未知配列のポリヌクレオチド領域５０を含有する鋳型を支持体、例えば、ビーズに結合させる。好ましい実施形態において、図５Ｂに示すように、結合領域は、支持体への結合点と反対の鋳型の末端に位置する。伸長可能な末端（この場合は、遊離５’リン酸基）を有する初期オリゴヌクレオチド３０を、結合領域４０にアニーリングさせる。伸長プローブ６０をポリヌクレオチド領域５０内の鋳型にハイブリダイズさせる

。ヌクレオチドＸは、鋳型内の未知ヌクレオチドＹと相補塩基対を形成する。伸長プローブ６０を初期オリゴヌクレオチドにライゲートさせる（例えば、Ｔ４リガーゼを用いて）。ライゲーション後、伸長プローブ６０に結合された標識を検出する（図示せず）。標識は、ヌクレオチドＸの正体に対応する。したがって、ヌクレオチドＹが、ヌクレオチドＸに相補的なヌクレオチドとして同定される。次いで、伸長プローブ６０をホスホロチオレート結合で切断し（例えば、$AgNO_3$ またはAg^+ イオンを提供する別の塩を用いて）、伸長された二本鎖が得られる。切断により伸長可能な一リン酸基が伸長された二本鎖の５’末端に生じ、したがって、伸長可能な末端を生成させるためのさらなる工程を行なうことは不必要である。該プロセスを所望回数サイクル反復する。

【0184】

このスキームのいくつかの変形例が使用され得ることは認識されよう。例えば、プローブは６ヌクレオチドよりも短くても長くてもよく、標識は３’末端ヌクレオチド上である必要はなく、Ｐ－Ｓ連結は任意の２つの隣接するヌクレオチド間に位置し得るなどである。上記の実施形態では、伸長、ライゲーション、検出および切断の連続的サイクルにより、隣接して位置するヌクレオチドの同定がもたらされる。しかしながら、Ｐ－Ｓ連結を伸長プローブの遠位端（すなわち、ライゲーションが起こる末端と反対の末端）に対してより近くに配置することによって、上記ならびに図１および６に示すように、連続的に同定されるヌクレオチドは鋳型に沿って間隔があく。

【0185】

図６Ａ～６Ｆは、単一の鋳型において連続的に行なわれるいくつかの配列決定反応のより詳細な概略図である。配列決定は、３’→５’方向に３’－Ｓ－Ｐ－Ｏ－５’連結を含有する伸長プローブを用いて行なわれる。各配列決定反応は、伸長、ライゲーション、検出および切断の多数のサイクルを含む。該反応では、鋳型の異なる部分に結合する初期オリゴヌクレオチドを用いる。伸長プローブは８ヌクレオチド長であり、プローブの３’末端から数えて６番目と７番目のヌクレオチド間に位置するホスホロチオレート結合を含有する。ヌクレオチド２～６は、各反応で、鋳型に沿って間隔があいている複数のヌクレオチドの同定が可能となるようにスペーサーとしての機能を果たす。多数の反応を順次行ない、各反応で得られた部分配列情報を適切に組み合わせることにより、鋳型の一部分完全な配列が決定される。

【0186】

図６Ａは、伸長可能な二本鎖を得るための、鋳型内のアダプター配列（図６Ａ～６Ｆでは結合領域という）にハイブリダイズさせた第１の初期オリゴヌクレオチド（図６Ａ～６Ｆではプライマーという）を用いた初期化を示す。図６Ｂ～６Ｄは、ヌクレオチド同定のいくつかのサイクルを示し、この場合、鋳型は６塩基ごとに読み出しされる。図６Ｂでは、第１の未知鋳型内のヌクレオチド配列に相補的な３’末端ヌクレオチドを有する第１の伸長プローブが鋳型に結合し、プライマーの伸長可能な末端にライゲートされる。伸長プローブに結合された標識により、プローブは３’末端ヌクレオチドとしてＡを有すると同定され、したがって、鋳型配列内の第１の未知ヌクレオチドはＡであると同定される。図６Ｃは、ホスホロチオレート結合での$AgNO_3$ による伸長オリゴヌクレオチドの切断および伸長プローブの標識が結合された部分の放出を示す。図６Ｄは、伸長、ライゲーションおよび切断のさらなるサイクルを示す。プローブは５ヌクレオチド長のスペーサーを含むため、配列決定反応により、鋳型は６ヌクレオチドごとに同定される。

【0187】

所望回数サイクル後、第１の初期化ヌクレオチドを含む伸長鎖が除去され、第１の初期オリゴヌクレオチドが結合した部分と異なる結合領域の部分に結合する第２の初期オリゴヌクレオチドが、鋳型にハイブリダイズされる。図６Ｅは、第２の配列決定反応を示し、この場合、初期化は、第２の初期オリゴヌクレオチドにより行なわれ、その後、数回サイクルのヌクレオチド同定が行なわれる。図６Ｆは、第３の初期オリゴヌクレオチドを用いた初期化後、数回サイクルのヌクレオチド同定を示す。第２の初期オリゴヌクレオチドからの伸長により、第１の配列決定反応同定されたヌクレオチドと異なる「フレーム」内で

６塩基ごとの同定が可能になる。

【0188】

ホスホロチオレート結合を含む伸長プローブは、本発明のある特定の実施形態において好ましいが、さまざまな他の切れやすい連結が好都合に使用され得る。例えば、天然に存在する核酸に見られるＯ－Ｐ－Ｏ連結に対する多数のバリエーションが知られている（例えば、Ｍｉｃｋｌｅｆｉｅｌｄ，　Ｊ．Ｃｕｒｒ．Ｍｅｄ．Ｃｈｅｍ．，８：１１５７－１１７９，２００１を参照）。本明細書に記載されたＰ－Ｏ結合を含む任意の構造は、切れやすいＰ－Ｓ結合を含むように修飾され得る。例えば、ＮＨ－Ｐ－Ｏ結合をＮＨ－Ｐ－Ｓ結合に変更してもよい。

【0189】

本発明のある一部の実施形態において、伸長プローブは、核酸を、切断剤またはその組合せによる切断に対して感受性とする（任意選択で、修飾剤によるトリガー残基の修飾後）トリガー残基を含む。特に、本発明者らは、ＤＮＡ修復に関与する酵素は、伸長、ライゲーション、検出および切断の連続的サイクルによる配列決定のための方法の実施における使用のための好都合な切断試薬であることを見出した。一般に、伸長プローブ内における損傷塩基または無塩基残基などのトリガー残基の存在により、プローブは、１種類以上のＤＮＡ修復酵素による切断に対して感受性となり得る（任意選択で、ＤＮＡグリコシラーゼによる修飾後）。したがって、ＡＰエンドヌクレアーゼなどのＤＮＡ修復に関与する酵素による切断の基質である連結を含む伸長プローブは、本発明において有用である。修飾によりプローブがＡＰエンドヌクレアーゼによる切断に対して感受性となるＤＮＡグリコシラーゼなどのＤＮＡ修復に関与する酵素による修飾の基質である残基を含有する伸長プローブもまた、本発明に特に有用である。ある一部の実施形態において、伸長プローブは、無塩基残基を含む、すなわちプリンまたはピリミジン塩基がない。無塩基残基と隣接ヌクレオシド間の連結は、ＡＰエンドヌクレアーゼによって切断に対して感受性であり、したがって、切れやすい連結である。本発明のある特定の実施形態において、無塩基残基は２’デオキシリボースを含む。ある一部の実施形態において、伸長プローブは損傷塩基を含む。損傷塩基は、ＤＮＡグリコシラーゼなどの損傷塩基を除去する酵素の基質である。損傷塩基の除去後、得られる無塩基残基と隣接ヌクレオシド間の連結は、ＡＰエンドヌクレアーゼによる切断に対して感受性であり、したがって、本発明に従う切れやすい連結とみなされる。

【0190】

多くの異なるＡＰエンドヌクレアーゼが本発明における切断試薬として有用である。ＡＰエンドヌクレアーゼの２つの主な類型が、無塩基残基に隣接する連結を切断する機構に基づいて識別されている。類型ＩのＡＰエンドヌクレアーゼ、例えば、大腸菌のエンドヌクレアーゼＩＩＩ（Ｅｎｄｏ　ＩＩＩ）およびエンドヌクレアーゼＶＩＩＩ（Ｅｎｄｏ　ＶＩＩＩ）ならびにヒト類縁体ｈＮＴＨ１、ＮＥＩＬ１、ＮＥＩＬ２およびＮＥＩＬ３などは、ＡＰ残基の３’側のＤＮＡを切断し、３’末端リン酸基を有する５’部分および５’末端リン酸基を担持する３’部分をもたらすＡＰリアーゼである。類型ＩＩのＡＰエンドヌクレアーゼ、例えば、例えば、大腸菌のエンドヌクレアーゼＩＶ（Ｅｎｄｏ　ＩＶ）およびエクソヌクレアーゼＩＩＩ（Ｅｘｏ　ＩＩＩ）などは、ＡＰ部位の５’のＤＮＡを切断し、３’ＯＨおよび５’デオキシリボースリン酸基部分を生じる断片の末端に生成させる。種々の類型Ｉおよび類型ＩＩのＡＰエンドヌクレアーゼならびに損傷塩基のＤＮＡからのを除去および／または無塩基残基含有ＤＮＡの切断の条件のさらなる論考については、例えば、Ｄｏｕｂｌｉｅ，　Ｓ．ら、Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ　１０１（２８），１０２８４－１０２８９，２００４；Ｈａｌｔｉｗａｎｇｅｒ，　Ｂ．Ｍ．ら，　Ｂｉｏｃｈｅｍ　Ｊ．，３４５，８５－８９，２０００；Ｌｅｖｉｎ，　Ｊ．ａｎｄ　Ｄｅｍｐｌｅ，　Ｂ．，　Ｎｕｃｌ．Ａｃｉｄｓ．Ｒｅｓ，１８（１７），１９９０、および前記文献中の参考文献を参照のこと。当業者には、これらの酵素のさまざまな類縁体が、他の生物体（例えば、酵母）に存在し、本発明において有用であることが認識されよう。

【0191】
ある種の酵素は、グリコシラーゼ活性を有し、損傷塩基を除去してＡＰ残基を生成させ、また、グリコシラーゼ活性によって生成されたＡＰ部位の３’側でホスホジエステル主鎖を切断するリアーゼ活性を示すという両方の点で二官能性である。したがって、このような二重活性酵素は、ＡＰエンドヌクレアーゼとＤＮＡグリコシラーゼの両方である。例えば、Ｅｎｄｏ　ＶＩＩＩは、Ｎ－グリコシラーゼとＡＰ－リアーゼの両方として作用する。Ｎ－グリコシラーゼ活性により損傷ピリミジンが二本鎖ＤＮＡから放出され、無プリン（ＡＰ部位）をもたらされる。ＡＰ－リアーゼ活性により、ＡＰ部位の３’および５’側が切断され、５’リン酸基および３’リン酸基が生じる。エンドヌクレアーゼＶＩＩＩによって認識および除去される損傷塩基としては、尿素、５，６－ジヒドロキシチミン、チミングリコール、５－ヒドロキシ－５－メチルヒダントン、ウラシルグリコール、６－ヒドロキシ－５、６－ジヒドロチミンおよびメチルタートロニル（ｔａｒｔｒｏｎｙｌ）尿素が挙げられる。例えば、Ｄｉｚｄａｒｏｇｌｕ，　Ｍ．ら、Ｂｉｏｃｈｅｍｉｓｔｒｙ，３２，１２１０５－１２１１１，１９９３およびＨａｔａｈｅｔ，　Ｚ．ら、Ｊ　Ｂｉｏｌ．Ｃｈｅｍ．，２６９，１８８１４－１８８２０，１９９４；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．，　２７２（５１），３２２２０－３２２２９，１９９７；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂａｃｔ，１７９（１１），　３７７３－３７８２，　１９９７を参照のこと。
【0192】
Ｆｐｇ（ホルムアミドピリミジン［ｆａｐｙ］－ＤＮＡグリコシラーゼ）（８－オキソグアニンＤＮＡグリコシラーゼとしても知られる）もまた、Ｎ－グリコシラーゼとＡＰ－リアーゼの両方として作用する。Ｎ－グリコシラーゼ活性により損傷プリンが二本鎖ＤＮＡから放出され、無プリン（ＡＰ部位）がもたらされる。ＡＰ－リアーゼ活性により、ＡＰ部位の３’および５’側両方が切断され、それによりＡＰ部位が除去され、１塩基ギャップが生じる。Ｆｐｇ認識および除去される損傷塩基の一例としては、７，８－ジヒドロ－８－オキソグアニン（８－オキソグアニン）、８－オキソアデニン、ｆａｐｙ－グアニン、メチル－ｆａｐｙ－グアニン、ｆａｐｙ－アデニン、アフラトキシンＢ１－ｆａｐｙ－グアニン、５－ヒドロキシ－シトシンおよび５－ヒドロキシ－ウラシルが挙げられる。例えば、Ｔｃｈｏｕ，　Ｊ．ら．Ｊ．Ｂｉｏｌ　Ｃｈｅｍ．，２６９，１５３１８－１５３２４，１９９４；Ｈａｔａｈｅｔ，　Ｚ．ら．Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．，２６９，１８８１４－１８８２０，　１９９４；Ｂｏｉｔｅｕｘ，　Ｓ．ら，　ＥＭＢＯ　Ｊ．，５，３１７７－３１８３，１９８７；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．，２７２（５１），３２２２０－３２２２９，１９９７；Ｊｉａｎｇ，　Ｄ．ら、Ｊ．Ｂａｃｔ，　１７９（１１），３７７３－３７８２，１９９７を参照のこと。
【0193】
いくつかのＤＮＡグリコシラーゼおよびＡＰエンドヌクレアーゼは、例えば、Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ，　Ｉｐｓｗｉｃｈ，　ＭＡから市販されている。
【0194】
本発明のある一部の実施形態において、ＡＰエンドヌクレアーゼによる切断のための基質である部位を含む伸長プローブが、ホスホロチオレート結合を含有する伸長プローブの上記のような配列決定法、または配列決定法ＡＢ（下記参照）に使用される。任意のこれらの方法では、伸長プローブのライゲーションにより核酸鎖を成長させた後、伸長プローブをＡＰエンドヌクレアーゼを用いて切断し、プローブの標識を含む部分を除去する。
【0195】
具体的なＡＰエンドヌクレアーゼに応じて、および配列決定が３’→５’または５’→３’方向のどちらで行なわれるかに応じて、切断後、伸長された二本鎖をポリヌクレオチドキナーゼまたはホスファターゼで処理し、伸長可能なプローブ末端を伸長された二本鎖上に生成させることが必要または望ましいことがある（伸長可能なプローブ末端を示す図５Ａおよび５Ｂ参照）。したがって、本発明のある特定の方法では、伸長可能な末端を、ポリヌクレオチドキナーゼまたはホスファターゼでの処理によって生成させる。当業者に

は、種々の酵素に適切なバッファーが使用され、酵素を除去し、該方法の後続の工程に適切な条件を提供するための洗浄するさらなる工程を含め得ることが認識されよう。

【0196】

他の実施形態において、伸長プローブは、ＤＮＡグリコシラーゼによる除去のための基質である損傷塩基を含む。広範な細胞傷害性および変異原性ＤＮＡ塩基が、ＤＮＡの損傷後に塩基切除修復経路を起始させる種々のＤＮＡグリコシラーゼによって除去される（Ｋｒｏｋａｎ，　Ｈ．Ｅ．ら，　Ｂｉｏｃｈｅｍ　Ｊ，３２５（Ｐｔ　１）：１－１６，１９９７）。ＤＮＡグリコシラーゼは、損傷塩基とデオキシリボース間のＮ－グリコシド結合を切断し、したがって、遊離塩基を放出させ無プリン／無ピリミジン（ＡＰ）部位をもたらす。ある一部の実施形態において、伸長プローブは、ウラシル－ＤＮＡグリコシラーゼ（ＵＤＧ）によって除去されるウラシル残基を含む。ＵＤＧは、これまでに研究されたあらゆる生きた生物体に見られ、これらの酵素の多くが当該技術分野で知られており、本発明に有用である（Ｆｒｅｄｅｒｉｃａら，　Ｂｉｏｃｈｅｍｉｓｔｒｙ，２９，　２３５３－２５３７，１９９０；Ｋｒｏｋａｎ、前掲）。例えば、哺乳動物は、少なくとも４種類のＵＤＧ：ミトコンドリアＵＮＧ１および核ＵＮＧ２、ＳＭＵＧ１、ＴＤＧおよびＭＢＤ４を含む（Ｋｒｏｋａｎら、Ｏｎｃｏｇｅｎｅ，２１，８９３５－８９４８，２００２）。ＵＮＧ１およびＵＮＧ２は、大腸菌Ｕｎｇに代表される高度に保存されたファミリーに属する。伸長プローブが損傷塩基を含む実施形態では、伸長可能なプローブ末端への伸長プローブのライゲーション後、伸長された二本鎖を、損傷塩基除去するグリコシラーゼと接触させ、それにより無塩基残基を生成させる。グリコシラーゼによる除去に供された損傷塩基を含む伸長プローブは、「切れやすい連結を含むように容易に修飾可能である」とみなされる。次いで、伸長された二本鎖を、上記のような無塩基残基と隣接ヌクレオシド間の連結を切断するＡＰエンドヌクレアーゼと接触させる。本発明のある特定の実施形態において、ＤＮＡグリコシラーゼとＡＰエンドヌクレアーゼの両方である二重活性酵素は、これらの反応の両方を行うために使用される。ある一部の実施形態では、損傷塩基を含有する伸長された二本鎖を、ＤＮＡグリコシラーゼおよびＡＰエンドヌクレアーゼと接触させる。これらの酵素は、本発明の種々の実施形態において、組合せて、または連続的に（すなわち、グリコシラーゼの後エンドヌクレアーゼ）使用され得る。

【0197】

本発明のある一部の実施形態において、伸長プローブは、デオキシイノシンであるトリガー残基を含む。上記のように、大腸菌エンドヌクレアーゼＶ（Ｅｎｄｏ　Ｖ）（デオキシイノシン３’エンドヌクレアーゼともいわれる）およびその類縁体は、デオキシイノシン含有する核酸を、デオキシイノシン残基の３’側の第２のホスホジエステル結合で切断し、３’ＯＨおよび５’リン酸基末端をもたらす。したがって、この結合は、伸長プローブ内の切れやすい連結としての機能を果たす。Ｅｎｄｏ　Ｖおよびその切断特性は、当該技術分野で知られている（Ｙａｏ，　Ｍ．およびＫｏｗ　Ｙ．Ｗ．，　Ｊ　Ｂｉｏｌ．Ｃｈｅｍ．，２７１，３０６７２－３０６７３（１９９６）；Ｙａｏ，　Ｍ．およびＫｏｗ　Ｙ．Ｗ．，　Ｊ　Ｂｉｏｌ．Ｃｈｅｍ．，２７０，２８６０９－２８６１６（１９９５）；Ｈｅ，　Ｂら、Ｍｕｔａｔ　Ｒｅｓ．，　４５９，　１０９－１１４（２０００）。デオキシイノシンに加え、Ｅｎｄｏ　Ｖはまた、デオキシウリジン、デオキシキサントシンおよびデオキシオキサノシンを認識する（Ｈｉｔｃｈｃｏｃｋ，　Ｔ．ら、Ｎｕｃ．Ａｃｉｄｓ　Ｒｅｓ．，３２（１３），３２（１３）（２００４）。ｍＥｎｄｏ　Ｖなどの哺乳動物の類縁体もまた切断活性を示す（Ｍｏｅ，　Ａ．ら、Ｎｕｃ．Ａｃｉｄｓ　Ｒｅｓ．，３１（１４），３８９３－３９００（２００４）。Ｅｎｄｏ　Ｖは、デオキシイノシンを含むプローブの好ましい切断剤であるが、他の切断試薬もまた、デオキシイノシンを含むプローブを切断するために使用され得る。例えば、損傷塩基としてヒポキサンチンが、適切なＤＮＡグリコシラーゼによる除去に供され得、生じた無塩基残基含有伸長プローブが、次いで、エンドヌクレアーゼによる切断に供される。

【0198】

デオキシイノシンがトリガー残基として使用される場合、プローブ内のどこか、特に、

伸長可能なプローブ末端にライゲートされる末端とトリガー残基間の位置でのデオキシイノシンの使用を回避することが望ましいことがあり得ることは認識されよう。したがって、プローブが１つ以上の普遍塩基を含む場合、デオキシイノシン以外のヌクレオシドが使用され得る。また、トリガー残基を含有する核酸を、ある特定の切断剤による切断に感受性にするトリガー残基が伸長プローブ内に使用される場合、同じ切断剤による切断を誘発し得る他の残基をプローブ内に（または該伸長プローブとともに配列決定反応に使用され得る他のプローブ内に）を含めることを回避することが望ましいことがあり得ることも認識されよう。

【0199】

本発明は、トリガー残基を含む核酸を切断する任意の酵素の使用を包含する。さらなる酵素は、Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ（登録商標），　Ｉｎｃ．などの酵素供給業者のカタログを調べることにより特定され得る。Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓカタログ，　２００５版（Ｎｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ，　Ｉｐｓｗｉｃｈ，　ＭＡ　０１９３８－２７２３）は、引用により本明細書に組み込まれ、本発明では、そこに開示されたトリガー残基を含有する核酸を切断する任意の酵素、またはかかる酵素の類縁体の使用が想定される。有用な他の酵素としては、例えば、ｈＯＧＧ１およびその類縁体が挙げられる（Ｒａｄｉｃｅｌｌａ，　ＪＰら、Ｐｒｏｃ　Ｎａｔｌ　Ａｃａｄ　Ｓｅｉ　ＵＳＡ．，９４（１５）：８０１０－５，１９９７）。

【0200】

損傷塩基、無塩基残基などのトリガー残基を含有するオリゴヌクレオチドを合成するための方法は、当該技術分野で知られている。ＡＰエンドヌクレアーゼの基質である部位を含有するオリゴヌクレオチド、例えば、無塩基残基を含有するオリゴヌクレオチド合成するための方法は、当該技術分野で知られており、一般的に自動固相オリゴヌクレオチド合成に適している。ある一部の実施形態において、ウリジンを無塩基残基の所望の位置に含有するオリゴヌクレオチドを合成する。次いで、該オリゴヌクレオチドを、ＵＤＧなどのウラシルを除去する酵素で処理し、それにより、該オリゴヌクレオチド内のどこかにウリジンが存在した無塩基残基を生成させる。

【0201】

本発明のある一部の実施形態において、オリゴヌクレオチドプローブは、Ｎａｕｗｅｌａｅｒｔｓ，　Ｋ．ら，　Ｎｕｃ．Ａｃｉｄｓ．Ｒｅｓ．，３１（２３），２００３に記載された二糖ヌクレオシドを含む。ライゲーション後、伸長された二本鎖を、過ヨウ素酸塩（$NaIO_4$）を用いて切断した後、塩基（例えば、ＮａＯＨ）で処理して標識を除去し、遊離３’ＯＨおよびＰ５－ＯＰＯ$_3$Ｈ$_2$基を生成させる。配列決定が３’→５’または５’→３’方向のどちらで行なわれるかに応じて、伸長された二本鎖をポリヌクレオチドキナーゼまたはホスファターゼで処理し、伸長可能な末端を生成させることが必要または望ましいことがある。したがって、本発明のある特定の方法では、伸長可能な末端は、ポリヌクレオチドキナーゼまたはホスファターゼでの処理によって生成される。

【0202】

二糖ヌクレオシドを含むポリヌクレオチドは、無塩基残基を含むとみなされる。例えば、一方のヌクレオチドの３’ＯＨと次のヌクレオチドの５’リン酸基との間に挿入されたリボース残基を含有するポリヌクレオチドは、無塩基残基を含むとみなされる。

【0203】

キャッピング

場合によっては、伸長可能な末端を有するプローブの全部未満が、伸長、ライゲーションおよび切断の各サイクルにおいて成功裏のライゲーション反応に関与する。かかるプローブが継続されるサイクルに関与する場合、各ヌクレオチド同定工程の精度が進行的に低下し得ることは認識されよう。本発明者らは、ホスホロチオレート連結を含有する伸長プローブの使用により、高効率でのライゲーションが可能になることを示したが、本発明のある特定の実施形態では、ライゲーションを行なわない伸長可能な末端が後続のサイクルに関与するのを抑制するためにキャッピング工程を含める。３’－Ｏ－Ｐ－Ｓ－５’ホス

ホロチオレート結合を含有する伸長プローブを用いる５’→３’方向における配列決定の場合、キャッピングは、例えばライゲーションまたは検出工程後に、ライゲートされない伸長可能な末端をＤＮＡポリメラーゼで伸長させ、伸長不可能な部分（例えば、ジデオキシヌクレオチドまたはヌクレオチドなどの連鎖停止ヌクレオチド）にブロッキング部分を結合させることにより行なわれ得る。３’－Ｓ－Ｐ－Ｏ－５’ホスホロチオレート結合が伸長プローブを用いる３’→５’方向の配列決定の場合、キャッピングは、例えばライゲーションまたは検出工程後に、例えば、鋳型をホスファターゼで処理することにより行なわれ得る。他のキャッピング方法もまた使用され得る。

【0204】

Ｈ．オリゴヌクレオチドプローブファミリーを用いる配列決定

上記の配列決定法を集合的に「方法Ａ」といい、該方法では、任意の特定の伸長プローブ結合された標識と、プローブの近位末端（すなわち、伸長された二本鎖の伸長可能なプローブ末端ライゲートされた末端）の１つ以上のヌクレオチドの正体との間に直接的で既知の対応がある。したがって、新たにライゲートされた伸長プローブの標識を同定することは、鋳型内の１つ以上ヌクレオチドを同定するのに充分である。本発明は、集合的に「方法ＡＢ」と称し、これもまた、伸長、ライゲーションおよび、好ましくは切断の連続的サイクルを伴い、ヌクレオチド同定に対する異なるアプローチが採用された、さらなる配列決定法を提供する。

【0205】

本発明は、少なくとも２つの識別可能に標識されたオリゴヌクレオチドプローブファミリーのコレクションを使用する配列決定法ＡＢを提供する。各プローブファミリーは、標識に基づいた名称、例えば、「赤色」、「青色」、「黄色」、「緑色」に割り当てられる。上記の方法のように、伸長は、初期オリゴヌクレオチドおよび鋳型によって形成された二本鎖から開始される。初期オリゴヌクレオチドは、オリゴヌクレオチドプローブをその末端にライゲートして伸長された二本鎖を形成させ、次いで、これを、ライゲーションの連続的サイクルによって反復的に伸長させることにより伸長される。伸長された二本鎖のたった１回の伸長が１サイクルで起こるように、プローブは伸長不可能な部分を、末端位置に（二本鎖の成長核酸鎖にライゲートされたヌクレオチドと反対のプローブ末端に）有する。各サイクル中、成功裏にライゲートされたプローブ上の、またはこれと会合させた標識が検出され、伸長不可能な部分が除去または修飾されて、伸長可能な末端が生成する。標識の検出により、プローブが属するプローブファミリー名が特定される。

【0206】

伸長、ライゲーションおよび検出の連続的なサイクルにより、一連の標識名の序列がもたらされる。標識は、連続的な位置の鋳型にハイブリダイズする成功裏にライゲートされたプローブが属するプローブファミリーに対応する。プローブは、ライゲーション後、鋳型内の異なるヌクレオチドと反対の位置に位置する近位末端を有する。したがって、プローブファミリー名の順序と鋳型内のヌクレオチドの順序との間に対応がある。

【0207】

切れやすい連結が、伸長プローブ内の近位ヌクレオシドと隣接するヌクレオシド間に位置する本発明の実施形態では、プローブファミリーの序列リスト名は、伸長されたオリゴヌクレオチドプローブが、各サイクルで１つのヌクレオチドによって伸長されるため、単一の初期オリゴヌクレオチドから開始される伸長、ライゲーション、検出および切断の連続的サイクルによって得られ得る。切れやすい連結がその他の２つのヌクレオシド間に位置する場合は、プローブファミリーの序列リスト名は、配列決定法Ａで記載のような、結合反応において異なる位置にハイブリダイズする初期オリゴヌクレオチドが使用される複数の配列決定反応から得られる結果から合成される。

【0208】

新たにライゲートされたプローブがどのプローブファミリーに属するかがわかることは、それ自体では、鋳型内のヌクレオチドの正体を決定するのに充分でない。それどころか、それどころか、プローブファミリー名が決定されることは、ヌクレオチドのある特定の

組合せが、プローブの少なくとも一部分の配列である可能性を排除するが、各ヌクレオチドの正体に少なくとも２つの可能性が残る。したがって、さらなる情報の非存在下で、プローブファミリー名がわかることにより、新たにライゲートされたプローブ内のヌクレオチドと反対の位置に位置する鋳型内のヌクレオチドの正体に少なくとも２つの可能性が残されたままになる。したがって、伸長、ライゲーション、検出（および、任意選択で切断）任意の１サイクルは、それ自体では、任意の鋳型内のヌクレオチドが同定されない。しかしながら、鋳型の配列の１つ以上の可能性の排除は可能にし、それにより配列に関する情報が提供される。本発明のある特定の実施形態において、後述のようなプローブおよびプローブファミリーの適切な設計により、鋳型の配列が、さらに決定され得る。したがって、本発明のある特定の実施形態において、配列決定法ＡＢは、２つの段階：プローブファミリーの序列リスト名を得る第１の段階、および序列リストをデコード化して鋳型の配列を決定する第２の段階を含む。

【0209】

特に記載のない限り、配列決定法ＡおよびＡＢでは、一般的に、プローブの合成、鋳型の調製、ならびに伸長、ライゲーション、切断および検出の工程の実施に、類似した方法を用いる。

【0210】

配列決定方法ＡＢのためのオリゴヌクレオチド伸長プローブおよびプローブファミリーの特徴

配列決定法ＡＢにおける使用のためのプローブファミリーは、各プローブファミリーが、異なる配列の複数の標識されたオリゴヌクレオチドプローブを配列内の各位置に含み、プローブファミリーが、該位置に異なる塩基を有する少なくとも２つのプローブを含むことを特徴とする。各プローブファミリー内のプローブは同じ標識を含む。好ましくは、プローブは切れやすいヌクレオシド間結合を含む。切れやすい連結は、プローブ内の任意の場所に位置し得る。好ましくは、プローブは、リガーゼによって伸長可能でない部分を一方の末端に有する。好ましくは、プローブは、切れやすい連結と、リガーゼによって伸長可能でない部分との間の位置で標識されており、その結果、伸長可能なプローブ末端へのプローブのライゲーション後、切れやすい連結の切断により、伸長可能なプローブ末端にライゲートされる非標識部分、および非標識部分にはもはや結合されない標識された部分がもたらされる。

【0211】

各プローブファミリー内のプローブは、好ましくは、少なくともｊ個のヌクレオシドＸを含み、ここで、ｊは少なくとも２であり、このとき各Ｘは、プローブファミリー内のプローブにおいて少なくとも２重に縮重している。各プローブファミリー内のプローブは、さらに、少なくともｋ個のヌクレオシドＮを含み、ここで、ｋは少なくとも２であり、Ｎは、任意のヌクレオシド表す。一般に、ｊ＋ｋは１００以下であり、典型的には３０以下である。ヌクレオシドＸは、プローブ内の任意の場所に位置し得る。ヌクレオシドＸは、連続的な位置する必要はない。同様に、ヌクレオシドＮは、連続的な位置する必要はない。換言すると、ヌクレオシドＸおよびＮは散在させ得る。それにもかかわらず、ヌクレオシドＸは、５’→３’配列を有するとみなされ得る。ヌクレオシドは連続的である必要はないと理解され得る。例えば、構造X_AＮＸ$_G$ＮＮＸ$_C$ Ｎのプローブ内のヌクレオシドＸは、配列ＡＧＣを有するとみなされ得る。同様に、ヌクレオシドＮは、配列を有するとみなされ得る。

【0212】

ヌクレオシドＸ同一であっても異なっていてもよいが、独立して選択されない、すなわち、各Ｘの正体は、プローブ内の１つ以上他のヌクレオシドＸの正体によって拘束される。したがって、一般的に、ヌクレオシドＸのある特定の組合せのみが、任意の特定のプローブおよび任意の特定のプローブファミリー内のプローブにおいて存在する。換言すると、各プローブにおいて、ヌクレオシドＸの配列は、長さｊの可能な全ての配列の部分集団のみを表し得る。したがって、Ｘの１つ以上のヌクレオチドの正体により、他方のヌクレ