DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 17476707 Document Date: 09/16/2021

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- Drawing

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Form Revision Date: March 1, 2019

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SCORE Placeholder Sheet for IFW Content

Application Number: 17476707 Document Date: 09/16/2021

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- Sequence Listing

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Form Revision Date: March 1, 2019

PTO/AIA/15 (10-17)
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UTILITY PATENT APPLICATION TRANSMITTAL

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

Attorney Docket No.	NATE-037/C01US 321329-2719
First Named Inventor	Joseph M. BEECHEM
Title	CHEMICAL COMPOSITIONS AND USES THEREOF
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* 106]
   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* 40]
6. **Inventor's Oath or Declaration** [*Total Pages* 6]
   (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. [X] Computer Readable Form (CRF)
   b. [ ] Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] Paper
   c. [X] Statements verifying identity of above copies

ADDRESS TO:

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

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. [ ] **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. [X] **Other:** Petition to Accept Color Drawings Under 37 C.F.R. § 1.84(A)(2)
    Certification and Request for Prioritized Examination Under 37 CFR 1.102(e)

***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).

19. CORRESPONDENCE ADDRESS

[X] The address associated with Customer Number: 141429 **OR** [ ] Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/ Matthew Pavao /	Date	September 16, 2021
Name (Print/Type)	Matthew Pavao	Registration No. (Attorney/Agent)	50,572

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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	BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN EXPRESSION

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 16/272,487 filed on 02/11/2019.

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:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Joseph M. BEECHEM Date (Optional): 4 Sept 2019

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.

NanoString EX2011.004

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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	BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN EXPRESSION

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 16/272,487 filed on 02/11/2019.

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.

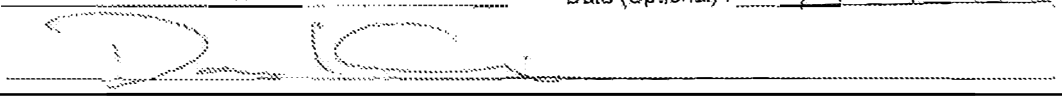
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LEGAL NAME OF INVENTOR

Inventor: Dae KIM Date (Optional): 5/4/2017

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

Title of Invention	BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN EXPRESSION

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 16/272,487 filed on 02/11/2019

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

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LEGAL NAME OF INVENTOR

Inventor: Margaret HOANG Date (Optional): 3/5/2019

Signature: Margaret Hoang

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

Title of Invention	BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN EXPRESSION

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 16/272,487 filed on 02/11/2019.

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.

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LEGAL NAME OF INVENTOR

Inventor: Mark GREGORY Date (Optional): 3/4/19

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

Title of Invention	BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN EXPRESSION

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 16/272,487 filed on 02/11/2019.

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.

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LEGAL NAME OF INVENTOR

Inventor: Erin PIAZZA Date (Optional): 3/1/2019

Signature: Erin Piazza

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.

NanoString EX2011.008

PTO/AIA/01 (08-12)
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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	BIOMOLECULAR PROBES AND METHODS OF DETECTING GENE AND PROTEIN EXPRESSION

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 16/272,487 filed on 02/11/2019.

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.

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LEGAL NAME OF INVENTOR

Inventor: Denise ZHOU Date (Optional): March 5, 2019

Signature: Denise Zhou

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.

NanoString EX2011.009

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:	Joseph M. BEECHEM	Nonprovisional Application Number (if known):	Not Yet Assigned
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF		

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	/ Matthew Pavao /	Date	September 16, 2021
Name (Print/Typed)	Matthew Pavao	Practitioner Registration Number	50,572

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.

256833111 v1

NanoString EX2011.010

Docket No.: NATE-037/C01US 321329-2719
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Joseph M. BEECHEM	Confirmation No.:	N/A
Application No.:	Not Yet Assigned	Group Art Unit:	N/A
Filed:	Concurrently Herewith	Examiner:	Not Yet Assigned

For: CHEMICAL COMPOSITIONS AND USES THEREOF

Via EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PETITION TO ACCEPT COLOR DRAWINGS UNDER 37 C.F.R. § 1.84(A)(2)

In compliance with 37 C.F.R. § 1.84(a)(2), Applicant respectfully requests and petitions for the acceptance of color drawings in the above-referenced application.

As directed by 37 C.F.R. § 1.84(a)(2), Applicant submits herewith the following:

(i) The petition fee to accept color drawings in a utility application of $140.00 under 37 CFR 1.17(h);

(ii) One set of color drawings (as the color drawings are being filed electronically via EFS-Web, only one set of the drawing is submitted); and

(iii) The following language is contained in the first paragraph of the brief description of the drawings in the specification as filed herewith:

> "The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee."

NanoString EX2011.011

The color drawings are necessary as the only practical medium by which aspects of the claimed subject matter may be accurately conveyed and are necessary to illustrate features of the claims. More specifically, the color drawings are necessary for reasons including, but not limited to the following:

1. FIG. 23 shows fluorescent micrographs of stained tissue samples. The use of color in the depictions of these fluorescent micrographs allows the reader to readily appreciate distinct domains within the tissue sample that are characterized by the expression of a particular cellular maker (*e.g.* PanCK, CD3E, Ki67 and SYT083). It would be nearly impossible for the reader to discern these distinct domains, let alone their spatial distribution within the tissue sample, in a black and white depiction of these fluorescent micrographs.

Applicant believes no additional fee is due with this response. However, if an additional fee is due, please charge our Deposit Account No. 50-1283, under Order No. NATE-037/C01US 321329-2719 from which the undersigned is authorized to draw.

Dated: September 16, 2021

Respectfully submitted,
COOLEY LLP

USPTO CUSTOMER NO. 141429

COOLEY LLP
ATTN: IP Docketing Department
1299 Pennsylvania Avenue NW, Suite 700
Washington, DC 20004

Tel: (617) 937-2300
Fax: (202) 842-7899

By: /Robert E. Powers/
Robert E. Powers
Reg. No. 77,802
Matthew Pavao
Reg. No. 50,572

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

PTO/AIA/82A (07-13)
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TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	Not Yet Assigned
Filing Date	Concurrently Herewith
First Named Inventor	Joseph M. BEECHEM
Title	CHEMICAL COMPOSITIONS AND USES THEREOF
Art Unit	N/A
Examiner Name	Not Yet Assigned
Attorney Docket Number	NATE-037/C01US 321329-2719

SIGNATURE of Applicant or Patent Practitioner

Signature	/Robert E. Powers/	Date (Optional)	
Name	Robert E. Powers	Registration Number	77,802
Title (if Applicant is a juristic entity)			

Applicant Name (if Applicant is a juristic entity)	

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

☐ *Total of 1 forms are submitted.

NanoString EX2011.013

DocuSign Envelope ID: 13F37F89-EA06-4020-B4E0-D1B4584CFB49

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

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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: 141429

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:

[x] The address associated with the above-mentioned Customer Number

OR

[ ] The address associated with Customer Number:

OR

[ ] Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

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

NanoString Technologies, Inc.

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

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[ ] 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	Bjorn Johnson	Date (Optional)	March 25, 2021
Name	Bjorn Johnson		
Title	Vice President, Legal Affairs		

DocuSigned by: B6:D0E86D07C4BA:

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.

[ ] Total of 1 forms are submitted.

Docket No.: NATE-037/C01US 321329-2719
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Joseph M. BEECHEM	Confirmation No.:	N/A
Application No.:	Not Yet Assigned	Group Art Unit:	N/A
Filed:	Concurrently Herewith	Examiner:	Not Yet Assigned

For: CHEMICAL COMPOSITIONS AND USES THEREOF

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

STATEMENT IN SUPPORT OF COMPUTER READABLE FORM SUBMISSION UNDER 37 C.F.R. § 1.821

I hereby state that the contents of the computer readable form of the Sequence Listing, submitted in the above-identified application in accordance with 37 C.F.R. § 1.821, does not include any new matter that goes beyond the disclosure of the application as filed. The Sequence Listing is supported by the specification and references incorporated therein. Therefore, no new matter is added.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 50-1283, under Order No. NATE-037/C01US 321329-2719 from which the undersigned is authorized to draw.

Dated: September 16, 2021

Respectfully submitted,
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Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Filer:	Matthew Pavao/Francesca Vandermaelen
Attorney Docket Number:	NATE-037C01US 321329-2719

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:				
UTILITY APPL SIZE FEE PER 50 SHEETS >100	1081	1	420	420
Claims:				
Miscellaneous-Filing:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL	1504	1	0	0
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140
Petition:				
PETITION FEE- 37 CFR 1.17(H) (GROUP III)	1464	1	140	140
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**6720**

Electronic Acknowledgement Receipt

EFS ID:	43779700
Application Number:	17476707
International Application Number:	
Confirmation Number:	5473
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Customer Number:	141429
Filer:	Matthew Pavao/Francesca Vandermaelen
Filer Authorized By:	Matthew Pavao
Attorney Docket Number:	NATE-037C01US 321329-2719
Receipt Date:	16-SEP-2021
Filing Date:	
Time Stamp:	12:53:01
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$6720
RAM confirmation Number	E20219FC53170366
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		NATE-037_C01US_Specification.pdf	465087 d7f4e5b85ce9be51e3bd70f64690bd4ee480be7a	yes	106

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Specification	1	102
	Claims	103	105
	Abstract	106	106

Warnings:

Information:

2	Drawings-other than black and white line drawings	NATE-037_C01US_Drawings.pdf	12822729 76b0891cbc6ba5aaba7b817def3de721afdc0c83	no	40

Warnings:

Information:

3	Application Data Sheet	NATE-037_C01US_ADS.pdf	1256219 65821aaf2a05879069fbf5182157fa8f5a331098	no	10

Warnings:

Information:

4	Transmittal of New Application	NATE-037_C01US_Transmittal.pdf	105482 f15cde349e6b198bab3919584fe7dedabf3e52c6	no	1

Warnings:

Information:

5	Oath or Declaration filed	NATE-037_C01US_Declarations.pdf	2229468 8f1dc49bebb33867f280715498346e243dd0ba3b	no	6

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Warnings:					
Information:					
6	TrackOne Request	NATE-037_C01US_Request.pdf	123130 5b53cdb1dd04bfec151467f28d5c189ed6382e43	no	1
Warnings:					
Information:					
7	Petition for review by the Office of Petitions	NATE-037_C01US_Petition.pdf	116080 724431e055ae7b71a5ee07ccd90be3f475594374	no	2
Warnings:					
Information:					
8	Sequence Listing (Text File)	NATE-037_C01US_SeqList.txt	50018	no	-
Warnings:					
Information:					
9	Power of Attorney	NATE-037_C01US_POA.pdf	139483 28ee650c925ba135060ddb128464784832 68db40	no	2
Warnings:					
Information:					
10	Sequence Listing	NATE-037_C01US_Statement.pdf	96706 09af6675cddcfaa3a2278808643da4975bf0b9f6	no	1
Warnings:					
Information:					
11	Fee Worksheet (SB06)	fee-info.pdf	55868 3df0f0071c582620fabc4b9da14f7a0072589ff6	no	2
Warnings:					
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			Total Files Size (in bytes):	17460270	

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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.

CHEMICAL COMPOSITIONS AND USES THEREOF

BACKGROUND OF THE INVENTION

[0001] This application is a continuation of U.S. Patent Application No. 16/272,487, filed February 11, 2019. U.S. Patent Application No. 16/272,487 claims priority to, and the benefit of, U.S. Provisional Application No. 62/629,180, filed February 12, 2018 and U.S. Provisional Application No. 62/771,212, filed November 26, 2018. The contents of each of the aforementioned patent applications are incorporated herein by reference in their entireties.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on September 15, 2021, is named "NATE-037_C01US_SeqList.txt" and is about 50,062 bytes in size.

BACKGROUND OF THE INVENTION

[0003] Standard immunohistochemical and *in situ* hybridization methods allow for simultaneous detection of, at most, six to ten protein or nucleic acid targets, with three to four targets being typical. There exists a need for probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell. Furthermore, there is a need for such systems to be adaptable for use with existing sequencing technologies that are already available to a large number of end users.

SUMMARY OF THE INVENTION

[0004] The present disclosure relates to probes, compositions, methods, and kits for simultaneous, multiplexed, spatial detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell.

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[0005] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0006] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises: a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias, and wherein at least one of the first or second amplification primer binding sites identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0007] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises: a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0008] The nucleic acid adapter of step (4) can be a partially double-stranded nucleic acid molecule. A partially double-stranded nucleic acid adapter can comprise a double-stranded annealed region, a first single-stranded mismatched region and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region can be present on opposing sides of the double-stranded annealed region.

[0009] The nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

[0010] The constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

[0011] A unique molecular identifier can be present in at least one of the first or second single-stranded mismatched regions of a partially double-stranded nucleic acid adapter.

[0012] The first amplification primer binding site can be present in the first single-stranded mismatched region of a partially double-stranded nucleic acid adapter and the second amplification primer binding site can be present in the second single-stranded mismatched region of the same partially double-stranded nucleic acid adapter.

[0013] The methods of the present disclosure described in the preceding can further comprise prior to step (4), performing an end repair reaction. The methods can also further comprise prior to step (4), performing a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide. The methods can further comprise, prior to step (4), performing an end repair reaction and a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide. The tailing reaction and the end repair reaction can be performed sequentially or concurrently.

[0014] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site, and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the ligation product produced in step (4); and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0015] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises: a first amplification primer binding site and a unique nucleic acid sequence which identifies the target

analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) ligating to the released identifier oligonucleotide at least one nucleic acid adapter, wherein the nucleic acid adapter comprises a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site, and optionally, a constant nucleic acid sequence to minimize ligation bias; (5) amplifying the extension product produced in step (4) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; and (6) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0016] The nucleic acid adapter of step (4) can be a partially double-stranded nucleic acid molecule. A partially double-stranded nucleic acid adapter can comprise a double-stranded annealed region and a single-stranded mismatched region.

[0017] The nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter.

[0018] The constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of a partially double-stranded nucleic acid adapter. The constant nucleic acid sequence can also comprise a cleavable moiety. The cleavable moiety can be an enzymatically cleavable moiety. The enzymatically cleavable moiety can be a USER sequence.

[0019] A unique molecular identifier can be present in the single-stranded mismatched region of a partially double-stranded nucleic acid adapter.

[0020] The second amplification primer binding site can be present in the single-stranded mismatched region of a partially double-stranded nucleic acid adapter.

[0021] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first

amplification primer binding site, and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a nucleic acid sequence complementary to a second amplification primer binding site and optionally, an affinity molecule; (5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the unique molecular identifier, and the second amplification primer binding site; (6) amplifying the extension product produced in step (5) using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0022] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier, a nucleic acid

sequence complementary to a second amplification primer binding site and optionally, an affinity molecule; (5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, the nucleic acid sequence complementary to the unique molecular identifier and the second amplification primer binding site; (6) amplifying the extension product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0023] The single stranded nucleic acid template can further comprise an affinity molecule. In aspects in which the single stranded nucleic acid template comprises an affinity molecule, the methods of the present disclosure described in the preceding can further comprise an affinity purification step between steps (4) and (5).

[0024] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site, and wherein the first the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying

the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0025] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a second amplification primer binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0026] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier

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oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0027] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a first amplification primer binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide and a second amplification primer binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes

hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0028] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is located on the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be located 5' to the first amplification primer binding site.

[0029] In aspects in which a unique molecular identifier is located on the second nucleic acid probe, the unique molecular identifier can be located 3' to the second amplification primer binding site.

[0030] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier are present in the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier can be located 5' to the first amplification primer binding site.

[0031] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a unique molecular identifier are present in the second nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the unique molecular identifier can be located 3' to the second amplification primer binding site.

[0032] In aspects in which a unique molecular identifier is present in the first nucleic acid probe and the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released is present in the second nucleic acid probe, the unique molecular identifier can be located 5' to the first amplification primer binding site and the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be located 3' to the second amplification binding site.

[0033] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier, a nucleic acid sequence comprising a third unique molecular identifier and a second flow cell binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0034] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to a portion of the

identifier oligonucleotide, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier and a second flow cell binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) performing nick repair such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0035] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier, a nucleic acid sequence comprising a third unique molecular identifier and a second flow cell binding site, and wherein the first and the second nucleic acid

probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0036] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a first amplification primer binding site, a nucleic acid sequence comprising a first unique molecular identifier and a first flow cell binding site, and wherein the second nucleic acid probe comprises a nucleic acid complementary to the identifier oligonucleotide, a nucleic acid sequence comprising a second unique molecular identifier and a second flow cell binding site, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence comprising a third unique molecular identifier, and wherein at least one of the first or second nucleic acid probes comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping; (5) performing a gap extension and nick repair reaction such that the hybridized first and second nucleic acid probes are ligated together; (6) amplifying the ligation product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0037] In aspects in which the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the first unique molecular identifier are present in the first nucleic acid probe, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the first unique molecular identifier can be located 5' to the first flow cell binding site.

[0038] In aspects in which the second and the third unique molecular identifiers are present in the second nucleic acid probe, the second and the third unique molecular identifiers can be located 3' to the second flow cell binding site.

[0039] In some aspects, the first unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site. In other aspects, the second unique molecular identifier can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

[0040] In some aspects, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site.

[0041] In some aspects, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and the third unique molecular identifier can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

[0042] In some aspect, the third unique molecular identifier can be present in the first nucleic acid probe and can be located 5' to the first flow cell binding site. In this same aspect, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can be present in the second nucleic acid probe and can be located 3' to the second flow cell binding site.

[0043] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding

domain, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, and a second amplification primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released; (5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0044] The present disclosure also provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a first amplification primer binding site and a second amplification primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site, wherein at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, and wherein at least one of the amplification primers comprises a nucleic acid sequence comprising a unique molecular identifier; and (5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0045] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding

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domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain and a capture probe binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a capture probe, wherein the capture probe comprises an affinity molecule and a region complementary to the capture probe binding site; and (5) Identifying the released identifier oligonucleotide by sequencing the amplified hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0046] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a capture probe binding site and a multiplexing probe binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a capture probe and a multiplexing probe, wherein the capture probe comprises an affinity molecule and a region complementary to the capture probe binding site, and wherein the multiplexing probe comprises a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site; and (5) Identifying the released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0047] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the

released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe, wherein the first nucleic acid probe comprises: a nucleic acid complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, and wherein the second nucleic acid probe comprises: a nucleic acid complementary to a portion of the identifier oligonucleotide, and a second amplification primer binding site, and wherein the first and the second nucleic acid probes hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent but not overlapping; (5) ligating the hybridized first and second nucleic acid probes together; (6) amplifying the ligation product produced in step (5); and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0048] The present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises: a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a nucleic acid sequence comprising a unique molecular identifier, a first amplification primer binding site, and a second amplification primer binding site; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) amplifying the collected identifier oligonucleotide; (5) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[0049] A In all methods of the present disclosure, the ligation process can be a nick ligation process. The nick ligation process can be a nick repair process.

[0050] In all methods of the present disclosure, the sequencing can be an enzyme free sequencing method.

[0051] In all methods of the present disclosure, the identifier oligonucleotide can be double-stranded. In aspects in which the identifier oligonucleotide is double-stranded, at least one of the two strands of the identifier oligonucleotide can comprise at least two separate nucleic acid

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molecules. Alternatively, at least one 3' end of an identifier oligonucleotide can comprise a single nucleotide overhang.

[0052] In all methods of the present disclosure, the identifier oligonucleotide can be single-stranded.

[0053] In all methods of the present disclosure, the unique nucleic acid sequence which identifies the target analyte bound to a target binding domain can comprise between about 5 nucleotides and about 40 nucleotides preferably about 35 nucleotides, preferably still about 10 nucleotides.

[0054] In all methods of the present disclosure, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can comprise between about 6 nucleotides and about 15 nucleotides, preferably about 12 nucleotides, preferably still about 10 nucleotides.

[0055] In all methods of the present disclosure, at least one of a first nucleic acid probe or a second nucleic acid probe can comprise an affinity molecule. For example, at least one of a first nucleic acid probe or a second nucleic acid probe can comprise a biotin.

[0056] In all methods of the present disclosure, an amplification primer binding site can comprise between about 18 nucleotides and about 40 nucleotides, preferably about 32 nucleotides, preferably still about 25 nucleotides. An amplification primer binding site can comprise an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1. An amplification primer binding site can comprise an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

[0057] In all methods of the present disclosure, an amplification primer can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. An amplification primer can comprise a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. An amplification primer can comprise a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

[0058] In all methods of the present disclosure, a flow cell binding site can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. A flow cell binding site can comprise a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. A flow cell binding site can

comprise a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

[0059] In all methods of the present invention, at least one of the amplification primers can comprise an affinity molecule. For example, at least one of the amplification primers cam comprise a biotin.

[0060] In all methods of the present disclosure, amplification can comprise performing PCR. Performing PCR can comprise an amplification primer.

[0061] An amplification primer can comprise a flow cell binding site. An amplification primer can comprise a nucleic sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released. An amplification primer can comprise a nucleic acid sequence complementary to an amplification primer binding site.

[0062] Any of the above aspects can be combined with any other aspect.

[0063] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. In the Specification, the singular forms also include the plural unless the context clearly dictates otherwise; as examples, the terms “a,” “an,” and “the” are understood to be singular or plural and the term “or” is understood to be inclusive. By way of example, “an element” means one or more element. Throughout the specification the word “comprising,” or variations such as “comprises” or “comprising,” will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term “about.”

[0064] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. The references cited herein are not admitted to be prior art to the claimed invention. In the case of conflict, the present Specification, including definitions, will control. In addition, the materials, methods, and examples are

illustrative only and are not intended to be limiting. Other features and advantages of the disclosure will be apparent from the following detailed description and claim.

BRIEF DESCRIPTION OF THE DRAWINGS

[0065] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0066] The above and further features will be more clearly appreciated from the following detailed description when taken in conjunction with the accompanying drawings.

[0067] Figure 1 is a schematic of a two-ended adapter ligation method of the present disclosure.

[0068] Figure 2 is a schematic of a one-ended adapter ligation method of the present disclosure.

[0069] Figure 3 is a schematic of a templated-primer extension method of the present disclosure.

[0070] Figure 4 is a schematic of a template-extended identifier oligonucleotide of the present disclosure.

[0071] Figure 5 is a schematic of a short probe hybridization method of the present disclosure.

[0072] Figure 6 is a schematic of a short probe hybridization method of the present disclosure.

[0073] Figure 7 is a schematic of a short probe hybridization method of the present disclosure.

[0074] Figure 8 is a schematic of a long probe hybridization method of the present disclosure.

[0075] Figure 9 is a schematic of a long probe hybridization method of the present disclosure.

[0076] Figure 10 is a schematic of a long probe hybridization method of the present disclosure.

[0077] Figure 11 is a schematic of a direct-PCR method of the present disclosure.

[0078] Figure 12 is a schematic of an enzyme free method of the present disclosure.

[0079] Figure 13 is a schematic of a multiplexed enzyme free method of the present disclosure.

[0080] Figure 14 is a schematic of a probe of the present disclosure indirectly binding to a target nucleic acid.

[0081] Figure 15 is a schematic of an identifier oligonucleotide-short nucleic acid probe complex of the present disclosure.

[0082] Figure 16 is a schematic of a short probe hybridization method of the present disclosure.

[0083] Figure 17 is a schematic of an identifier oligonucleotide-short nucleic acid probe complex of the present disclosure.

[0084] Figure 18 is a schematic of a short probe hybridization method of the present disclosure.

[0085] Figure 19 is a schematic of a direct-PCR method of the present disclosure.

[0086] Figure 20 is a schematic overview of the methods of the present disclosure.

[0087] Figure 21A, Figure 21B, Figure 21C and Figure 21D show the spatial detection of protein target analytes using the methods of the present disclosure.

[0088] Figure 22A, Figure 22B, Figure 22C and Figure 22D show the spatial detection of RNA target analytes using the methods of the present disclosure.

[0089] Figure 23 shows the spatial detection of protein target analytes using the methods of the present disclosure.

[0090] Figure 24 shows the spatial detection of RNA target analytes using the methods of the present disclosure.

[0091] Figure 25 shows the spatial detection of protein target analytes using the methods of the present disclosure.

[0092] Figure 26 is a schematic of a probe of the present disclosure. The nucleic acid sequence shown in Figure 26 corresponds to SEQ ID NO: 175.

[0093] Figure 27 shows the use of probe tiling in the methods of the present disclosure.

[0094] Figure 28 shows the regions of interest selected on a tissue microarray.

[0095] Figure 29 is a series of graphs showing the read depth achieved using the methods of the present disclosure.

[0096] Figure 30 is a series of graphs showing the spatial detection of RNA target analytes in negative control samples using the methods of the present disclosure.

[0097] Figure 31 is a series of graphs showing the spatial detection of RNA target analytes in a HEK293 sample (top panel) and a Jurkat cell sample (bottom panel) using the methods of the present disclosure.

[0098] Figure 32 is a series of graphs showing the spatial detection of RNA target analytes in sixteen FFPE samples using the methods of the present disclosure.

[0099] Figure 33 is a graph showing the spatial detection of RNA target analytes in a HEK293 sample using the methods of the present disclosure.

[00100] Figure 34 is a graph showing the spatial detection of RNA target analytes in a Jurkat cell sample using the methods of the present disclosure.

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DETAILED DESCRIPTION OF THE INVENTION

[00101] The present disclosure is based in part on probes, compositions, methods, and kits for simultaneous, multiplexed spatial detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell using existing sequencing methods.

[00102] The present disclosure provides a comparison of the identity and abundance of target proteins and/or target nucleic acids present in a first region of interest (*e.g.*, tissue type, a cell (including normal and abnormal cells), and a subcellular structure within a cell) and the identity and abundance of target proteins and/or target nucleic acids present in a second region of interest. There is no pre-defined upper limit to the number of regions of interest and comparisons that can be made; the upper limit relates to the size of the region of interest relative the size of the sample. As examples, when a single cell represents a region of interest, then a section may have hundreds to thousands of regions of interest; however, if a tissue section includes only two cell types, then the section may have only two regions of interest (each including only one cell type).

[00103] The present disclosure provides a higher degree of multiplexing than is possible with standard immunohistochemical or ***in situ*** hybridization methods. Standard immunohistochemical methods allow for maximal simultaneous detection of six to ten protein targets, with three to four protein targets being more typical. Similarly, ***in situ*** hybridization methods are limited to simultaneous detection of fewer than ten nucleic acid targets. The present disclosure provides detection of large combinations of nucleic acid targets and/or protein targets from a defined region of a sample. The present disclosure provides an increase in objective measurements by digital quantification and increased reliability and consistency, thereby enabling comparison of results among multiple centers.

[00104] Various compositions and methods of the present disclosure are described in full detail herein.

[00105] In one aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "two-ended adapter ligation method".

[00106] A two ended-adapter ligation method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The probes and samples of the present disclosure are described in further detail herein. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain.

[00107] Following contacting the at least on target analyte with the at least one probe, a two-ended adapter ligation method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00108] Following release of the identifier oligonucleotide, a two-ended adapter ligation method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location, thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00109] Following collection of the released identifier oligonucleotide, a two-ended adapter ligation method can further comprise: (4) ligating to the released identifier oligonucleotide collected in step (3) at least one nucleic acid adapter.

[00110] The nucleic acid adapter can comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated

"ROI #1", the nucleic acid adapter would comprise a nucleic acid sequence that corresponds to "ROI #1".

[00111] The nucleic acid adapter can also comprise a unique molecular identifier.

[00112] The nucleic acid adapter can also comprise a first amplification primer binding site. In other aspects, the nucleic acid adapter can also comprise a second amplification primer binding site.

[00113] In some aspects, the nucleic acid adapter can also comprise a constant nucleic acid sequence to minimize ligation bias caused by differences in sequences of particular identifier oligonucleotides.

[00114] The nucleic acid adapter can be a partially double-stranded nucleic acid molecule. In aspects in which the nucleic acid adapter is partially double-stranded, the nucleic acid adapter comprises a double-stranded annealed region, a first single-stranded mismatched region, and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region can be present on opposing sides of the double-stranded annealed region.

[00115] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of the nucleic acid adapter.

[00116] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a constant nucleic acid sequence to minimize ligation bias, the constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of the nucleic acid adapter.

[00117] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a unique molecular identifier, the unique molecular identifier can be present in at least one of the first or second single-stranded mismatched regions of the nucleic acid adapter.

[00118] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a first and a second amplification primer binding site, the first amplification primer binding site can be present in the first single-stranded mismatched region of the nucleic acid adapter and the

second amplification primer binding site can be present in the second single-stranded mismatched region of the nucleic acid adapter.

[00119] After ligation of the at least one nucleic acid adapter, a two-ended adapter ligation method can further comprise: (5) amplifying the ligation product produced in step (4) using amplification primers that bind to the first and second amplification primer binding sites; and (6) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

[00120] A two-ended adapter ligation method of the present disclosure can further comprise, prior to step (4), performing an end repair reaction using methods known in the art. The method can also further comprise, prior to step (4), performing a tailing reaction to attach a single nucleotide overhang to the 3' ends of the identifier oligonucleotide using methods known in the art. In aspects, the end repair reaction and the tailing reaction can be performed sequentially or concurrently.

[00121] In preferred aspects of a two-ended adapter ligation method, a nucleic acid adapter is ligated to both ends of the released and collected identifier oligonucleotide.

[00122] In other aspects of a two-ended adapter ligation method, at least one of the amplification primers used in step (5) to amplify the ligation product produced in step (4) comprises a nucleic acid sequence which identifies the specific location of the tissue sample form which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to "ROI #1".

[00123] Figure 1 shows a schematic of a preferred aspect of a two-ended adapter ligation method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising an antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In the upper right panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein bound to the target binding domain. In the bottom panel, a nucleic acid adapter is ligated to both ends of the identifier oligonucleotide. In this non-limiting example, the nucleic acid adapter is partially double-stranded and comprises a double-stranded annealed region, a first single-stranded mismatched region, and a second single-stranded mismatched region. Present in

the double-stranded annealed region is a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. Present in the first single-stranded mismatched region is a first amplification primer binding site. Present in the second single-stranded mismatched region is a unique molecular identifier and a second amplification primer binding site. Following ligation of the nucleic acid adapters to the identifier oligonucleotide, the product is amplified using amplification primers that bind the first and the second amplification primer binding sites and sequenced to identify the target protein bound by the probe.

[00124] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide dually ligated to two nucleic acid adapters for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide dually ligated to two nucleic acid adapters comprises an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which is capable of identifying a target analyte in a sample. Each end of the identifier oligonucleotide is attached to a nucleic acid adapter molecule, wherein the nucleic acid adapter molecule is partially double-stranded and comprises a double-stranded annealed region, a first single-stranded mismatched region and a second single-stranded mismatched region. The first single-stranded mismatched region and the second single stranded mismatched region are present on opposing sides of the double-stranded annealed region. The double-stranded mismatch region comprises a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence nucleic acid sequence which is capable of identifying a specific location of a sample. The first single-stranded mismatched region comprises a first amplification primer binding site. The second single-stranded mismatched region comprises a second amplification primer binding site and a nucleic acid sequence comprising a unique molecular identifier. A schematic of an identifier oligonucleotide dually ligated to two nucleic acid adapters is shown in the bottom panel of Figure 1.

[00125] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "one-ended adapter ligation method".

[00126] A one-ended adapter ligation method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that

the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a first amplification primer binding site. In some aspects, the identifier oligonucleotide also comprises at least one 3' end with a single nucleotide overhang.

[00127] Following contacting the at least one target analyte with the at least one probe, a one-ended adapter ligation method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00128] Following release of the identifier oligonucleotide, a one-ended adapter ligation method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00129] Following collection of the released identifier oligonucleotide, a one-ended adapter ligation method can further comprise: (4) ligating to the released oligonucleotide collected in step (3) at least one nucleic acid adapter;

[00130] The nucleic acid adapter can comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", the nucleic acid adapter would comprise a nucleic acid sequence that corresponds to "ROI #1". The nucleic acid adapter can also comprise a unique molecular identifier. The nucleic acid adapter can also comprise a second amplification primer binding site.

[00131] In some aspects, the nucleic acid adapter can also comprise a constant nucleic acid sequence to minimize ligation bias caused by differences in sequences of particular identifier oligonucleotides. The constant nucleic acid sequence can comprise a cleavable moiety. The cleavable moiety can be enzymatically cleavable. In a non-limiting example, the enzymatically cleavable moiety can be a USER sequence, wherein the USER sequence comprises the sequence GUGUATUG.

[00132] The nucleic acid adapter can comprise any combination of the features described above.

[00133] The nucleic acid adapter can be a partially double-stranded nucleic acid molecule. In aspects in which the nucleic acid adapter is partially double-stranded, the nucleic acid adapter comprises a double-stranded annealed region and a single-stranded mismatched region.

[00134] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released can be present in the double-stranded annealed region of the nucleic acid adapter.

[00135] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a constant nucleic acid sequence to minimize ligation bias, the constant nucleic acid sequence to minimize ligation bias can be present in the double-stranded annealed region of the nucleic acid adapter.

[00136] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a unique molecular identifier, the unique molecular identifier can be present in the single-stranded mismatched region.

[00137] In aspects in which the nucleic acid adapter is partially double-stranded and comprises a second amplification primer binding site, the second amplification primer binding site can be present in the single-stranded mismatched region of the nucleic acid adapter.

[00138] After ligation of the at least one nucleic acid adapter, a two-ended adapter ligation method can further comprise: (5) amplifying the ligation product produced in step (4); and (6) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

[00139] In other aspects of a one-ended adapter ligation method of the present disclosure, at least one of the amplification primers used in step (5) to amplify the ligation product produced in step

(4) comprises a nucleic acid sequence which identifies the specific location of the tissue sample form which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to "ROI #1".

[00140] Figure 2 shows a schematic of a preferred aspect of a one-ended adapter ligation method of the present disclosure. In this aspect, the probe comprises a target binding domain that is an antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In upper right panel, the UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein and a first amplification primer binding site. In this non-limiting example, the identifier oligonucleotide is double-stranded with one strand that comprises three separate nucleic acid molecules. The identifier oligonucleotide also comprises one 3' end with a single nucleotide overhang.

[00141] In the bottom panel of Figure 2, a nucleic acid adapter is ligated to the end of the identifier oligonucleotide that comprises the 3' single nucleotide overhang. In this non-limiting example, the nucleic acid adapter is partially double-stranded and comprises a double-stranded annealed region and a single-stranded mismatched region. Present in the double-stranded annealed region is a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. Present in the single-stranded mismatched region is a unique molecular identifier and a second amplification primer binding site. Following ligation of the nucleic acid adapter to the identifier oligonucleotide, the product is amplified using amplification primers that bind to the first and the second amplification primer binding sites and sequenced to identify the target protein bound by the probe.

[00142] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide ligated to one nucleic acid adapter for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide ligated to one nucleic acid adapter comprises an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a unique nucleic acid sequence which is capable of identifying a target analyte in a sample and a first amplification primer binding site. One end of the identifier oligonucleotide is attached to a nucleic acid adapter molecule,

wherein the nucleic acid adapter molecule is partially double-stranded and comprises a double-stranded annealed region and a single-stranded mismatched region and a second single-stranded mismatched region. The double-stranded mismatch region comprises a constant nucleic acid sequence to minimize ligation bias and a nucleic acid sequence nucleic acid sequence which is capable of identifying a specific location of a sample. The single-stranded mismatched region comprises a second amplification primer binding site. A schematic of an identifier oligonucleotide ligated to one nucleic acid adapter is shown in the bottom panel of Figure 2.

[00143] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "templated-primer extension method".

[00144] A templated-primer extension method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a first amplification primer binding site.

[00145] Following contacting the at least one target analyte with the at least one probe, a templated-primer extension method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00146] Following release of the identifier oligonucleotide, a templated-primer extension method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier

oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00147] Following collection of the released identifier oligonucleotide, a templated-primer extension method can further comprise: (4) hybridizing to the released identifier oligonucleotide collected in step (3) a single stranded nucleic acid template.

[00148] The single stranded nucleic acid template can comprise a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, thereby allowing for the hybridization of the single stranded nucleic acid template and the collected identifier oligonucleotide.

[00149] The single stranded nucleic acid template can also comprise a nucleic acid sequence comprising a unique molecular identifier.

[00150] The single stranded nucleic acid template can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released.

[00151] The single stranded nucleic acid template can also comprise a nucleic acid sequence that is complementary to a second amplification primer binding site.

[00152] The single stranded nucleic acid template can comprise any combination of the features described above.

[00153] Following hybridization of the identifier oligonucleotide to the single stranded nucleic acid template, a templated-primer extension method can further comprise: (5) extending the identifier oligonucleotide of step (4) to form an extension produce complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide and the sequence complementary to the single stranded nucleic acid template; (6) amplifying the extension product of step (6) using amplification primers that hybridize to the first and second amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

[00154] In some aspects, the single stranded nucleic acid template can comprise an affinity molecule. In aspects in which the single stranded nucleic acid template comprises an affinity

molecule, a templated-primer extension method can further comprise an affinity purification step between steps (4) and (5).

[00155] Figure 3 shows a schematic of a preferred aspect of a templated-primer extension method of the present disclosure. In this aspect, the probe comprises a target binding domain that is an antibody that binds to a target protein. In the upper left panel, the probe binds to the target protein. In upper right panel, the UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target protein and a first amplification primer binding site. In the lower right panel, the identifier oligonucleotide is hybridized to a single stranded nucleic acid template. In this non-limiting example, the single-stranded nucleic acid template comprises an affinity molecule, a nucleic acid sequence complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a first unique molecular identifier, and a sequence complementary to a second amplification primer biding site. The identifier oligonucleotide is extended to form an extension product complementary to the single stranded nucleic acid template. As shown in the lower left panel, the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the first unique molecular identifier, and the second amplification primer binding site. Following the extension reaction, the primer extension product is amplified using amplification primers that bind to the first and the second amplification primer binding sites. In this non-limiting example, one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The amplified product is then sequenced to identify the target protein bound by the probe.

[00156] In one aspect, the present disclosure provides a composition of a template-extended identifier oligonucleotide for spatially detecting at least one target analyte in a sample. The template-extended identifier oligonucleotide comprises a first flow cell adapter sequence suitable for sequencing, followed by a first unique molecular identifier, followed by an identifier oligonucleotide, followed by a second unique molecular identifier, followed by a second amplification primer binding site, followed by a third unique molecular identifier, followed by a second flow cell adapter sequence suitable for sequencing. The identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which is

capable of identifying a target analyte in a sample. A schematic of a template-extended identifier oligonucleotide is shown in the bottom panel of Figure 4.

[00157] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "short probe hybridization method".

[00158] A short probe hybridization method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain.

[00159] Following contacting the at least one target analyte with the at least one probe, a short probe hybridization method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00160] Following release of the identifier oligonucleotide, a short probe hybridization method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00161] Following collection of the released identifier oligonucleotide, a short probe hybridization method can further comprise: (4) hybridizing to the release identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe.

[00162] The first or the second nucleic acid probe can comprise a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The first or the second nucleic acid probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The first or the second nucleic acid probe can also comprise a nucleic acid sequence comprising unique molecular identifier. The first nucleic acid probe can comprise a first amplification primer binding site. The second nucleic acid probe can comprise a second amplification primer binding site.

[00163] The first or the second nucleic acid probe can comprise any combination of the features described above. In a preferred aspect depicted in Figure 5, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released is located 5' to the first amplification primer binding site and the unique molecular identifier is located 3' to the second amplification primer binding site.

[00164] In another preferred aspect depicted in Figure 6, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 5' to the first amplification primer binding site.

[00165] In another preferred aspect depicted in Figure 7, the first nucleic acid probe comprises a first amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe

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comprises a second amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the unique molecular identifier are located 3' to the second amplification primer binding site.

[00166] In another preferred aspect depicted in Figure 15, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence comprising a unique molecular identifier is located 3' to the first amplification binding site.

[00167] In another preferred aspect depicted in Figure 17, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence comprising a unique molecular identifier is located 5' to the first amplification binding site.

[00168] The first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Alternatively, the first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping.

[00169] Following hybridization of the first and the second nucleic acid probe to the identifier oligonucleotide, a short probe hybridization method can further comprise: (5) in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping, ligating the first and the second nucleic acid probes together, for example, by performing a nick repair

reaction. Alternatively, in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping, the method comprises ligating the first and the second nucleic acid probes together, for example, by performing a gap extension reaction and a nick repair reaction, such that the first and the second nucleic acid probes are ligated together.

[00170] Following ligation of the first and the second nucleic acid probe, a short probe hybridization method can further comprise: (6) amplifying the ligation product produced in step (5) using amplification primers that hybridize to the first and second amplification primer binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

[00171] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-short nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-short nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first amplification primer binding site, followed by a unique nucleic acid sequence capable of identifying a specific location in a sample, followed by a region complementary to the identifier oligonucleotide. The second nucleic acid probe comprises a second amplification primer binding site, followed by a nucleic acid sequence comprising a unique molecular identifier, followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in Figure 5.

[00172] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-short nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-short nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first amplification primer binding site, followed by a nucleic acid sequence comprising a unique molecular identifier, followed by a region complementary to the identifier oligonucleotide,

wherein the nucleic acid sequence comprising a unique molecular identifier is located 3' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in Figure 15.

[00173] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-short nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-short nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first amplification primer binding site, followed by a nucleic acid sequence comprising a unique molecular identifier, followed by a region complementary to the identifier oligonucleotide, wherein the nucleic acid sequence comprising a unique molecular identifier is located 5' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in Figure 17.

[00174] Figure 16 shows a schematic overview of an exemplary short probe hybridization method of the present disclosure. First at least one target analyte in a sample is contacted with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain.

[00175] Following contacting the at least one target analyte with the at least one probe, a force is then provided to a location of the sample sufficient to release the identifier oligonucleotide. The identifier oligonucleotide is collected following release, as shown in the top panel of Figure 16.

[00176] As shown in the second panel from the top of Figure 16, the released identifier oligonucleotide is then hybridized to a first nucleic acid probe and a second nucleic acid probe. In this non-limiting example, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The nucleic acid sequence comprising the unique molecular identifier is located 3' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this non-limiting example, the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Following hybridization to the identifier oligonucleotide, the first and second probe are ligated together, for example, by performing a nick repair reaction.

[00177] Following ligation of the first and second nucleic acid probes, the ligation product is amplified via PCR using amplification primers that hybridize to the first and second amplification primer binding sites. As shown in the second panel from the bottom of Figure 16, the amplification primer that hybridizes to the second amplification primer binding site comprises a first flow cell binding site, a first nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the second amplification primer binding site. The amplification primer that hybridizes to the first amplification primer binding site comprises a second flow cell binding site, a second nucleic acid sequence which identifies the specific location from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the first amplification primer binding site. The PCR product shown in the bottom panel of Figure 16 is then sequenced to identify the released oligonucleotide, thereby spatially detecting the at least one target analyte in the sample.

[00178] Figure 18 shows a schematic overview of an exemplary short probe hybridization method of the present disclosure. First, at least one target analyte in a sample is contacted with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target

nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target domain.

[00179] Following contacting the at least one target analyte with the at least one probe, a force is then provided to a location of the sample sufficient to release the identifier oligonucleotide. The identifier oligonucleotide is collected following release, as shown in the top panel of Figure 18.

[00180] As shown in the second panel from the top of Figure 18, the released identifier oligonucleotide is then hybridized to a first nucleic acid probe and a second nucleic acid probe. In this non-limiting example, the first nucleic acid probe comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The nucleic acid sequence comprising the unique molecular identifier is located 5' to the first amplification primer binding site. The second nucleic acid probe comprises a second amplification primer binding site and a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. In this non-limiting example, the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Following hybridization to the identifier oligonucleotide, the first and second probe are ligated together, for example, by performing a nick repair reaction.

[00181] Following ligation of the first and second nucleic acid probes, the ligation product is amplified via PCR using amplification primers that hybridize to the first and second amplification primer binding sites. As shown in the second panel from the bottom of Figure 18, the amplification primer that hybridizes to the second amplification primer binding site comprises a first flow cell binding site, a first nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the second amplification primer binding site. The amplification primer that hybridizes to the first amplification primer binding site comprises a second flow cell binding site, a second nucleic acid sequence which identifies the specific location from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the first amplification primer binding site. The PCR product shown in the bottom panel Figure 18 is then

sequenced to identify the released oligonucleotide, thereby spatially detecting the at least one target analyte in the sample.

[00182] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "long probe hybridization method".

[00183] A long probe hybridization method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain.

[00184] Following contacting the at least one target analyte with the at least one probe, a long probe hybridization method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00185] Following release of the identifier oligonucleotide, a long probe hybridization method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00186] Following collection of the released identifier oligonucleotide(s), a long probe hybridization method can further comprise: (4) hybridizing to the released identifier oligonucleotide a first nucleic acid probe and a second nucleic acid probe.

[00187] The first or the second nucleic acid probe can comprise a nucleic acid sequence complementary to a portion of the identifier oligonucleotide. The first or the second nucleic acid probe can also comprise a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released.

[00188] The first or the second nucleic acid probe can also comprise a first unique molecular identifier. The first or the second nucleic acid probe can also comprise a second unique molecular identifier. The first or the second nucleic acid probe can also comprise a third unique molecular identifier.

[00189] The first nucleic acid probe can comprise a first amplification primer binding site.

[00190] The first nucleic acid probe can also comprise a first flow cell binding site. The second nucleic acid probe can comprise a second flow cell binding site.

[00191] The first and the second nucleic acid probes can comprise any combination of the features described above. In a preferred aspect depicted in Figure 8, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a second unique molecular identifier, a third unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and the first unique molecular identifier are located 5' to the first flow cell binding site and the second and the third unique molecular identifiers are located 3' to the second flow cell binding site.

[00192] In another preferred aspect depicted in Figure 9, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a second unique molecular identifier, a first amplification primer binding site, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In the same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a third unique molecular identifier and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the first unique molecular identifier, the second unique molecular

identifier and the nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released are located 5' to the first flow cell binding site and the third unique molecular identifier is located 3' to the second flow cell binding site.

[00193] In another preferred aspect depicted in Figure 10, the first nucleic acid probe comprises a first flow cell binding site, a first unique molecular identifier, a first amplification primer binding site and a nucleic acid sequence complementary to the identifier oligonucleotide. In this same preferred aspect, the second nucleic acid probe comprises a second flow cell binding site, a second unique molecular identifier, a third unique molecular identifier, a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the identifier oligonucleotide. In this preferred aspect, the first unique molecular identifier is located 5' to the first flow cell binding site and the second unique molecular identifier, the third unique molecular identifier, and the nucleic acid sequence which identifies the specific location of the sample form which the identifier oligonucleotide was released are located 3' to the second amplification primer binding site.

[00194] The first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping. Alternatively, the first nucleic acid probe and the second nucleic acid probe can hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping.

[00195] Following hybridization of the first and the second nucleic acid probe to the identifier oligonucleotide, a long probe hybridization method can further comprise: (5) in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are adjacent and are not overlapping, performing a nick repair reaction such that the first and the second nucleic acid probes are ligated together. Alternatively, in the aspect in which the first and the second nucleic acid probe hybridize to the identifier oligonucleotide such that the first and the second nucleic acid probes are not adjacent and are not overlapping, the method comprises performing a gap extension and a nick repair reaction such that the first and the second nucleic acid probes are ligated together.

[00196] The name method can further comprise: (6) amplifying the ligation product produced in step (5) using amplification primers that hybridize to the first and second amplification primer

binding sites; and (7) identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the sample.

[00197] In one aspect, the present disclosure provides a composition of an identifier oligonucleotide-long nucleic acid probe complex for spatially detecting at least one target analyte in a sample. An identifier oligonucleotide-long nucleic acid probe complex comprises an identifier oligonucleotide hybridized to a first nucleic acid probe and a second nucleic acid probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a target analyte in a sample. The first nucleic acid probe comprises a first flow cell binding site suitable for sequencing, followed by a first unique molecular identifier, followed by a first amplification primer binding site, followed by a unique nucleic acid sequence capable of identifying a specific location in a sample, followed by a region complementary to the identifier oligonucleotide. The second nucleic acid probe comprises a second flow cell binding site, followed by a second unique molecular identifier, followed by a third unique molecular identifier, followed by a region complementary to the identifier oligonucleotide. A schematic of an identifier oligonucleotide-short nucleic acid probe complex is depicted in Figure 8.

[00198] In another aspect, the present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "direct PCR method".

[00199] A direct PCR method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a first amplification primer binding site, a second amplification primer binding site, or a unique molecular identifier. The identifier oligonucleotide can comprise any combination of these features. Any of these features

NanoString EX2011.064

can also be flanked by regions comprising constant nucleic acid sequences of about 1 nucleotide to about 10 nucleotides.

[00200] Following contacting the at least one analyte with the at least one probe, a direct PCR method further comprises: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00201] A direct PCR method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00202] Following release of the identifier oligonucleotide, a direct PCR method can further comprise: (4) amplifying the released identifier oligonucleotide using a first amplification primer capable of binding to the first amplification primer binding site and a second amplification primer capable of binding to the second amplification primer binding site. In some aspects, at least one of the amplification primers comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. For example, if the identifier oligonucleotide was released from location of the sample designated "ROI #1", at least one of the amplification primers would comprise a nucleic acid sequence that corresponds to "ROI #1". In still other aspects, at least one of the amplification primers comprises a unique molecular identifier.

[00203] Following amplification, a direct PCR method of the present disclosure can further comprise: (5) identifying the released oligonucleotide by sequencing the amplified products produced in step (5), thereby spatially detecting the at least one target analyte in the sample.

[00204] Figure 11 shows a schematic of a preferred aspect of a direct PCR method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the upper panel, the probe hybridizes to the target nucleic acid. In the lower panel, a UV photo-cleavable linker located between the

target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a first amplification primer binding site, a second amplification primer binding site, a unique molecular identifier, and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. Located between these four features are constant spacer regions that are 3 nucleotides in length. The identifier oligonucleotide is double-stranded and comprises a strand that comprises 3 separate nucleic acid molecules. After release, the identifier oligonucleotide is amplified using a first amplification primer that hybridizes to the first amplification primer binding site and comprises a nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a second amplification primer that hybridizes to the second amplification primer binding site. The amplified product is then sequenced to identify the target nucleic acid bound by the probe.

[00205] Figure 19 shows a schematic of a preferred aspect of a direct PCR method of the present disclosure. In this aspect, the identifier oligonucleotide comprises a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain and a second amplification primer binding site, as shown in the top panel of Figure 19. The identifier oligonucleotide is amplified using amplification primers that hybridize to the first and second amplification primer binding sites. As shown in the middle panel of Figure 19, the amplification primer that hybridizes to the second amplification primer binding site comprises a first flow cell binding site, a first nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the second amplification primer binding site. The amplification primer that hybridizes to the first amplification primer binding site comprises a second flow cell binding site, a second nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a nucleic acid sequence complementary to the first amplification primer binding site. The PCR product shown in the bottom panel of Figure 19 is sequenced to identify the released oligonucleotide, thereby spatially detecting the at least one target analyte in the sample.

[00206] In one aspect, the present disclosure provides a composition of a direct-PCR compatible identifier oligonucleotide for spatially detecting at least one target analyte in a sample. A direct-

PCR compatible identifier oligonucleotide comprises a first amplification primer binding site, followed a unique nucleic acid sequence which is capable of identifying a target analyte in a sample, followed by a unique molecular identifier, followed by a second amplification primer binding site. A schematic of a direct-PCR compatible identifier oligonucleotide is depicted in the lower panel of Figure 11.

[00207] In one aspect, the present disclosure provides a composition of a direct-PCR compatible identifier oligonucleotide for spatially detecting at least one target analyte in a sample. A direct-PCR compatible identifier oligonucleotide comprises a first amplification primer binding site, followed a nucleic acid sequence comprising a unique molecular identifier, followed by a unique nucleic acid sequence which is capable of identifying a target analyte in a sample, followed by a second amplification primer binding site. A schematic of a direct-PCR compatible identifier oligonucleotide is shown in the top panel of Figure 19.

[00208] In some aspects of the methods of the present disclosure at least one, or at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten, or at least eleven, or at least twelve, or at least thirteen, or at least fourteen, or at least fifteen, or at least sixteen, or at least seventeen, or at least eighteen, or at least nineteen, or at least twenty, or at least thirty, or at least forty, or at least fifty, or at least sixty, or at least seventy, or at least eighty, or at least ninety, or at least one hundred probes can been to a single target analyte. As used herein, the term "tiling" is used to describe when more than one probe of the present disclosure is bound to a target analyte. The top panel of Figure 27 shows the tiling of probes onto a target RNA. Tiling multiple probes onto a target analyte means that each target analyte will be individually detected multiple times, increasing the overall accuracy of the measurement. In a non-limiting example, as shown in the bottom panel of Figure 27, in the case where 10 probes are tiled onto a single target RNA, one of the probes may be incorrectly detected too many times (outlier high count probe), while another probe may be incorrectly detected too few times (outlier low count probe). However, the other 8 probes may be detected at a similar level, indicating that the two outliers should be discarded during analysis and the signals from the 8 probes used to generate a more accurate measurement of the abundance of the target RNA.

[00209] The present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as an "enzyme free method".

[00210] An enzyme free method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. An identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. An identifier oligonucleotide can also comprise a capture probe binding site.

[00211] Following contacting at least one target analyte with at least one probe, an enzyme free method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00212] Following release of an identifier oligonucleotide, an enzyme free method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00213] Following collection of a released identifier oligonucleotide, an enzyme free method can further comprise: (4) hybridizing to a released identifier oligonucleotide a capture probe.

[00214] A capture probe can comprise a region complementary to the capture probe binding site. A capture probe can also comprise an affinity molecule.

[00215] Following hybridization of a capture probe, an enzyme free method can further comprise: (5) Identifying a released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[00216] A hybridized product produced in step (4) can be sequenced using an enzyme free method of sequencing. Enzyme-free methods of sequencing have been described in, e.g., US2014946386 and US15/819,151 (US Patent No. 10,415,080), each of which is incorporated herein by reference in its entirety.

[00217] Figure 12 shows a schematic of a preferred aspect of an enzyme free method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the top panel, the probe hybridizes to the target nucleic acid. In the middle panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target nucleic acid bound to the target binding domain and a capture probe binding site. After release, the identifier oligonucleotide is hybridized to a capture probe, as depicted in the bottom panel. The capture probe comprises a nucleic acid sequence complementary to the capture probe binding site and an affinity molecule. The hybridized product is then sequenced using enzyme free sequencing methods to identify the target nucleic acid bound by the probe.

[00218] In one aspect, the present disclosure provides a composition of a hybridized identifier oligonucleotide-capture probe complex for spatially detecting at least one target analyte in a sample. A hybridized identifier oligonucleotide-capture probe complex comprises an identifier oligonucleotide hybridized to a capture probe. An identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a specific a target analyte in a sample and a capture probe binding site. A capture probe comprises an affinity molecule and a region complementary to the capture probe binding site. A schematic of a hybridized identifier oligonucleotide-capture probe complex is depicted in the bottom of panel Figure 12.

[00219] The present disclosure provides compositions and methods for spatially detecting at least one target analyte in a sample using the probes of the present disclosure in a method herein referred to as a "multiplexed enzyme free method".

[00220] A multiplexed enzyme free method of the present disclosure can comprise: (1) contacting at least one target analyte in a sample with at least one probe of the present disclosure. The at least one target analyte can be a target protein or a target nucleic acid. In the aspect that the at least one target analyte is a target protein, the probe can comprise a target binding domain that is a target protein-binding region that can specifically bind to a target protein of interest. In the aspect that the at least one target analyte is a target nucleic acid, the probe can comprise a target nucleic acid-binding region that can directly or indirectly hybridize to a target nucleic acid of interest. The probe further comprises an identifier oligonucleotide. The identifier oligonucleotide can comprise a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain. The identifier oligonucleotide can also comprise a capture probe binding site. The identifier oligonucleotide can also comprise a multiplexing probe binding site.

[00221] Following contacting the at least one target analyte with the at least one probe, a multiplexed enzyme free method can further comprise: (2) providing a force to a location of the sample sufficient to release the identifier oligonucleotide. In a non-limiting example, in aspects in which the probe comprises a photo-cleavable linker between the identifier oligonucleotide and the target binding domain, a region-of-interest (ROI) is excited with light of a sufficient wavelength capable of cleaving the photo-cleavable linker.

[00222] Following release of the identifier oligonucleotide, a multiplexed enzyme free method can further comprise: (3) collecting the released identifier oligonucleotide. By directing the force only to a specific location in step (2), identifier oligonucleotides are only released from probes within that location and not from probes located outside that location. Thus, identifier oligonucleotides are collected only for probes that are bound to targets within that location in step (3), thereby permitting detection of the identities and quantities of the targets (proteins and/or nucleic acids) located only within that location.

[00223] Following collection of the released identifier oligonucleotide, a multiplexed enzyme free method can further comprise: (4) hybridizing to the released identifier oligonucleotide a capture probe and a multiplexing probe.

[00224] A capture probe can comprise a region complementary to the capture probe binding site. A capture probe can also comprise an affinity molecule.

[00225] A multiplexing probe can comprise a region complementary to the multiplexing probe binding site. A multiplexing probe can also comprise a nucleic acid sequence which identifies

the specific location of the sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site.

[00226] Following hybridization of a capture probe and a multiplexing probe, a multiplexed enzyme free method can further comprise: (5) Identifying the released identifier oligonucleotide by sequencing the hybridized product produced in step (4), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample.

[00227] A hybridized product produced in step (4) can be sequenced using an enzyme free method of sequencing. Enzyme-free methods of sequencing have been described in, e.g., US2014946386 and US15/819,151, each of which is incorporated herein by reference in its entirety.

[00228] Figure 13 shows a schematic of a preferred aspect of a multiplexed enzyme free method of the present disclosure. In this aspect, the probe comprises a target binding domain comprising a nucleic acid sequence that is complementary to a target nucleic acid. In the top panel, the probe hybridizes to the target nucleic acid. In the middle panel, a UV photo-cleavable linker located between the target binding domain and the identifier oligonucleotide is cleaved, releasing the identifier oligonucleotide. The identifier oligonucleotide comprises a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain, a capture probe binding site, and a multiplexing probe binding site, as shown in the middle panel. After release, the identifier oligonucleotide is hybridized to a capture probe and a multiplexing probe as shown in the lower panel. The capture probe comprises a nucleic acid sequence complementary to the capture probe binding site and an affinity molecule. The multiplexing probe comprises a nucleic acid sequence complementary to the multiplexing probe binding site and a nucleic acid sequence which identifies the specific location of a sample from which the identifier oligonucleotide was released. The hybridized product is then sequenced using enzyme free sequencing methods to identify the target nucleic acid bound by the probe.

[00229] In one aspect, the present disclosure provides a composition of a hybridized identifier oligonucleotide-capture probe-multiplex probe complex for spatially detecting at least one target analyte in a sample. A hybridized identifier oligonucleotide-capture probe-multiplex probe complex comprises an identifier oligonucleotide hybridized to a capture probe and a multiplexing probe. The identifier oligonucleotide comprises a unique nucleic acid sequence capable of identifying a specific a target analyte in a sample, a capture probe binding site and a

multiplexing probe binding site. The capture probe comprises an affinity molecule and a region complementary to the capture probe binding site. The multiplexing probe comprises a nucleic acid sequence which identifies the specific location of a sample from which the identifier oligonucleotide was released and a region complementary to the multiplexing probe binding site. A schematic of a hybridized identifier oligonucleotide-capture probe-multiplex probe complex is depicted in the bottom panel of Figure 13.

[00230] Figure 20 is an exemplary schematic of overview of the methods of the present disclosure. First, a sample on a microscope slide is contacted with a plurality of probes of the present disclosure (step 1 in Figure 20). The slide is then imaged and particular regions of interest (ROIs) are selected (step 2 in Figure 20). A specific ROI is then illuminated by UV light to release identifier oligonucleotides from probes bound within the ROI. The released identifier oligonucleotides are then collected via aspiration with a microcapillary. Following aspiration, the identifier oligonucleotides are transferred to a particular well within a 96 well plate. Steps 4 and 5 are then repeated for each ROI identified in step 2. After all ROIs have been illuminated and all released identifier oligonucleotides collected, the identifier oligonucleotides are sequenced using next generation sequencing methods to spatially detect at least one target analyte in the sample.

[00231] As described in the preceding, the present disclosure provides probes for the compositions and methods of spatially detecting at least one target analyte in a sample. The present disclosure provides probes comprising a target binding domain and an identifier oligonucleotide. The target binding domain is a region of the probe that specifically binds to at least one target analyte in a sample.

[00232] Probes of the present disclosure can be used for spatially detecting a target nucleic acid. In this aspect, the target binding domain can be a target nucleic acid-binding region. The target nucleic acid-binding region is preferably at least 15 nucleotides in length, and more preferably is at least 20 nucleotides in length. In specific aspects, the target nucleic acid-binding region is approximately 10 to 500, 20 to 400, 25, 30 to 300, 35, 40 to 200, or 50 to 100 nucleotides in length. Probes and methods for binding and identifying a target nucleic acid have been described in, *e.g.*, US2003/0013091, US2007/0166708, US2010/0015607, US2010/0261026, US2010/0262374, US2010/0112710, US2010/0047924, and US2014/0371088, each of which is incorporated herein by reference in its entirety.

[00233] The target nucleic acid-binding region can directly hybridize to a target nucleic acid present in a sample. Alternatively, the probes of the present disclosure can indirectly hybridize to a target nucleic acid present in a sample (via an intermediary oligonucleotide). Figure 14 illustrates a probe (or composition) of this aspect. The probe includes a target nucleic-acid binding domain which binds to a synthetic oligonucleotide (the intermediary oligonucleotide) that in turn binds to a target nucleic acid in a biological sample. It could be said that the intermediary oligonucleotide is a probe, as defined herein, since it comprises a nucleic acid backbone and is capable of binding a target nucleic acid. In these aspects, a probe's target nucleic acid-binding region hybridizes to a region of an intermediary oligonucleotide (*i.e.*, a synthetic oligonucleotide) which is different from the target nucleic acid present in a sample. Thus, the probe's target binding region is independent of the ultimate target nucleic acid in the sample. This allows economical and rapid flexibility in an assay design, as the target (present in a sample)-specific components of the assay are included in inexpensive and widely-available synthetic DNA oligonucleotides rather than the more expensive probes. Such synthetic oligonucleotides are simply designed by including a region that hybridizes to the target nucleic acid present in a sample and a region that hybridizes to a probe. Therefore, a single set of indirectly-binding probes can be used to detect an infinite variety of target nucleic acids (present in a sample) in different experiments simply by replacing the target-specific (synthetic) oligonucleotide portion of the assay.

[00234] A target nucleic acid may be DNA or RNA and preferably messenger RNA (mRNA) or miRNA.

[00235] Probes of the present disclosure can be used for detecting a target protein. In this aspect, the target binding domain can be a target protein-binding region. A target protein-binding region includes molecules or assembles that are designed to bind to at least one target protein, at least one target protein surrogate, or both and can, under appropriate conditions, form a molecular complex comprising the probe and the target protein. The target-protein binding region can include an antibody, a peptide, an aptamer, or a peptoid. The antibody can be obtained from a variety of sources, including but not limited to polyclonal antibody, monoclonal antibody, monospecific antibody, recombinantly expressed antibody, humanized antibody, plantibodies, and the like. The terms protein, polypeptide, peptide, and amino acid sequence are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be

linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids or synthetic amino acids. The terms also encompass an amino acid polymer that has been modified, for example, by disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein the term amino acid refers to either natural and/or unnatural or synthetic amino acids, including but not limited to glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. Probes and methods for binding and identifying a target protein have been described, *e.g.*, in US2011/0086774, the contents of which is incorporated herein by reference in its entirety.

[00236] An identifier oligonucleotide is a nucleic acid molecule that identifies the target analyte bound to the target binding domain. The identifier oligonucleotide comprises a unique nucleic acid sequence that identifies the target analyte bound to the target binding domain of the probe. In a non-limiting example, a probe with a target binding domain that binds to the protein P53 comprises an identifier oligonucleotide with a unique nucleic acid sequence that corresponds to P53, while a probe with a target binding domain that binds to the protein P97 comprises an identifier oligonucleotide with a unique nucleic acid sequence that corresponds to P97.

[00237] An identifier oligonucleotide can be DNA, RNA, or a combination of DNA and RNA.

[00238] In some aspects, an identifier oligonucleotide can comprise at least one amplification primer binding site. An amplification primer binding site is a nucleic acid sequence capable of binding to an amplification primer. An amplification primer can be used to amplify the nucleic molecule to which it is bound using methods known in the art, including, but not limited to, polymerase chain reaction (PCR).

[00239] In some aspects, an identifier oligonucleotide can comprise at least one unique molecular identifier.

[00240] An identifier oligonucleotide can be a single-stranded, a double-stranded, or a partially double-stranded nucleic acid molecule. In the aspects in which an identifier oligonucleotide is double-stranded or partially double-stranded, at least one of the two strands can comprise at least two separate nucleic acid molecules which, without being bound by theory, allows for denaturing of the identifier oligonucleotide at lower temperatures.

[00241] An identifier oligonucleotide can also comprise at least one 3' end that comprises a single nucleotide overhang.

[00242] An identifier oligonucleotide can also comprise a capture probe binding site. A capture probe binding site is a nucleic acid sequence to which a capture probe can bind.

[00243] A capture probe of the present disclosure can comprise a nucleic acid sequence complementary to a capture probe binding site. A capture probe can also comprise an affinity molecule.

[00244] An identifier oligonucleotide can also comprise a multiplexing probe binding site. A multiplexing probe binding site is a nucleic acid sequence to which a multiplexing probe can bind.

[00245] A multiplexing probe of the present disclosure can comprise a nucleic acid sequence complementary to a multiplexing probe binding site. A multiplexing probe can also comprise a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released.

[00246] A probe of the present disclosure can include a region which permits the release of an identifier oligonucleotide following the application of a suitable force. In one non-limited example, the region is a cleavable motif (*e.g.*, a restriction enzyme site or cleavable linker). The cleavable motif allows release of an identifier oligonucleotide from a bound target nucleic acid or protein and the identifier oligonucleotide can then be collected and detected. The region which permits the release of an identifier oligonucleotide can be positioned between the target-binding domain and the identifier oligonucleotide, allowing for the release of the identifier oligonucleotide from the target binding domain. An identifier oligonucleotide is said to be releasable when it can be separated (*i.e.*, cleaved and released) from the remainder of the probe. Examples of cleavable motives include but are not limited to photo-cleavable linkers. Photo-cleavable linkers can be cleaved by light provided by a suitable coherent light source (*e.g.*, a laser and a UV light source) or a suitable incoherent light source (*e.g.*, an arc-lamp and a light-emitting diode (LED)).

[00247] In some aspects, the identifier oligonucleotide is collected from a solution proximal to, e.g., at least immediately above or surrounding, the point at which the identifier oligonucleotide is released or the at least one cell. The proximal solution may be collected by aspirating, e.g., via a pipette, a capillary tube, a microarray pin, a flow cell comprising holes, or another suitable aspirating system known in the art or any combination thereof. The capillary tube may comprise an optical device capable of transmitting a light force, e.g., UV light, to the at least one cell. The

pipette or a microarray pin may be attached to an array comprising a plurality of pipettes or microarray pins. The proximal solution may comprise an anionic polymer, e.g., dextran sulfate, and/or salmon sperm DNA and/or the collected signal oligonucleotide may be added to a solution comprising an anionic polymer, e.g., dextran sulfate, and/or salmon sperm DNA. Other non-specific blocking agents known in the art in addition to or instead of salmon sperm DNA may be used.

[00248] In some aspects, the identifier oligonucleotide is collected from a tissue, at least one cell or proximal to the point at which the identifier oligonucleotide is released via liquid laminar, turbulent, or transitional flow. The flow may be via a channel, e.g., having 25 to 500 m depth between the tissue and a fluidic device or impermeable barrier placed over the tissue.

[00249] In aspects where the target-binding domain of a probe is an antibody, the probe can be prepared using a cysteine bioconjugation method that is stable, site-specific to, preferably, the antibody's hinge-region heavy-chain. This preparation method provides relatively controllable identifier oligonucleotides to antibody stoichiometric ratios. A probe can comprise a plurality (i.e., more than one, e.g., 2, 3, 4, 5, or more) identifier oligonucleotides per antibody. Generally, "heavier" probes, which comprise 3 or 4 identifier oligonucleotides per antibody, are significantly less sensitive than antibodies lacking an identifier oligonucleotide or "lighter" probes, which comprise 1 or 2 identifier oligonucleotides per antibody.

[00250] In aspects, probes are provided to a sample at concentrations typically less than that used for immunohistochemistry (IHC) or for in situ hybridization (ISH). Alternately, the concentration may be significantly less than that used for IHC or ISH. For example, the probe concentration may be 2 fold less, 5 fold less, 10 fold less, 20 fold less, 25 fold less, 30 fold less, 50 fold less, 60 fold less, 70 fold less, 80 fold less, 90 fold less, 100 fold less, 200 fold less, 300 fold less, 400 fold less, 500 fold less, 600 fold less, 700 fold less, 800 fold less, 900 fold less, 1000 fold less, 2000 fold less, or less and any number in between. In aspects, probes are provided at a concentration of 100 nM, 70 nM, 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM, 9 nM, 8 nM, 7 nM, 6 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.9 nM, 0.8 nM, 0.7 nM, 0.6 nM, 0.5 nM, 0.4 nM, 0.3 nM, 0.2 nM, 0.1 nM, 0.09 nM, 0.08 nM, 0.07 nM, 0.06 nM, 0.05 nM, 0.04 nM, 0.03 nM, 0.02 nM, 0.01 nM, and less and any concentration in between.

[00251] Background noise, during protein detection, can be reduced by performing a negative purification of the intact probe molecule. This can be done by conducting an affinity purification

of the antibody or photo-cleavable linker after collection of eluate from a region of interest. Normally, released signal oligonucleotides will not be pulled out of solution. A protein-G or -O mechanism in a pipet tip, tube, or plate can be employed for this step. Such devices and reagents commercially available.

[00252] Background noise, during nucleic acid detection, can be reduced by performing a negative purification of the intact probe molecule. This can be done by conducting an affinity purification of the target binding domain or photo-cleavable linker after collection of eluate from a region of interest. Normally, released signal oligonucleotides will not be pulled out of solution. To assist in the negative purification, a universal purification sequence may be included in a probe, e.g., in the target binding domain.

[00253] Protein-targeting probes and nucleic acid-targeting probes may be applied simultaneously as long as conditions allow for binding of both a protein target and a nucleic acid target. Alternately, protein-targeting probes and nucleic acid-targeting probes may be applied sequentially when conditions allowing for binding of both a protein target and a nucleic acid target are not possible.

[00254] A set of probes is synonymous with a composition of probes. A set of probes includes at least one species of probes, *i.e.*, directed to one target. A set of probes preferably includes at least two, e.g., 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more species of probes. A probe set may include one or multiple copies of each species of probe.

[00255] A first set of probes only may be applied to a sample. Alternately, a second set (or higher number) of probes may be later applied to the sample. The first set and second (or higher number) may target only nucleic acids, only proteins, or a combination thereof.

[00256] In the present disclosure, two or more targets (*i.e.*, proteins, nucleic acids, or a combination thereof) are detected; 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 or more targets, and any number there between, are detected.

[00257] A set of probes may be pre-defined based upon the cell type or tissue type to be targeted. For example, if the tissue is a breast cancer, then the set of probes will include probes directed to proteins relevant to breast cancer cells (e.g., Her2, EGFR, and PR) and/or probes directed to proteins relevant to normal breast tissues. Additionally, the set of probes may be pre-defined

based upon developmental status of a cell or tissue to be targeted. Alternately, the set of probes may be pre-defined based upon subcellular localizations of interest, e.g., nucleus, cytoplasm, and membrane. For example, antibodies directed to Foxp3, Histone H3, or P-S6 label the nucleus, antibodies directed to CD3, CD4, PD-1, or CD45RO label the cytoplasm, and antibodies directed to PD-L1 label membranes.

[00258] A probe may be chemically synthesized or may be produced biologically using a vector into which a nucleic acid encoding the probe has been cloned.

[00259] Any probe or set of probes described herein may be used in methods and kits of the present disclosure.

[00260] For the herein-described probes, association of a unique nucleic acid sequence to a specific target nucleic acid or target protein is not fixed.

[00261] As described in the preceding, probes of the present disclosure can be used to detect a target nucleic acid or target protein present in any sample, *e.g.*, a biological sample. As will be appreciated by those in the art, the sample may comprise any number of things, including, but not limited to: cells (including both primary cells and cultured cell lines) and tissues (including cultured or explanted). In aspects, a tissue sample (fixed or unfixed) is embedded, serially sectioned, and immobilized onto a microscope slide. As is well known, a pair of serial sections will include at least one cell that is present in both serial sections. Structures and cell types, located on a first serial section will have a similar location on an adjacent serial section. The sample can be cultured cells or dissociated cells (fixed or unfixed) that have been immobilized onto a slide. A sample can be a formalin-fixed paraffin-embedded (FFPE) tissue sample.

[00262] In aspects, a tissue sample is a biopsied tumor or a portion thereof, i.e., a clinically-relevant tissue sample. For example, the tumor may be from a breast cancer. The sample may be an excised lymph node.

[00263] The sample can be obtained from virtually any organism including multicellular organisms, *e.g.*, of the plant, fungus, and animal kingdoms; preferably, the sample is obtained from an animal, *e.g.*, a mammal. Human samples are particularly preferred.

[00264] In some aspects, the probes, compositions, methods, and kits described herein are used in the diagnosis of a condition. As used herein the term diagnose or diagnosis of a condition includes predicting or diagnosing the condition, determining predisposition to the condition, monitoring treatment of the condition, diagnosing a therapeutic response of the disease, and

prognosis of the condition, condition progression, and response to particular treatment of the condition. For example, a tissue sample can be assayed according to any of the probes, methods, or kits described herein to determine the presence and/or quantity of markers of a disease or malignant cell type in the sample (relative to the non-diseased condition), thereby diagnosing or staging a disease or a cancer.

[00265] In general, samples attached to a slide can be first imaged using fluorescence (e.g., fluorescent antibodies or fluorescent stains (e.g., DAPI)) to identify morphology, regions of interest, cell types of interest, and single cells and then expression of proteins and/or nucleic acids can be digitally counted from the sample on the same slide.

[00266] Compositions and kits of the present disclosure can include probes and other reagents, for example, buffers and other reagents known in the art to facilitate binding of a protein and/or a nucleic acid in a sample, *i.e.*, for performing hybridization reactions.

[00267] A kit also will include instructions for using the components of the kit, including, but not limited to, information necessary to hybridize labeled oligonucleotides to a probe, to hybridize a probe to a target-specific oligonucleotide, to hybridize a target-specific oligonucleotide to a target nucleic acid and/or to hybridize a probe to target protein.

[00268] A region of interest may be a tissue type present in a sample, a cell type, a cell, or a subcellular structure within a cell.

[00269] Together, a comparison of the identity and abundance of the target proteins and/or target nucleic acids present in a first region of interest (*e.g.*, tissue type, a cell type (including normal and abnormal cells), and a subcellular structure within a cell) and the identity and abundance of the target proteins and/or target nucleic acids present in second region of interest or more regions of interest can be made using the methods of the present disclosure.

[00270] As described in the preceding, the products produced by the methods of the present disclosure can be used for nucleic acid amplification. In a preferred aspect, the nucleic acid amplification can be solid-phase nucleic acid amplification. Thus, in further aspects the invention provides a method of solid-phase nucleic acid amplification of template polynucleotide molecules which comprises: preparing a library of template polynucleotide molecules which have common sequences at their 5' and 3' ends using the methods of the present disclosure and carrying out a solid-phase nucleic acid amplification reaction wherein said template polynucleotide molecules are amplified. Compositions and methods for nucleic acid

amplification and sequencing have been described in, e.g., US9376678, which is incorporated herein by reference in its entirety.

[00271] The term "solid-phase amplification" as used herein refers to any nucleic acid amplification reaction carried out on or in association with a solid support such that all or a portion of the amplified products are immobilized on the solid support as they are formed. In particular, the term encompasses solid-phase polymerase chain reaction (solid-phase PCR), which is a reaction analogous to standard solution phase PCR, except that one or both of the forward and reverse amplification primers is/are immobilized on the solid support.

[00272] Although the invention encompasses "solid-phase" amplification methods in which only one amplification primer is immobilized (the other primer usually being present in free solution), it is preferred for the solid support to be provided with both the forward and the reverse primers immobilized. In practice, there will be a "plurality" of identical forward primers and/or a "plurality" of identical reverse primers immobilized on the solid support, since the PCR process requires an excess of primers to sustain amplification. References herein to forward and reverse primers are to be interpreted accordingly as encompassing a "plurality" of such primers unless the context indicates otherwise.

[00273] As will be appreciated by the skilled reader, any given PCR reaction requires at least one type of forward primer and at least one type of reverse primer specific for the template to be amplified. However, in certain aspects the forward and reverse primers may comprise template-specific portions of identical sequence, and may have entirely identical nucleotide sequence and structure (including any non-nucleotide modifications). In other words, it is possible to carry out solid-phase amplification using only one type of primer, and such single-primer methods are encompassed within the scope of the invention. Other aspects may use forward and reverse primers which contain identical template-specific sequences but which differ in some other structural features. For example one type of primer may contain a non-nucleotide modification which is not present in the other.

[00274] In other aspects of the invention the forward and reverse primers may contain template-specific portions of different sequence.

[00275] Amplification primers for solid-phase PCR are preferably immobilized by covalent attachment to the solid support at or near the 5' end of the primer, leaving the template-specific portion of the primer free for annealing to its cognate template and the 3' hydroxyl group free for

primer extension. Any suitable covalent attachment means known in the art may be used for this purpose. The chosen attachment chemistry will depend on the nature of the solid support, and any derivatization or functionalization applied to it. The primer itself may include a moiety, which may be a non-nucleotide chemical modification, to facilitate attachment. In one particularly preferred aspect the primer may include a sulphur-containing nucleophile, such as phosphorothioate or thiophosphate, at the 5' end. In the case of solid-supported polyacrylamide hydrogels (as described below), this nucleophile will bind to a "C" group present in the hydrogel. The most preferred means of attaching primers and templates to a solid support is via 5' phosphorothioate attachment to a hydrogel comprised of polymerised acrylamide and N-(5-bromoacetamidylpentyl)acrylamide (BRAPA).

[00276] The terms "cluster" and "colony" are used interchangeably herein to refer to a discrete site on a solid support comprised of a plurality of identical immobilized nucleic acid strands and a plurality of identical immobilized complementary nucleic acid strands. The term "clustered array" refers to an array formed from such clusters or colonies. In this context the term "array" is not to be understood as requiring an ordered arrangement of clusters.

[00277] The invention also encompasses methods of sequencing the amplified nucleic acids generated by solid-phase amplification. Thus, the invention provides a method of nucleic acid sequencing comprising amplifying a library of nucleic acid templates by the methods of the present disclosure described above, using solid-phase amplification as described above to amplify this library on a solid support, and carrying out a nucleic acid sequencing reaction to determine the sequence of the whole or a part of at least one amplified nucleic acid strand produced in the solid-phase amplification reaction.

[00278] Sequencing, as referred to herein, can be carried out using any suitable "sequencing-by-synthesis" technique, wherein nucleotides are added successively to a free 3' hydroxyl group, resulting in synthesis of a polynucleotide chain in the 5' to 3' direction. The nature of the nucleotide added is preferably determined after each nucleotide addition.

[00279] The initiation point for the sequencing reaction may be provided by annealing of a sequencing primer to a product of the whole genome or solid-phase amplification reaction. In this connection, one or both of the adapters added during formation of the template library may include a nucleotide sequence which permits annealing of a sequencing primer to amplified products derived by whole genome or solid-phase amplification of the template library.

[00280] The products of solid-phase amplification reactions wherein both forward and reverse amplification primers are covalently immobilized on the solid surface are so-called "bridged" structures formed by annealing of pairs of immobilized polynucleotide strands and immobilized complementary strands, both strands being attached to the solid support at the 5' end. Arrays comprised of such bridged structures provide inefficient templates for nucleic acid sequencing, since hybridization of a conventional sequencing primer to one of the immobilized strands is not favored compared to annealing of this strand to its immobilized complementary strand under standard conditions for hybridization.

[00281] In order to provide more suitable templates for nucleic acid sequencing it is preferred to remove substantially all or at least a portion of one of the immobilized strands in the "bridged" structure in order to generate a template which is at least partially single-stranded. The portion of the template which is single-stranded will thus be available for hybridization to a sequencing primer. The process of removing all or a portion of one immobilized strand in a "bridged" double-stranded nucleic acid structure may be referred to herein as "linearization".

[00282] Bridged template structures may be linearized by cleavage of one or both strands with a restriction endonuclease or by cleavage of one strand with a nicking endonuclease. Other methods of cleavage can be used as an alternative to restriction enzymes or nicking enzymes, including inter alfa chemical cleavage (e.g. cleavage of a diol linkage with periodate), cleavage of abasic sites by cleavage with endonuclease, or by exposure to heat or alkali, cleavage of ribonucleotides incorporated into amplification products otherwise comprised of deoxyribonucleotides, photochemical cleavage or cleavage of a peptide linker.

[00283] It will be appreciated that a linearization step may not be essential if the solid-phase amplification reaction is performed with only one primer covalently immobilized and the other in free solution.

[00284] In order to generate a linearized template suitable for sequencing it is necessary to remove "unequal" amounts of the complementary strands in the bridged structure formed by amplification so as to leave behind a linearized template for sequencing which is fully or partially single stranded. Most preferably one strand of the bridged structure is substantially or completely removed.

[00285] Following the cleavage step, regardless of the method used for cleavage, the product of the cleavage reaction may be subjected to denaturing conditions in order to remove the portion(s)

of the cleaved strand(s) that are not attached to the solid support. Suitable denaturing conditions will be apparent to the skilled reader with reference to standard molecular biology protocols.

[00286] Denaturation (and subsequent re-annealing of the cleaved strands) results in the production of a sequencing template which is partially or substantially single-stranded. A sequencing reaction may then be initiated by hybridization of a sequencing primer to the single-stranded portion of the template.

[00287] Thus, the nucleic acid sequencing reaction may comprise hybridizing a sequencing primer to a single-stranded region of a linearized amplification product, sequentially incorporating one or more nucleotides into a polynucleotide strand complementary to the region of amplified template strand to be sequenced, identifying the base present in one or more of the incorporated nucleotide(s) and thereby determining the sequence of a region of the template strand.

[00288] One preferred sequencing method which can be used in accordance with the invention relies on the use of modified nucleotides that can act as chain terminators. Once the modified nucleotide has been incorporated into the growing polynucleotide chain complementary to the region of the template being sequenced there is no free 3'-OH group available to direct further sequence extension and therefore the polymerase cannot add further nucleotides. Once the nature of the base incorporated into the growing chain has been determined, the 3' block may be removed to allow addition of the next successive nucleotide. By ordering the products derived using these modified nucleotides it is possible to deduce the DNA sequence of the DNA template. Such reactions can be done in a single experiment if each of the modified nucleotides has attached a different label, known to correspond to the particular base, to facilitate discrimination between the bases added at each incorporation step. Alternatively, a separate reaction may be carried out containing each of the modified nucleotides separately.

[00289] The modified nucleotides may carry a label to facilitate their detection. Preferably this is a fluorescent label. Each nucleotide type may carry a different fluorescent label. However the detectable label need not be a fluorescent label. Any label can be used which allows the detection of an incorporated nucleotide.

[00290] One method for detecting fluorescently labelled nucleotides comprises using laser light of a wavelength specific for the labelled nucleotides, or the use of other suitable sources of

illumination. The fluorescence from the label on the nucleotide may be detected by a CCD camera or other suitable detection means.

[00291] The invention is not intended to be limited to use of the sequencing method outlined above, as essentially any sequencing methodology which relies on successive incorporation of nucleotides into a polynucleotide chain can be used. Suitable alternative techniques include, for example, Pyrosequencing, FISSEQ (fluorescent in situ sequencing), MPSS (massively parallel signature sequencing) and sequencing by ligation-based methods.

[00292] In methods of the present disclosure, the unique nucleic acid sequence present in the identifier oligonucleotide of a probe which identifies the target analyte bound to the target binding domain of the probe can comprise between about 5 nucleotides and about 50 nucleotides. Preferably, the sequence comprises between about 20 nucleotides and about 40 nucleotides. Even more preferably, the sequence comprises about 35 nucleotides. In some preferred aspects, the sequence comprises 10 nucleotides.

[00293] In methods of the present disclosure, the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released comprises between about 6 nucleotides and about 15 nucleotides. Preferably, the sequence comprises about 12 nucleotides.

[00294] In methods of the present disclosure, an amplification primer binding sites comprises between about 18 nucleotides and about 40 nucleotides. Preferably, an amplification primer binding sites comprises about 32 nucleotides.

[00295] In some aspects of the methods of the present disclosure, an amplification primer binding site can comprise an i7 sequence, wherein the i7 sequence comprises the sequence set forth in SEQ ID NO: 1.

[00296] In some aspects of the methods of the present disclosure, an amplification primer binding site can comprise an i5 sequence, wherein the i5 sequence comprises the sequence set forth in SEQ ID NO: 2.

[00297] In some aspects of the methods of the present disclosure, an amplification primer can comprise a flow cell adapter sequence, wherein the flow cell adapter sequence is suitable for sequencing. Preferably, at least one amplification primer used in the methods of the present disclosure comprises a P5 flow cell adapter sequence, wherein the P5 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 3. Preferably still, at least one amplification

primer used in the methods of the present disclosure comprises a P7 flow cell adapter sequence, wherein the P7 flow cell adapter sequence comprises the sequence set forth in SEQ ID NO: 4.

[00298] In methods of the present disclosure, a unique molecular identifier can comprise between about 6 nucleotides and about 30 nucleotides. Preferably, a unique molecular identifier can comprise about 15 nucleotides. The terms unique molecular identifier and random molecular tags are used interchangeably herein. Using methods known in that art, unique molecular identifiers are random sequences that can be used to correct for biases in amplification prior to sequencing.

[00299] In methods of the present disclosure, a constant nucleic acid sequence to minimize ligation bias comprises between about 1 nucleotide and about 15 nucleotides. Preferably, the constant sequence comprises about 8 nucleotides.

[00300] In some aspects, a flow cell binding site can comprise between about 15 to about 40 nucleotides. A flow cell binding site can comprise about 29 nucleotides. A flow cell binding site can comprise about 24 nucleotides.

[00301] In some aspects, a target binding domain can comprise between about 10 to about 70 nucleotides. A target binding domain can comprise between about 30 to about 55 nucleotides. A target binding domain can comprise between about 35 to about 50 nucleotides.

[00302] In some aspects, a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain can comprise between about 20 to about 40. A unique nucleic acid sequence which identifies the target analyte bound to the target binding domain can comprise about 25 nucleotides, or about 35 nucleotides, or about 12 nucleotides.

[00303] In some aspects, an amplification primer binding site can comprise between about 20 to about 50 nucleotides. An amplification primer binding site can comprise about 33 nucleotides, or about 34 nucleotides.

[00304] In some aspects, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can comprise between about 1 to about 20 nucleotides. A nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released can comprise about 8 nucleotides.

[00305] In some aspects, a nucleic acid sequence comprising a unique molecular identifier can comprise between about 5 to about 20 nucleotides. A nucleic acid sequence comprising a unique molecular identifier can comprise about 14 nucleotides.

[00306] As used herein, the terms "region of interest" and "ROI" are used in their broadest sense to refer to a specific location within a sample that is to be analyzed using the methods of the present disclosure.

[00307] As used herein, the term "adjacent" can mean within about 1 nucleotide, or within about 2 nucleotides, or within about 3 nucleotides, or within about 4 nucleotides, or within about 5 nucleotides, or within about 6 nucleotides, or within about 7 nucleotides, or within about 8 nucleotides, or within about 9 nucleotides, or within about 10 nucleotides, or within about 11 nucleotides, or within about 12 nucleotides, or within about 13 nucleotides, or within about 14 nucleotides, or within about 15 nucleotides, or within about 16 nucleotides, or within about 17 nucleotides, or within about 18 nucleotides, or within about 19 nucleotides, or within about 20 nucleotides, or within about 21 nucleotides, or within about 22 nucleotides, or within about 23 nucleotides, or within about 24 nucleotides, or within about 25 nucleotides, or within about 26 nucleotides, or within about 27 nucleotides, or within about 28 nucleotides, or within about 29 nucleotides, or within about 30 nucleotides, or within about 40 nucleotides, or within about nucleotides, or within about 50 nucleotides, or within about 60 nucleotides, or within about 70 nucleotides, or within about 80 nucleotides, or within about 90 nucleotides, or within about 100 nucleotides.

[00308] As used herein, the term "spatially detecting" is used in its broadest sense to refer to the identification of the presence of a specific target analyte within a specific region of interest in a sample. Spatially detecting can comprise quantifying the amount of a specific target analyte present within a specific region of interest in a sample. Spatially detecting can further comprise quantifying the relative amount of a first target analyte within a specific region of interest in a sample as compared to the amount of at least a second target analyte within a specific region of interest in a sample. Spatially detecting can also comprise quantifying the relative amount of a specific target analyte within a first region of interest in a sample compared to the amount of the same target analyte in at least a second region of interest in the same sample or different sample.

[00309] In some aspects of the methods and compositions of the present disclosure, a target analyte can be any molecule within a sample that is to be spatially detected. Target analytes include, but are not limited to, nucleic acid molecules and protein molecules. When the target analyte is a protein, the protein can be referred to as a target protein. When the target analyte is a nucleic acid, the nucleic acid can be referred to as a target nucleic acid. Target nucleic acids can include, but

are not limited to, mRNA molecules, micro RNA (miRNA) molecules, tRNA molecules, rRNA molecules, gDNA or any other nucleic acid present within a sample.

[00310] In some aspects of the methods and compositions of the present disclosure, the term target binding domain is used in its broadest sense to refer to a portion of a probe of the present disclosure that binds to, either directly or indirectly, a target analyte located in a sample. A target binding domain can comprise nucleic acid, protein, at least one antibody, an aptamer, or any combination thereof. A target binding domain can comprise DNA, RNA or any combination thereof. A target binding domain can comprise any number of modified nucleotides and/or nucleic acid analogues.

[00311] In the aspect that the target analyte to be spatially detected is a target protein, a target binding domain can be a protein-target binding domain. A protein-target binding domain can comprise an antibody or antibody fragment that binds to the target protein.

[00312] In the aspect that the target analyte to be spatially detected is a target nucleic acid, a target binding domain can be a target nucleic acid-binding region. A target nucleic acid-binding region can comprise a nucleic acid that is complementary to the target nucleic acid to be spatially detected. A target nucleic acid-binding region can comprise a nucleic acid that hybridizes to the target nucleic acid to be detected.

[00313] As used herein, the term "hybridize" is used in its broadest sense to mean the formation of a stable nucleic acid duplex. In one aspect, "stable duplex" means that a duplex structure is not destroyed by a stringent wash under conditions such as, for example, a temperature of either about 5 °C below or about 5 °C above the Tm of a strand of the duplex and low monovalent salt concentration, *e.g.,* less than 0.2 M, or less than 0.1 M or salt concentrations known to those of skill in the art. A duplex can be "perfectly matched", such that the polynucleotide and/or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. The term "duplex" comprises, but is not limited to, the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, PNAs, and the like, that can be employed. A duplex can comprise at least one mismatch, wherein the term "mismatch" means that a pair of nucleotides in the duplex fail to undergo Watson-Crick bonding.

[00314] As used herein, the term "hybridization conditions," will typically include salt concentrations of less than about 1 M, more usually less than about 500 mM and even more usually less than about 200 mM. Hybridization temperatures can be as low as 5 °C, but are typically greater

than 22 °C, more typically greater than about 30 °C, and often in excess of about 37 °C. Hybridizations are usually performed under stringent conditions, *e.g.,* conditions under which a probe will specifically hybridize to its target analyte. Stringent conditions are sequence-dependent and are different in different circumstances. Longer fragments can require higher hybridization temperatures for specific hybridization. As other factors can affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one alone. Certain hybridization conditions will promote the formation of a duplex between the entire length of a target binding domain and the target analyte. Other hybridization conditions will promote the formation of a duplex only along certain portions of the target binding domain.

[00315] In some aspects of the methods and compositions of the present disclosure, a probe can comprise a target binding domain directly, or indirectly linked to an identifier oligonucleotide. In the context of a probe, an identifier oligonucleotide is a polynucleotide that comprises a nucleic acid sequence that identifies the target analyte bound to the target binding domain of that probe. That is to say, the identifier oligonucleotide comprises a specific nucleic acid sequence that is *a priori* assigned to the specific target analyte bound to the target binding to which the identifier oligonucleotide is attached. In a non-limiting example, a probe designated as "probe *X*" designed to spatially detect "target analyte *X*" comprises a target binding domain designated "target binding domain *X*" linked to an identifier oligonucleotide designated "identifier oligonucleotide *X*". Target binding domain *X* binds to target analyte *X* and identifier oligonucleotide *X* comprises a nucleic acid sequence, designated as "nucleic acid sequence *X*", which corresponds to target analyte *X*. Thus, if a skilled artisan practicing the methods of the present disclosure were to collect identifier oligonucleotides released from a region of interest in sample and obtain nucleic acid sequence *X* after sequencing, the skilled artisan would understand that to indicate that target analyte *X* was present in that region of interest. The amount, or number of sequencing reads, of nucleic acid sequence *X* can be used to determine the quantify, in absolute or relative terms, the amount of target analyte *X* within the region of interest.

[00316] As used herein, the term "amplification primer binding site" is used in its broadest sense to refer to a nucleic acid sequence that is complementary to, or at least partially complementary to at least one amplification primer, wherein the amplification primer is a short single-stranded or

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partially single-stranded oligonucleotide that is sufficient to prime DNA and/or RNA synthesis, for example, by PCR.

[00317] In some aspects of the methods and compositions of the present disclosure, a target binding domain can be linked to an identifier oligonucleotide by a cleavable linker. Suitable cleavable linkers include, but are not limited to, chemically cleavable linkers (*e.g.* a linker that is cleaved when exposed to a particular chemical, combination of chemicals or reaction conditions), a photo-cleavable linker (*e.g.* a linker that is cleaved when exposed to light of a sufficient wavelength or light comprising a sufficient range of wavelengths), or an enzymatically cleavable linker (*e.g.* a linker that is cleaved by a specific enzyme or class of enzymes). Thus, as used herein the phrase "providing a force to a location of the sample sufficient to release an identifier oligonucleotide" is used in its broadest sense to describe changing the conditions within a certain region of interest in a sample such that, for any probe bound to a target analyte within that region of interest, the linker between the target binding domain of the probe and the identifier oligonucleotide of the probe is cleaved, thereby separating the identifier oligonucleotide from the target binding domain so that the identifier oligonucleotide can be subsequently collected from solution. For example, in aspects wherein a probe comprises a chemically cleavable linker between the target binding domain and the identifier oligonucleotide, providing a force to a location of the sample sufficient to release an identifier oligonucleotide can comprise exposing that location of the sample to the specific chemical, combination of chemicals or reaction conditions that catalyze the cleavage of the linker. In another non-limiting example, in aspects wherein a probe comprises a photo-cleavable linker between the target binding domain and the identifier oligonucleotide, providing a force to a location of the sample sufficient to release an identifier oligonucleotide can comprise exposing/exciting that location of the sample with light of a sufficient wavelength capable of cleaving the photo-cleavable linker. In another non-limiting example, in aspects wherein a probe comprises an enzymatically cleavable linker between the target binding domain and the identifier oligonucleotide, providing a force to a location of the sample sufficient to release an identifier oligonucleotide can comprise exposing that location of the sample to an amount of enzyme sufficient to catalyze the cleavage of the linker.

[00318] Providing a force to a location of the sample sufficient to release an identifier oligonucleotide can result in at least about 10%, or at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least

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about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 99% of probes bound to target analytes within that location of the sample to undergo cleavage of the linker connecting the target binding domain and the identifier oligonucleotide.

[00319] As would be appreciated by one skilled in the art, the term "unique molecular identifier" or "UMI" refer to short nucleic acid sequences that are used to quantify and reduce quantitative bias caused by nucleic acid amplification prior to sequencing reactions.

[00320] In some aspects of the methods and compositions of the present disclosure, an affinity moiety can comprise biotin, avidin, streptavidin, nucleic acid, or any combination thereof.

[00321] In some aspects of the methods and compositions of the present disclosure, a probe can comprise at least at least about 5, about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

[00322] In some aspects of the methods and compositions of the present disclosure, a target binding domain can comprise at least at least about 5, about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

[00323] In some aspects of the methods and compositions of the present disclosure, an identifier oligonucleotide can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

[00324] In some aspects of the methods and compositions of the present disclosure, an amplification primer can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

[00325] In some aspects of the methods and compositions of the present disclosure, a nucleic acid probe can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150, at least about 160, at least about 170, at least about 180, at least about 190 or at least about 200 nucleotides.

[00326] In some aspects of the methods and compositions of the present disclosure, a nucleic acid complementary to a portion of a identifier oligonucleotide can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95 or at least about 100 nucleotides.

[00327] In some aspects of the methods and compositions of the present disclosure, a nucleic acid sequence comprising a molecular identifier can comprise at least about 5, or at least about 10 nucleotides, or at least about 15, or at least about 20, or at least about 25, or at least about 30, or at least about 35, or at least about 40, or at least about 45, or at least about 50 nucleotides.

[00328] In some aspects of the methods and compositions of the present disclosure, an amplification primer binding site can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65 or at least about 70 nucleotides.

[00329] In some aspects of the methods and compositions of the present disclosure, a flow cell adapter sequence suitable for sequencing can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35 at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 85, at least about 90, at least about 95 or at least about 100 nucleotides.

[00330] In some aspects of the methods and compositions of the present disclosure, a flow cell binding site can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 90, at least about 95 or at least about 100 nucleotides.

[00331] In some aspects of the methods and compositions of the present disclosure, a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released can comprise at least about 5, or at least about 10 nucleotides, or at least about 15, or at least about 20, or at least about 25, or at least about 30, or at least about 35, or at least about 40, or at least about 45, or at least about 50 nucleotides.

[00332] In some aspects of the methods and compositions of the present disclosure, a unique nucleic acid sequence which identifies the target analyte bound to a target binding domain can comprise at least about 5, at least about 10, at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, at least about 65, at least about 70, at least about 75, at least about 80, at least about 90, at least about 95 or at least about 100 nucleotides.

[00333] In some aspects of the methods and compositions of the present disclosure, a probe, a target binding domain, an identifier oligonucleotide, an amplification primer, a nucleic acid probe, a nucleic acid complementary to a portion of a identifier oligonucleotide, a nucleic acid sequence comprising a molecular identifier, an amplification primer binding site, a flow cell adapter sequence, a flow cell binding site, a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released, a unique nucleic acid sequence which identifies the target analyte bound to a target binding domain or any combination thereof can comprise at least one natural base, can comprise no natural bases, can comprise at least one modified nucleotide or nucleic acid analog, can comprise no modified

nucleotides or nucleic acid analogs, can comprise at least one universal base, can comprise no universal bases, can comprise at least one degenerate base or can comprise no degenerate bases.

[00334] In some aspects of the methods and compositions of the present disclosure, a probe, a target binding domain, an identifier oligonucleotide, an amplification primer, a nucleic acid probe, a nucleic acid complementary to a portion of a identifier oligonucleotide, a nucleic acid sequence comprising a molecular identifier, an amplification primer binding site, a flow cell adapter sequence, a flow cell binding site, a nucleic acid sequence which identifies the specific location of the tissue sample from which an identifier oligonucleotide was released, a unique nucleic acid sequence which identifies the target analyte bound to a target binding domain or any combination thereof can comprise any combination natural bases (*e.g.* 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more natural bases), modified nucleotides or nucleic acid analogs (*e.g.* 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more modified or analog nucleotides), universal bases (*e.g.* 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more universal bases), or degenerate bases (*e.g.* 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more degenerative bases). When present in a combination, the natural bases, modified nucleotides or nucleic acid analogs, universal bases and degenerate bases can be arranged in any order.

[00335] The terms "modified nucleotides" or "nucleic acid analogues" include, but are not limited to, locked nucleic acids (LNA), bridged nucleic acids (BNA), propyne-modified nucleic acids, zip nucleic acids (ZNA●), isoguanine and isocytosine. Preferably, the modified nucleotides or nucleic acid analogues are locked nucleic acids (LNAs).

[00336] The term "locked nucleic acids (LNA)" as used herein includes, but is not limited to, a modified RNA nucleotide in which the ribose moiety comprises a methylene bridge connecting the 2' oxygen and the 4' carbon. This methylene bridge locks the ribose in the 3'-endo confirmation, also known as the north confirmation, that is found in A-form RNA duplexes. The term inaccessible RNA can be used interchangeably with LNA. The term "bridged nucleic acids (BNA)" as used herein includes, but is not limited to, modified RNA molecules that comprise a five-membered or six-membered bridged structure with a fixed 3'-endo confirmation, also known as the north confirmation. The bridged structure connects the 2' oxygen of the ribose to the 4' carbon of the ribose. Various different bridge structures are possible containing carbon, nitrogen, and hydrogen atoms. The term "propyne-modified nucleic acids" as used herein includes, but is not limited to, pyrimidines, namely cytosine and thymine/uracil, that comprise a propyne modification at the C5 position of the nucleic acid base. The term "zip nucleic acids

(ZNA●)" as used herein includes, but is not limited to, oligonucleotides that are conjugated with cationic spermine moieties.

[00337] The term "universal base" as used herein includes, but is not limited to, a nucleotide base does not follow Watson-Crick base pair rules but rather can bind to any of the four canonical bases (A, T/U, C, G) located on the target nucleic acid. The term "degenerate base" as used herein includes, but is not limited to, a nucleotide base that does not follow Watson-Crick base pair rules but rather can bind to at least two of the four canonical bases A, T/U, C, G), but not all four. A degenerate base can also be termed a Wobble base; these terms are used interchangeably herein.

[00338] As used in this Specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

[00339] Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive and covers both "or" and "and".

[00340] Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term "about."

[00341] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although other probes, compositions, methods, and kits similar, or equivalent, to those described herein can be used in the practice of the present disclosure, the preferred materials and methods are described herein. It is to be understood that the terminology used herein is for the purpose of describing particular aspects only, and is not intended to be limiting.

EXAMPLES

[00342] Example 1—two-ended adapter ligation method for 96 multiplexed samples

[00343] In this example, a two-ended adapter ligation method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The nucleic acid adapters used in this experiment were partially double-stranded. The nucleic acid adapters comprised a first strand and a second strand. The first strand comprised a 5' phosphate moiety for ligation. The first strand also comprised a constant nucleic acid sequence to minimize ligation

bias (GCGTAGTG), a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a first amplification primer binding site (SEQ ID NO: 2). The second strand comprised a single overhanging thymine nucleotide at the 3' end, a sequence complementary to the constant nucleic acid sequence to minimize ligation bias present in the first strand, a sequence complementary to the unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released present in the first strand and a second amplification primer binding site (SEQ ID NO: 1).

[00344] To form the partially double stranded nucleic acid adapters, first strand oligonucleotides and second strand oligonucleotides were combined in equimolar proportion for a final total oligonucleotide concentration of 28 μM in buffer comprising 50 mM NaCl. The oligonucleotide mixture was heated at 95°C for 2 minutes and cooled at ambient temperature for 30 minutes, thereby annealing the first stand and second strand oligonucleotides together to form the partially double-stranded nucleic acid adapters. Annealed nucleic acid adaptors were diluted to final concentration ranging between 0.02 μM to 0.002 μM in a solution of 10 mM Tris pH 8 and 0.05% Tween20.

[00345] Collected identifier oligonucleotides were end repaired and A-tailed using NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) with a modified protocol. End repair/A-tail master mix was prepared by combining the following: 627.8 μL of PCR-grade H2O, 143.9 μL of NEBNext Ultra II End Prep Reaction Buffer, and 61.7 μL of NEBNext Ultra II End Prep Enzyme Mix. 8.3 μL of end repair/A-tail master mix was added to 4 μL of each sample of identifier oligonucleotides. The reaction was incubated for 30 minutes at 20°C with a heated lid of >75°C, followed by a second incubation for 30 minutes at 65°C. The repaired/A-tailed identifier oligonucleotide mixtures were then stored at 4°C.

[00346] Following end repair and A-tailing, the nucleic acid adaptors were ligated to the repaired/A-tailed identifier oligonucleotides by adding 6.4 μL of NEBNext Ultra II Ligation Master Mix, 0.2 μL of NEBNext Ligation Enhancer, and 1 μL of the nucleic acid adapter dilution to each repaired/A-tailed identifier oligonucleotide mixture. These reactions were incubated for 15 minutes at 20°C with the heated lid off and subsequently quenched with 1 μL

0.5M EDTA. All of the reactions were then pooled into a single 15 mL conical tube to form a pooled adapter-ligated sample.

[00347] The pooled adaptor-ligated sample was purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 μL of AMPure XP beads and 3.15 mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). AMPure XP bead cleanup was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20 to obtain purified adapter-ligated samples.

[00348] Following AMPure XP cleanup, PCR reactions with purified adaptor-ligated sample were prepared to amplify the adapter-ligated identifier oligonucleotides. To 6 μL of the purified adapter-ligated sample, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward and reverse primers, and 3.6 μL of PCR-grade H2O was added. The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 1 provides the sequences of the forward primers used.

Table 1. Forward primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCCTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTTAAGACGACGTCGCTATGGCCTCTCC	12

[00349] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second

amplification primer binding site located on the second strand of the nucleic acid adapter. Table 2 provides the sequences of the reverse primers used.

Table 2. Reverse primers for two-ended adapter ligation

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	14
CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	17
CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	22
CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	24

[00350] The optimal number of PCR cycles was determined empirically with triplicate PCR reactions. Alternately, the optimal number of PCR cycles could have been determined using real-time/qPCR. The PCR program used comprised the following steps:

(1) 30 seconds at 98°C

(2) 10 seconds at 98°C

(3) 1 minute at 65°C

(4) Repeating steps (2) and (3) nine times

(5) 5 minutes at 65°C

[00351] The amplified products were purified using 18 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00352] The amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC (SEQ ID NO: 25).

[00353] Example 2—one-ended adapter ligation method for 96 multiplexed samples

[00354] In this example, a one-ended adapter ligation method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The nucleic acid adapters used in this experiment were partially double-stranded. The nucleic acid adapters comprised a first strand and a second strand. The first strand comprised a 5' phosphate moiety for ligation. The first strand also comprised a constant nucleic acid sequence to minimize ligation bias (CACTACGC), a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a first amplification primer binding site (SEQ ID NO: 1). The second strand comprised a single overhanging thymine nucleotide at the 3' end, a sequence complementary to the constant nucleic acid sequence to minimize ligation bias present in the first strand and a sequence complementary to the unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released present in the first strand.

[00355] To form the partially double stranded nucleic acid adapters, first strand oligonucleotides and second strand oligonucleotides were combined in equimolar proportion for a final total oligonucleotide concentration of 28μM in 50 mM NaCl. The oligonucleotide mixture was heated to 95°C for 2 minutes and cooled at ambient temperature for 30 minutes, thereby annealing the first stand and second strand oligonucleotides together to form the partially double-stranded nucleic acid adapters. Annealed nucleic acid adaptors were diluted to a final concentration ranging between 0.02 μM to 0.002 μM in a solution of 10 mM Tris pH 8 and 0.05% Tween20.

[00356] Nucleic acid adapters were ligated to the collected identifier oligonucleotides by addition of 10 μL of 2X rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics),

and 1 μL of annealed nucleic acid adapter dilutions to each sample of collected identifier oligonucleotides. Samples were incubated for 15 minutes at 20°C with the heated lid off and subsequently quenched with 1 μL 0.5M EDTA. All of the reactions were then pooled into a single 15 mL conical tube to form a pooled adapter-ligated sample.

[00357] The pooled adaptor-ligated sample was purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 μL of AMPure XP beads and 3.15 mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). AMPure XP bead cleanup was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20 to obtain purified adapter-ligated samples.

[00358] Following AMPure XP cleanup, PCR reactions with purified adaptor-ligated sample were prepared to amplify the adapter-ligated identifier oligonucleotides. To 6 μL of the purified adapter-ligated sample, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward and reverse primers, and 3.6 μL of PCR-grade H_2O was added. The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 3 provides the sequences of the forward primers used.

Table 3. Forward primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCCTACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	12

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[00359] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 4 provides the sequences of the reverse primers used.

Table 4. Reverse primers for two-ended adapter ligation.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	14
CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	17
CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	22
CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	23
CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	24

[00360] The optimal number of PCR cycles was determined empirically with triplicate PCR reactions. Alternately, the optimal number of PCR cycles could have been determined using real-time/qPCR. The PCR program used comprised the following steps:

(1) 30 seconds at 98°C

(2) 10 seconds at 98°C

(3) 1 minute at 65°C

(4) Repeating steps (2) and (3) nine times

(5) 5 minutes at 65°C

[00361] The amplified products were purified using 18 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00362] The amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC (SEQ ID NO: 25).

[00363] Example 3—templated-primer extension method for 96 multiplexed samples

[00364] In this example, a templated-primer extension method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. The single stranded nucleic acid templates used in this example comprised a 3' biotin moiety, a region complementary to the unique nucleic acid sequences present in the collected identifier oligonucleotides, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding sequence (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT, SEQ ID NO: 26). Table 5 provides the sequences of the single-stranded nucleic acid templates used in this example.

Table 5. Single stranded nucleic acid templates for templated-primer extension method

Single-stranded nucleic acid templates	SEQ ID NO
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTTGAAGCACACCGTTTTTCTTTCTTCTTTCACGG	27
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNACCCACAGGTTATACGGGATTATCCGGTTATCCA	28
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCGACACCGAGTTCGACCGTTATGTTGGTAGGATC	29
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCGGTGTGTAAGCGTAACGATGTTGGTGTCGCTCT	30
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCAGACACTGCGACAACTCACGATCATGACACAGA	31
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNATATTCTGTACTCAGTGCCTATCCACCTAATAGG	32
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTTCAGTTATAATGTGTCCAGCAGAAGCAGGAATT	33
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNGTCCTTTGTTGGGCGGACCGTAATGAGGAATTTG	34
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNGATGAGACTTCTACATGTCCGATGTTTTTGTGCT	35

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNACTC ACACATAGTACTGACACGTAAGATAGGATG	36
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTTAC CCTATCTCGTCTATGTACGTCAGGCTGAAT	37
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNATCA ACGTAGGGTAAGGTCATATTTTTACCTTAC	38
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTTCC CTCTTTCTCCGCTTATGGATGAAAGGACAG	39
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCCTG CACAGTGAGTTTCTTTCACTCTAACTCTCT	40
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTGTC GCTCTAGTGTGACTTTTCCACCTCGCATCT	41
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNATAT CTTTCTCGGGTAAAGATTAGGCGTCCGATA	42
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCGAT TAGCCGTAGACGCAACTCATTGCCGAAGAT	43
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTGTG AGCATTTCAGTACGAGTGATGCAGATAAAC	44
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTATA GTTACCAAGTACTATGGGTTGGTGGAAGCC	45
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCCAA TTATACTGTCTGTTATGTTCTCGGATAAGC	46
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTCAG GTGCTTGTAGGCTCATGATAGGGGTAATGC	47
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCTCT GCTGTAATCTCAGCTCCACTTGTTTCTAAG	48
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNGTGC ATATTGCAGCTGAGCCAGCTCAATTTGAAG	49
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCCGT TGATTTACGCAACAGCGGCTTATATAGCTC	50
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCATC ATCGACAGTTCGCAGCCCTATAACATGATA	51
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNATCG CAGGATGGTACAGCATCATACATGATGAGC	52
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCTGA TAAGTCGTAGGAATGTCGCTTAATACGGAT	53
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNATGG CGGTTTCGGGTCCTGCACTATTCCTAATAA	54
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNCCAG TACGGGTACTAATAAGTGTCATATCTATTG	55
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNNNNNTGTT GGAGAGGTTAGAGGTGAGGAGGCGAAGATA	56

[00365] Single stranded nucleic acid templates were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Individual single stranded nucleic acid templates were normalized to a standard concentration

and then pooled to be equimolar. The pool of single stranded nucleic acid templates was diluted to 0.83 nM in a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00366] The collected identifier oligonucleotides were hybridized to the single stranded nucleic acid templates and extended by addition of 10 μL of NEBNext Ultra II Q5 Master Mix (New England Biolabs), 4 μL of the diluted single stranded nucleic acid template pool and 4 μL of H_2O to 2 μL of each sample of identifier oligonucleotides. The following PCR program was used to extend the identifier oligonucleotides:

(1) 30 seconds at 98°C, 10×

(2) 1 minutes at 98°C,

(3) 1 minutes at 68°C

(4) 1 minutes at 72°C

(5) Repeating steps (2)-(4) ten times

(6) 2 minutes at 72°C

[00367] The extension products were stored at 4°C. Magnetic streptavidin beads (MyOne Streptavidin C1 beads, Thermo Fisher Scientific) were washed in 1X Binding and Washing Buffer (5 mM Tris-HCl, 0.5 mM EDTA, 1M NaCl), and 5 μL of streptavidin beads were added to each extension product sample. The extension product samples were incubated with the beads on an orbital mixer for a minimum of 15 minutes. Following incubation, the samples were heated to 95°C for 3 minutes and transferred to a magnetic plate. Supernatant was extracted immediately after sufficient bead pelleting to yield the purified extension product samples.

[00368] The purified extension product samples were amplified by adding to 7.5 μL of each purified extension product sample, 12.5 μL of NEBNext Ultra II Q5 Master Mix, 0.25 μL of 100 μM forward primer, 1 μL of 25 μM reverse primer and 3.8 μL of PCR-grade H_2O. The forward primer comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the identifier oligonucleotide. Table 6 provides the sequences of the forward primers used in this example.

Table 6. Forward primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCCTACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTTA AGACGACGTCGCTATGGCCTCTCC	12

[00369] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located single-stranded nucleic acid template. Table 7 provides the sequences of the reverse primers used.

Table 7. Reverse primers for templated-primer extension method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATGTCGGTAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	57
CAAGCAGAAGACGGCATACGAGATAGGTCACTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	58
CAAGCAGAAGACGGCATACGAGATGAATCCGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	59
CAAGCAGAAGACGGCATACGAGATGTACCTTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	60
CAAGCAGAAGACGGCATACGAGATCATGAGGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	61
CAAGCAGAAGACGGCATACGAGATTGACTGACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	62
CAAGCAGAAGACGGCATACGAGATCGTATTCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	63
CAAGCAGAAGACGGCATACGAGATCTCCTAGAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	64
CAAGCAGAAGACGGCATACGAGATTAGTTGCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	65

CAAGCAGAAGACGGCATACGAGATGAGATACGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	66
CAAGCAGAAGACGGCATACGAGATAGGTGTACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	67
CAAGCAGAAGACGGCATACGAGATTAATGCCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	68
CAAGCAGAAGACGGCATACGAGATTCAGACGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	69
CAAGCAGAAGACGGCATACGAGATGATAGGCTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	70
CAAGCAGAAGACGGCATACGAGATTGGTACAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	71
CAAGCAGAAGACGGCATACGAGATCAAGGTCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	72
CAAGCAGAAGACGGCATACGAGATGCTATCCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	73
CAAGCAGAAGACGGCATACGAGATATGGAAGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	74
CAAGCAGAAGACGGCATACGAGATTCAAGGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	75
CAAGCAGAAGACGGCATACGAGATGTTACGCAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	76
CAAGCAGAAGACGGCATACGAGATAGTCTGTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	77
CAAGCAGAAGACGGCATACGAGATGCACGTAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	78
CAAGCAGAAGACGGCATACGAGATAACCTTGGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	79
CAAGCAGAAGACGGCATACGAGATATTGCGTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	80
CAAGCAGAAGACGGCATACGAGATACCTGGAAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	81
CAAGCAGAAGACGGCATACGAGATGGAGATGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	82
CAAGCAGAAGACGGCATACGAGATGTACTCTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	83
CAAGCAGAAGACGGCATACGAGATGTAACGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	84
CAAGCAGAAGACGGCATACGAGATATTCCTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	85
CAAGCAGAAGACGGCATACGAGATGTGTTCCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	86
CAAGCAGAAGACGGCATACGAGATAAGCACTGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	87
CAAGCAGAAGACGGCATACGAGATCTAGCAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	88
CAAGCAGAAGACGGCATACGAGATTGCTTCCAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	89
CAAGCAGAAGACGGCATACGAGATGCTTAGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	90

CAAGCAGAAGACGGCATACGAGATAACCGTTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	91
CAAGCAGAAGACGGCATACGAGATGACATTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	92
CAAGCAGAAGACGGCATACGAGATAGACCGTAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	93
CAAGCAGAAGACGGCATACGAGATGATACTGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	94
CAAGCAGAAGACGGCATACGAGATTGCGTAGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	95
CAAGCAGAAGACGGCATACGAGATTCGGTTACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	96
CAAGCAGAAGACGGCATACGAGATATGACGTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	97
CAAGCAGAAGACGGCATACGAGATGCTGTAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	98
CAAGCAGAAGACGGCATACGAGATGCAATGGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	99
CAAGCAGAAGACGGCATACGAGATATCTCGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	100
CAAGCAGAAGACGGCATACGAGATGGCTATTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	101
CAAGCAGAAGACGGCATACGAGATGGTGTCTTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	102
CAAGCAGAAGACGGCATACGAGATTCAACTGGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	103
CAAGCAGAAGACGGCATACGAGATCTTCACCAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	104
CAAGCAGAAGACGGCATACGAGATACGGTCTTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	105
CAAGCAGAAGACGGCATACGAGATTCTCGCAAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	106
CAAGCAGAAGACGGCATACGAGATGGAATTGCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	107
CAAGCAGAAGACGGCATACGAGATACGGATTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	108
CAAGCAGAAGACGGCATACGAGATTTAAGCGGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	109
CAAGCAGAAGACGGCATACGAGATTGCAGGTAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	110
CAAGCAGAAGACGGCATACGAGATCAATCGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	111
CAAGCAGAAGACGGCATACGAGATGTGCCATAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	112
CAAGCAGAAGACGGCATACGAGATTGTTCGAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	113
CAAGCAGAAGACGGCATACGAGATTGGAGTTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	114
CAAGCAGAAGACGGCATACGAGATACGATGACGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	115

CAAGCAGAAGACGGCATACGAGATTGATGTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	116
CAAGCAGAAGACGGCATACGAGATTGAACCTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	117
CAAGCAGAAGACGGCATACGAGATCTTCGTTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	118
CAAGCAGAAGACGGCATACGAGATCTTCTGAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	119
CAAGCAGAAGACGGCATACGAGATTGCTCATGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	120
CAAGCAGAAGACGGCATACGAGATAGTTCGTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	121
CAAGCAGAAGACGGCATACGAGATTAGCGTCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	122
CAAGCAGAAGACGGCATACGAGATGGCGTTATGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	123
CAAGCAGAAGACGGCATACGAGATGGTGATTCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	124
CAAGCAGAAGACGGCATACGAGATAACTTGCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	125
CAAGCAGAAGACGGCATACGAGATGCAAGATCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	126
CAAGCAGAAGACGGCATACGAGATTCGCATTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	127
CAAGCAGAAGACGGCATACGAGATTGTACACCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	128
CAAGCAGAAGACGGCATACGAGATAGCTCCTAGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	129
CAAGCAGAAGACGGCATACGAGATGCAATTCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	130
CAAGCAGAAGACGGCATACGAGATCTTAGGACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	131
CAAGCAGAAGACGGCATACGAGATGTCCTAAGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	132
CAAGCAGAAGACGGCATACGAGATAACGTGGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	133
CAAGCAGAAGACGGCATACGAGATCTGTGTTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	134
CAAGCAGAAGACGGCATACGAGATGTTAAGGCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	135
CAAGCAGAAGACGGCATACGAGATCACCTTACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	136
CAAGCAGAAGACGGCATACGAGATTGGTAGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	137
CAAGCAGAAGACGGCATACGAGATCAGTGAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	138
CAAGCAGAAGACGGCATACGAGATGTTCAACCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	139
CAAGCAGAAGACGGCATACGAGATTGGCTATCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	140

CAAGCAGAAGACGGCATACGAGATCTGGAGTAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	141
CAAGCAGAAGACGGCATACGAGATTCTCTTCCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	142
CAAGCAGAAGACGGCATACGAGATTCTAACGCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	143
CAAGCAGAAGACGGCATACGAGATGGTCAGATGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	144
CAAGCAGAAGACGGCATACGAGATCTCTGGTTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	145
CAAGCAGAAGACGGCATACGAGATTGTGGTACGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	146
CAAGCAGAAGACGGCATACGAGATCCTATACCGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	147
CAAGCAGAAGACGGCATACGAGATTTCTCTCGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	148
CAAGCAGAAGACGGCATACGAGATGTATGCTGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	149
CAAGCAGAAGACGGCATACGAGATAAGTCGAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	150
CAAGCAGAAGACGGCATACGAGATAACCGAAGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCT	151
CAAGCAGAAGACGGCATACGAGATTGTTGTGGGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	152

[00370] The PCR program used to amplify the purified extension products comprised the following steps:

(1) 30 seconds at 98°C

(2) 10 seconds at 98°C

(3) 30 seconds at 65°C

(4) 30 seconds at 72°C

(5) Repeat steps (2)-(4) eighteen times

(6) 2 minutes at 72°C

[00371] The amplified extension products were stored at 4°C. 4 μL of each PCR reaction were combined into four pools, for 24 samples per pool.

[00372] The pooled PCR reactions were purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 100 μL of AMPure XP beads and 400 μL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). Purification was performed with 76.8 μL of diluted AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. Beads were retained and cleanup process was repeated

with 24 μL of AMPure XP buffer and eluted in 20 μL of buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00373] The purified PCR products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified PCR products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer comprising the nucleotide sequence ACACTCTTTAAGACGACGTCGCTATGGCCTCTCC (SEQ ID NO: 25).

[00374] Example 4—long probe hybridization method for 96 multiplexed samples

[00375] In this example, a long probe hybridization method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, the first nucleic acid probe comprises a 5' phosphate moiety, a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a first amplification primer binding site comprising an i7 sequence (SEQ ID NO: 1), unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released and a P7 flow cell adapter sequence (SEQ ID NO: 4). The second nucleic acid probe comprises a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier, a second amplification primer binding site comprising and i5 sequence (SEQ ID NO: 2) and a P5 flow cell adapter sequence (SEQ ID NO: 3).

[00376] The first and second nucleic acid probes were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Individual nucleic acid probes were normalized to a standard concentration, pooled to be equimolar, and diluted to 0.83 nM in a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The nucleic acid probes and the identifier oligonucleotides were hybridized by combining 0.5 μL of diluted nucleic acid probe pool with 2 μL of a mixture of identifier oligonucleotides collected from a sample solution in a buffer comprising 50 mM NaCl. This mixture was heated for 2 minutes at 95°C and cooled for 30 minutes at ambient temperature to yield an annealed identifier oligonucleotide-nucleic acid probe mixture.

[00377] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were not adjacent and were not overlapping, a gap extension reaction was performed. To 2.5 μL of each annealed

identifier oligonucleotide-nucleic acid probe mixture, 3.8 μL of NEBNext Ultra II Q5 Master mix and 1.3 μL of PCR-grade H_2O was added. The mixture was then subjected to the following Gap extension temperature cycle:

(1) 30 seconds at 98°C

(2) 1 minute at 98°C

(3) 1 minute at 68°C

(4) 1 minute at 72°C,

(5) Repeat steps (2)-(4) ten times

(6) 2 minutes at 72°C

[00378] The gap extension products were then stored at 4°C. The first and second nucleic acid probes were then ligated together by adding to 1 μL of the gap extension product, 10 μL of 2X rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 8 μL of PCR-grade H_2O. These ligation reactions were incubated for 15 minutes 20°C, subsequently quenched with 1 μL 0.5M EDTA, and pooled into a single 15 mL conical tube.

[00379] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were adjacent and were not overlapping, nick repair ligation reaction was performed. To 2.5 μL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 10 μL of 2X rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 1 μL of PCR-grade H_2O was added. These ligation reactions were incubated for 15 minutes at 20°C, subsequently quenched with 1 μL of 0.5M EDTA, and pooled into a single 15 mL conical tube.

[00380] The pools of quenched ligation reactions were then purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 μL of AMPure XP beads and 3.15 mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). The purification was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00381] To amplify the purified ligation products, PCR reactions with purified ligation products and primers were prepared. To 6 μL of purified ligation product, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward primer (CAAGCAGAAGACGGCATACGA, SEQ ID

NO: 153) and reverse primer (AATGATACGGCGACCACCGA, SEQ ID NO: 154) and 3.6 μL of PCR-grade H_2O was added. The PCR program used to amplify the purified extension products comprised the following steps:

(1) 30 seconds at 98°C

(2) 10 seconds at 98°C

(3) 30 seconds at 65°C

(4) 30 seconds at 72°C

(5) Repeat steps (2)-(4) eighteen times

(6) 2 minutes at 72°C

[00382] The amplified products were stored at 4°C. 4 μL of each PCR reaction were combined into six pools, for 16 samples per pool. The amplified products were further purified using an AMPure XP bead cleanup with 64 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00383] The purified amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with either standard sequencing primers or a custom spike-in Read1 primer (SEQ ID NO: 25).

[00384] Example 5—short probe hybridization method for 96 multiplexed samples

[00385] In this example, a short probe hybridization method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, the first nucleic acid probe comprises a 5' phosphate moiety, a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a first amplification primer binding site comprising an i7 sequence (SEQ ID NO: 1) and a unique nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released. The second nucleic acid probe comprises a nucleic acid sequence complementary to a portion of the identifier oligonucleotide, a nucleic acid sequence comprising a unique molecular identifier and a second amplification primer binding site comprising an i5 sequence (SEQ ID NO: 2).

[00386] The first and second nucleic acid probes were ordered from Integrated DNA Technologies, Inc. and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher

Scientific). Individual nucleic acid probes were normalized to a standard concentration, pooled to be equimolar, and diluted to 0.83 nM in a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The nucleic acid probes and the identifier oligonucleotides were hybridized by combining 0.5 μL of diluted nucleic acid probe pool with 2 μL of a mixture of identifier oligonucleotides collected from a sample solution in a buffer comprising 50 mM NaCl. This mixture was heated for 2 minutes at 95°C and cooled for 30 minutes at ambient temperature to yield an annealed identifier oligonucleotide-nucleic acid probe mixture.

[00387] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were not adjacent and were not overlapping, a gap extension reaction was performed. To 2.5 μL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 3.8 μL of NEBNext Ultra II Q5 Master mix and 1.3 μL of PCR-grade H_2O was added. The mixture was then subjected to the following Gap extension temperature cycle:

(1) 30 seconds at 98°C

(2) 1 minute at 98°C

(3) 1 minute at 68°C

(4) 1 minute at 72°C,

(5) Repeat steps (2)-(4) ten times

(6) 2 minutes at 72°C

[00388] The gap extension products were then stored at 4°C. The first and second nucleic acid probes were then ligated together by adding to 1 μL of the gap extension product, 10 μL of 2X rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 8 μL of PCR-grade H_2O. These ligation reactions were incubated 15 min 20°C, quenched with 1 μL 0.5M EDTA, and pooled into a single 15 mL conical tube.

[00389] In the case in which the first and the second nucleic acid probes hybridized to the identifier oligonucleotide such that the first and the second nucleic acid probes were adjacent and were not overlapping, nick repair ligation reaction was performed. To 2.5 μL of each annealed identifier oligonucleotide-nucleic acid probe mixture, 10 μL of 2X rapid ligation buffer (Enzymatics), 1 μL of T4 DNA Rapid Ligase (Enzymatics), and 1 μL of PCR-grade H_2O was added. These ligation reactions were incubated for 15 minutes at 20°C, subsequently quenched with 1 μL of 0.5M EDTA, and pooled into a single 15 mL conical tube.

[00390] The pools of quenched ligation reactions were then purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 350 μL of AMPure XP beads and 3.15 mL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). The purification was performed with 3.5 mL of diluted AMPure XP beads and eluted in 200 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20. The AMPure XP bead cleanup was then repeated with 400 μL of AMPure XP beads and eluted in 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00391] To amplify the purified ligation products, PCR reactions with purified ligation products and primers were prepared. To 6 μL of purified ligation product, 10 μL of NEBNext Ultra II Q5 Master Mix, 0.2 μL of 100 μM forward primer and reverse primer and 3.6 μL of PCR-grade H_2O was added. . The forward primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the first amplification primer binding site located on the first strand of the nucleic acid adapter. Table 8 provides the sequences of the forward primers used.

Table 8. Forward primers for short probe hybridization method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	13
CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	14
CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	15
CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	16
CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	17
CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	18
CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	19
CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	20
CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	21
CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	22
CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	23

CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGT GCTCTTCCGATCT	24

[00392] The reverse primers comprised a flow cell adapter sequence suitable for sequencing, a unique molecular identifier and a nucleic acid sequence complementary to the second amplification primer binding site located on the second strand of the nucleic acid adapter. Table 9 provides the sequences of the reverse primers used.

Table 9. Reverse primers for short probe hybridization method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACA CGACGCTCTTCCGATCT	155
AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTAC ACGACGCTCTTCCGATCT	156
AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACA CGACGCTCTTCCGATCT	157
AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACA CGACGCTCTTCCGATCT	158
AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTAC ACGACGCTCTTCCGATCT	159
AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACA CGACGCTCTTCCGATCT	160
AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACA CGACGCTCTTCCGATCT	161
AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACA CGACGCTCTTCCGATCT	162

[00393] The PCR program used to amplify the purified extension products comprised the following steps:

(1) 30 seconds at 98°C

(2) 10 seconds at 98°C

(3) 30 seconds at 65°C

(4) 30 seconds at 72°C

(5) Repeat steps (2)-(4) eighteen times

(6) 2 minutes at 72°C

[00394] The amplified products were stored at 4°C. 4 μL of each PCR reaction were combined into six pools, for 16 samples per pool. The amplified products were further purified using an AMPure XP bead cleanup with 64 μL of AMPure XP beads and eluting with 20 μL of a buffer comprising 10 mM Tris pH 8 and 0.05% Tween20.

[00395] The purified amplified products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics) and KAPA Library Quantification Kit for Illumina Platforms (Kapa Biosystems). The purified amplified products were also diluted to 15 pM for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with either standard sequencing primers or a custom spike-in Read1 primer (SEQ ID NO: 25).

[00396] Example 6—direct PCR method for 96 multiplexed samples

[00397] In this example, a direct PCR method of the present disclosure was used to sequence identifier oligonucleotides collected from 96 multiplexed samples. In this example, 8 species of forward amplification primers and 12 species of reverse amplification primers were used. The forward primers comprised a P5 flow cell adapter (SEQ ID NO: 3), a nucleic acid sequence comprising a unique molecular identifier and a region complementary to a first amplification primer binding site present on the identifier oligonucleotide. Table 10 provides the sequences of the forward amplification primers used.

Table 10. Forward amplification primers for direct PCR method.

Primer Sequence	SEQ ID NO
AATGATACGGCGACCACCGAGATCTACACGCTCAGATATAGCCTACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	5
AATGATACGGCGACCACCGAGATCTACACGCTCAGAATAGAGGCACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	6
AATGATACGGCGACCACCGAGATCTACACGCTCAGACCTATCCTACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	7
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGGCTCTGAACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	8
AATGATACGGCGACCACCGAGATCTACACGCTCAGAAGGCGAAGACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	9
AATGATACGGCGACCACCGAGATCTACACGCTCAGATAATCTTAACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	10
AATGATACGGCGACCACCGAGATCTACACGCTCAGACAGGACGTACACTCTT TAAGACGACGTCGCTATGGCCTCTCC	11
AATGATACGGCGACCACCGAGATCTACACGCTCAGAGTACTGACACACTCTTT AAGACGACGTCGCTATGGCCTCTCC	12

[00398] The reverse primers comprised a P7 flow cell adapter (SEQ ID NO: 4), a nucleic acid sequence comprising a unique molecular identifier and a region complementary to a second amplification primer binding site present on the identifier oligonucleotide. Table 11 provides the sequences of the reverse amplification primers used.

Table 11. Reverse amplification primers for direct PCR method.

Primer Sequence	SEQ ID NO
CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	163
CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	164
CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	165
CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	166
CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	167
CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	168
CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	169
CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	170
CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	171
CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	172
CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	173
CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGC TCTTCCGATCTNNNNNNNNNNNNNNAACGGACAGGATGCAGCAAAAT	174

[00399] In the case of 8 forward amplification primers and 12 reverse amplification primers, when the unique molecular identifiers from a pair of forward and reverse primers are combined, a total of 96 unique combinations can be obtained, allowing for the multiplexing of 96 samples.

[00400] To amplify the collected identifier oligonucleotides for sequencing, PCR reactions with collected identifier oligonucleotides and forward and reverse amplification primers were prepared on a 96-well plate with 2 μL of each identifier oligonucleotide sample, 10 μL of NEBNext Ultra II Q5 Master Mix, 2 μL of 10 μM forward amplification primer, 2 μL of reverse amplification primer, and 4 μL of PCR-grade H_2O. Each well in the 96-well plate contained an identifier oligonucleotide sample and a unique combination of forward and reverse amplification primers. The PCR program used comprised the following steps:

(1) 30 seconds at 98°C

(2) 10 seconds at 98°C

(3) 30 seconds at 65°C

(4) 30 seconds at 72°C

(5) Repeat steps (2)-(4) six to ten times

(6) 2 minutes at 72°C

[00401] The amplified products were stored at 4°C. 10 μL of each PCR reaction was combined into a single 15 mL conical tube.

[00402] The pooled PCR reactions were purified using diluted Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics Inc.), which were prepared by combining 115.2 μL of AMPure XP beads and 1036.8 μL of AMPure XP buffer (2.5M NaCl, 20% PEG8000). Purification was performed with 1152 μL of diluted AMPure XP beads and eluted in 60 μL of a buffer comprising 10 mM Tris pH 8. The purification process was repeated with 60 μL of AMPure XP beads and eluted in 70 μL of a buffer comprising 10 mM Tris pH 8.

[00403] Following AMPure XP cleanup, PCR reactions with universal primers were prepared with 9 μL of pooled direct PCR product, 15 μL of NEBNext Ultra II Q5 Master Mix, 3 μL of 10 μM universal P7 primer (SEQ ID NO: 153) and 2 μL of 10 μM universal P5 primer (SEQ ID NO: 154). The PCR program used was:

(1) 30 seconds at 98°C,

(2) 10 seconds at 98°C

(3) 30 seconds at 65°C

(4) 30 seconds at 72°C

(5) Repeat steps (2)-(4) 15 to 24 times

(6) 2 minutes at 72°C

[00404] Two rounds of AMPure XP bead cleanup was performed. The first round was performed with 30 μL of beads and eluted with 20 μL of a buffer comprising 10 mM Tris pH 8 and the second round was performed with 20 μL beads and eluted with 11 μL of a buffer comprising 10 mM Tris pH 8.

[00405] These purified PCR products were assessed using a High Sensitivity DNA chip on 2100 Bioanalyzer (Agilent Genomics). The purified PCR products were also diluted for sequencing on a MiSeq (Illumina) according to manufacturer's instructions (MiSeq Reagent Kit v3 2×75 bp) with a custom spike-in primer (SEQ ID NO: 25).

[00406] Example 7—spatially detecting target analytes in a FFPE sample

[00407] The methods of the present invention were used to spatially detect a plurality of different target analytes, including target proteins and target RNAs, in a sample of inflamed human tonsil tissue FFPE section.

[00408] In one experiment, 30 different target proteins were spatially detected using the methods of the present disclosure in two serial sections cut from the inflamed human tonsil tissue FFPE section. The 30 target proteins are put forth in Table 12. The 30 target proteins included IgG Rabbit isotype and IgG Mouse isotype as negative controls, as these target proteins should not have been present in the inflamed human tonsil sample and therefore should not have been detected.

Table 12. Target Proteins

Target Protein	Target Protein
AKT	FOXP3
B7-H3	GZMB
Bcl-2	Histone H3
Beta-2-microglobulin	Ki67
Beta-catenin	CD20
CD14	P-AKT
CD19	PanCK
CD3	PD1
CD4	PD-L1
CD44	S6
CD45	STAT3
CD45RO	P-STAT3
CD56	VISTA
CD68	IgG Rabbit isotype (negative control)
CD8A	IgG Mouse isotype (control)

[00409] To spatially detect the 30 target proteins, 30 different probes of the present disclosure were used. Each probe comprised a target binding domain comprising an antibody that specifically binds to one of the 30 target proteins in Table 12. The two serial sections were contacted with a plurality of the 30 different probes. Ninety-six regions of interest (ROI) were then identified. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a short probe hybridization method of the present disclosure, thereby spatially detecting the 30 target proteins in the two serial sections. As shown

NanoString EX2011.118

in Figures 21A-21D, the number of reads per target protein in each ROI for the two serial sections were well correlated, demonstrating that the method yields reproducible results.

[00410] In a second experiment, 20 different target RNAs were spatially detected using the methods of the present disclosure in two different serial sections cut from the inflamed human tonsil tissue FFPE section. The 20 different target RNAs are put forth in Table 13. The 20 target RNAs included 6 negative controls(Negative Probe) that should not have been detected in the sample.

Table 13. Target RNAs

Target RNA	Target RNA	Target RNA
CD3E	CD40	CTLA4
CD3G	CD45	GAPDH
CD4	CD74	KRT13
CD20	CD79A	PD1
PSA	RP56	Negative Probe #1
Negative Probe #2	Negative Probe #3	Negative Probe #4
Negative Probe #5	Negative Probe #6	

[00411] To spatially detect the 20 Target RNAs, 20 different probes of the present disclosure were used. Each probe comprised a target binding domain comprising a nucleic acid sequence complementary to at least one portion of one of the 20 target RNAs. The two serial sections were contacted with a plurality of the 20 different probes. Ninety-six regions of interest (ROI) were then identified. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a direct PCR method of the present disclosure, thereby spatially detecting the 20 target RNAs in the two serial sections. As shown in Figures 22A-22D, the number of reads per target RNA in each ROI for the two serial sections were well correlated, demonstrating that the method yields reproducible results.

[00412] Example 8—spatially detecting target proteins in a fluorescently stained FFPE sample

[00413] In another experiment, a 5 μm FFPE section of inflamed human tonsil tissue was stained with 4 fluorescent visualization markers: (1) CD3E, a T-cell marker; (2) PanCK, an epithelial cell marker; (3) Ki-67, a proliferation marker; and (4) SYTO83, a DNA stain, as shown in the left panel of Figure 23. The stained FFPE section was then contacted with the probes directed

against 30 target proteins, as described in Example 7. As shown in the left panel Figure 23, 96 regions of interest (ROIs) were selected. Each ROI was a circle with a 500 μm diameter. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a short probe hybridization method of the present disclosure, thereby spatially detecting the 30 target proteins in the FFPE section. As shown in the right panel of Figure 23, PanCK, CD3E and Ki67 were spatially detected in ROIs that correlated with their fluorescent visualization markers. Thus, the results generated by the methods of the present disclosure correlate with the results generated using established immunohistochemical method.

[00414] Example 9—spatially detecting target RNAs in a FFPE sample

[00415] In another experiment, a 5 μm section from an inflamed human tonsil tissue FFPE block was contacted with probes directed against 20 target RNAs, as described in Example 7. 96 regions of interest (ROIs) were then selected. Each ROI was a circle with a 500 μm diameter. For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a direct PCR method of the present disclosure, thereby spatially detecting the 20 target RNAs in the two serial sections. The total RNA from a 20 μm section from the same inflamed human tonsil tissue FFPE block was then isolated. The total RNA was analyzed using the NanoString nCounter® system. Figure 24 shows that the average number of counts for 11 different RNA targets recorded using the methods of the present disclosure were well correlated with the average number of counts for the same 11 different RNA targets recorded using the nCounter® system. Thus, the results generated using the methods of the present disclosure correlate with the results generated using established direct detection methods.

[00416] Example 10—spatially detecting target RNAs in specific sub-regions of an ROI

[00417] In another experiment, a 5 μm section from an inflamed human tonsil tissue FFPE block was contacted with probes directed against 30 target proteins, as described in Example 7. The same 5 μm section was also stained with 4 fluorescent visualization markers: (1) CD3E, a T-cell marker; (2) PanCK, an epithelial cell marker; (3) Ki-67, a proliferation marker; and (4) SYTO83, a DNA stain. As shown in Figure 25, 48 regions of interest (ROIs) were identified. For each ROI, two sub-regions were then identified based on the fluorescent staining. Areas of an ROI that were fluorescently stained positive for PanCK (PanCK+) were designated a "tumor" sub-

region and the areas of an ROI that lacked PanCK fluorescent staining were designated a "micro-environment" sub-region, as shown in Figure 25. For each ROI, the tumor sub-region and the micro-environment sub-region were separately illuminated with UV light to release the identifier oligonucleotides from the probes bound within each sub-region by creating a custom mask based on the intensity of PanCK fluorescent staining. The released identifier oligonucleotides were also separately collected. The collected identifier oligonucleotides were then analyzed using the short-probe hybridization method of the present disclosure and the NanoString nCounter® system. As shown in the bottom panel of Figure 25, the results using the NanoString nCounter® system and the short-probe hybridization method of the present disclosure were well correlated. Furthermore, in the tumor sub-regions, PanCK was detected at a significantly higher level as compared to the micro-environment sub-regions. Thus, the spatial detection results provided by the methods of the present disclosure are consistent with established fluorescent immunohistochemical methods and allows for the spatial detection within highly specific regions of a sample.

[00418] Example 11—96-plex human immuno-oncology panel

[00419] A 96-plex human immuno-oncology panel was designed for use in the direct-PCR methods of the present invention. The panel comprised a plurality of probes that could be used to spatially detect 96 different human target RNAs using the direct-PCR methods of the present disclosure. The 96 target RNAs are shown in Table 14.

Table 14. Target RNAs

Target	Target	Target	Target	Target	Target	Target	Target
AKT1	CD3E	CEACAM8	FOXP3	IFNG	KRT1	NCAM1	SOD2
ARG1	CD4	CMKLR1	GZMB	IFNGR1	KRT10	NKG7	SOX10
B2M	CD40	CSF1R	H3F3A	IL10	KRT14	NT5E	STAT1
BATF3	CD40LG	CTLA4	HAVCR2	IL12B	KRT17	PDCD1	STAT2
BCL2	CD44	CTNNB1	HIF1A	IL15	KRT18	PDCD1LG2	STAT3
BCL2L1	CD47	CXCL10	HLA_DQA1	IL1B	KRT19	PECAM1	TBX21
CCL5	CD68	CXCL9	HLA_DRB	IL6	KRT6A	PMEL	TIGIT
CCND1	CD74	CSCR6	HLA_E	ITGAM	KRT7	PSMB10	TNF
CD14	CD86	DKK2	ICAM1	ITGAV	LAG3	PTEN	TNFRSF9
CD27	CD8A	EPCAM	ICOSLG	ITGAX	LY6E	PRTPRC	TNFSF4

CD274	CEACAM1	FAS	IDO1	ITGB2	MKI67	RPS6	VEGFA
CD276	CEACAM6	FASLG	IFNAR1	ITGB8	MS4A1	S100B	VSIR

[00420] In total, the panel comprised 928 different probes. Each of the probes comprised an identifier oligonucleotide comprising a first amplification primer binding site, a nucleic acid sequence comprising a unique molecular identifier, a unique nucleic acid sequence which identified the target RNA bound to the target binding domain and a second amplification primer binding site. Figure 26 shows a schematic of the probes used in the panel. For each of the 96 target RNAs, there was at least one probe within the 928 probe set comprising a target binding domain that directly or indirectly hybridized to that target RNA. For most of the 96 target RNAs, there were 10 different probes that directly or indirectly hybridized to the specific target RNA. These 10 different probes directly or indirectly hybridized to different locations on the target RNA to create a "tiling" effect, as shown in the top panel of Figure 27. Tiling multiple probes onto a target RNA means that each target RNA will be individually detected multiple times, increasing the overall accuracy of the measurement. For example, as shown in the bottom panel of Figure 27, in the case where 10 probes are tiled onto a single target RNA, one of the probes may be incorrectly detected too many times (outlier high count probe), while another probe may be incorrectly detected too few times (outlier low count probe). However, the other 8 probes may be detected at a similar level, indicating that the two outliers should be discarded during analysis and the signals from the 8 probes used to generate a more accurate measurement of the abundance of the target RNA.

[00421] The set of 928 probes also comprised 80 negative control probes. Each of the 80 negative control probes comprised a target binding domain comprising a scrambled, non-specific nucleic acid sequence that was designed using guidelines from the External RNA Controls Consortium such that the target binding domain should not be complementary to RNA molecules present within a human sample. Thus, these 80 negative control probes should not be detected during analysis.

[00422] The 96-plex human immune-oncology panel was used to analyze a tissue microarray. The tissue microarray comprised FFPE samples of 22 common human cell lines, including normal and cancerous cell types. Some of the cell lines are shown in Table 15.

[00423] Table 15. Cell lines

Cell Line	Cell Line	Cell Line	Cell Line
CCRF-CEM	DAUDI	H596	H2228
HT29	HUT78	HUH7	JURKAT
M14	MDA-MB-468	MOLT4	RAJI
SKBR3	SUDHL1	SUDHL4	

[00424] The tissue microarray also comprised one mouse cell line (3T3) as a negative control. Each of the FFPE samples on the microarray was contacted with a plurality of the 928 different probes in the imunno-onocology panel. As shown in Figure 28, for each of the FFPE samples, at least three circular regions of interest (ROIs) with a diameter of 300 μm were selected. As a negative control, ROIs were also selected on regions of the microarray that did not comprise a FFPE sample (glass negative control). For each ROI, the ROI was illuminated with UV light to release the identifier oligonucleotides from the probes bound within the ROI. The released identifier oligonucleotides were then collected and identified using a direct PCR method of the present disclosure thereby spatially detecting the 96 target RNAs in each of the FFPE samples on the tissue microarray.

[00425] Figure 29 shows that a sufficient read depth was achieved using a MiSeq v3 flowcell. The top panel of Figure 30 shows that none of the target RNAs were spatially detected for the glass negative control ROIs. Likewise, the bottom panel of Figure 30 shows that nearly none of the target RNAs were detected in the negative control mouse 3T3 FFPE sample. Conversely, as shown in Figures 31, 33 and 34, specific target RNAs were successfully detected in the HEK293 (human embryonic kidney) FFPE sample and the Jurkat (human T-cell lymphocyte) FFPE sample. Figures 31, 33 and 34 show that clusters of "tiled" probes were detected for particular target RNAs, including AKT1, B2M, CD3E, HIF1A, PTEN, RPS6, STAT1, STAT2, STAT3, VEGF, PTPRC (CD45), and KRT1/10/18/19. These results indicated that there are certain target RNAs that are differentially transcribed in the two different cells lines. The results of this experiment were also verified using the NanoString nCounter system to identify the collected identifier oligonucleotides. As shown in Figure 27, the results using the direct-PCR method of the present disclosure were consistent with the results obtained using the NanoString nCounter system.

What is claimed is:

1. A method for spatially detecting at least one target analyte in a tissue sample comprising:

a) contacting the tissue sample with probes comprising a target binding domain that hybridizes to the at least one target analyte;

b) collecting the probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample;

c) collecting the probes, or portions thereof, bound to the at least one target analyte in an at least second location of the tissue sample;

d) performing an extension reaction to form extension products comprising the probes, or portions thereof, collected in step (b) and at least one nucleic acid sequence that identifies the first location of the tissue sample;

e) performing an extension reaction to form extension products comprising the probes, or portions thereof, collected in step (c) and at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and

f) identifying the extension products produced in step (d) and step (e) by sequencing, thereby spatially detecting the at least one target analyte in the first and the at least second location of the tissue sample.

2. The method of claim 1, wherein contacting the tissue sample with probes in step (a) comprises contacting the at least one target analyte with at least two probes, thereby tiling the probes on the at least one target analyte.

3. The method of claim 2, wherein step (a) further comprises ligating the at least two probes.

4. The method of claim 1, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.

5. The method of claim 4, wherein the tissue sample has been treated to facilitate the binding of the probes to the at least one target analyte.

6. The method of claim 1, wherein the tissue sample is immobilized onto a microscope slide.

7. The method of claim 6, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.

8. The method of claim 7, wherein the primers in the plurality of primers are immobilized on the microscope slide at their 5' ends.

9. The method of claim 8, wherein performing the extension reaction of step (d) and step (e) comprises performing solid-phase amplification, wherein the solid-phase amplification is carried out in association with the microscope slide using the plurality of primers immobilized on the microscope slide.

10. The method of claim 1, wherein the sequencing is next generation sequencing.

11. The method of claim 1, wherein the sequencing comprises amplifying a library using the extension products as templates.

12. The method of claim 1, wherein the extension products of step (d) further comprise at least one unique molecular identifier.

13. The method of claim 1, wherein the extension products of step (e) further comprise at least one unique molecular identifier.

14. The method of claim 2, wherein one of the at least two probes comprises an amplification primer binding site.

15. The method of claim 1, wherein the extension products of step (d) further comprise at least one amplification primer binding site.

16. The method of claim 1, wherein the extension products of step (e) further comprise at least one amplification primer binding site.

17. The method of claim 1, wherein the target binding domains comprise a single-stranded nucleic acid molecule.

18. The method of claim 1, wherein steps (b) and (c) are performed simultaneously.

19. The method of claim 1, wherein steps (d) and (e) are performed simultaneously.

ABSTRACT OF THE INVENTION

The present invention relates to, among other things, probes, compositions, methods, and kits for simultaneous, multiplexed detection and quantification of protein and/or nucleic acid expression in a user-defined region of a tissue, user-defined cell, and/or user-defined subcellular structure within a cell that are adaptable for use with existing sequencing technologies.

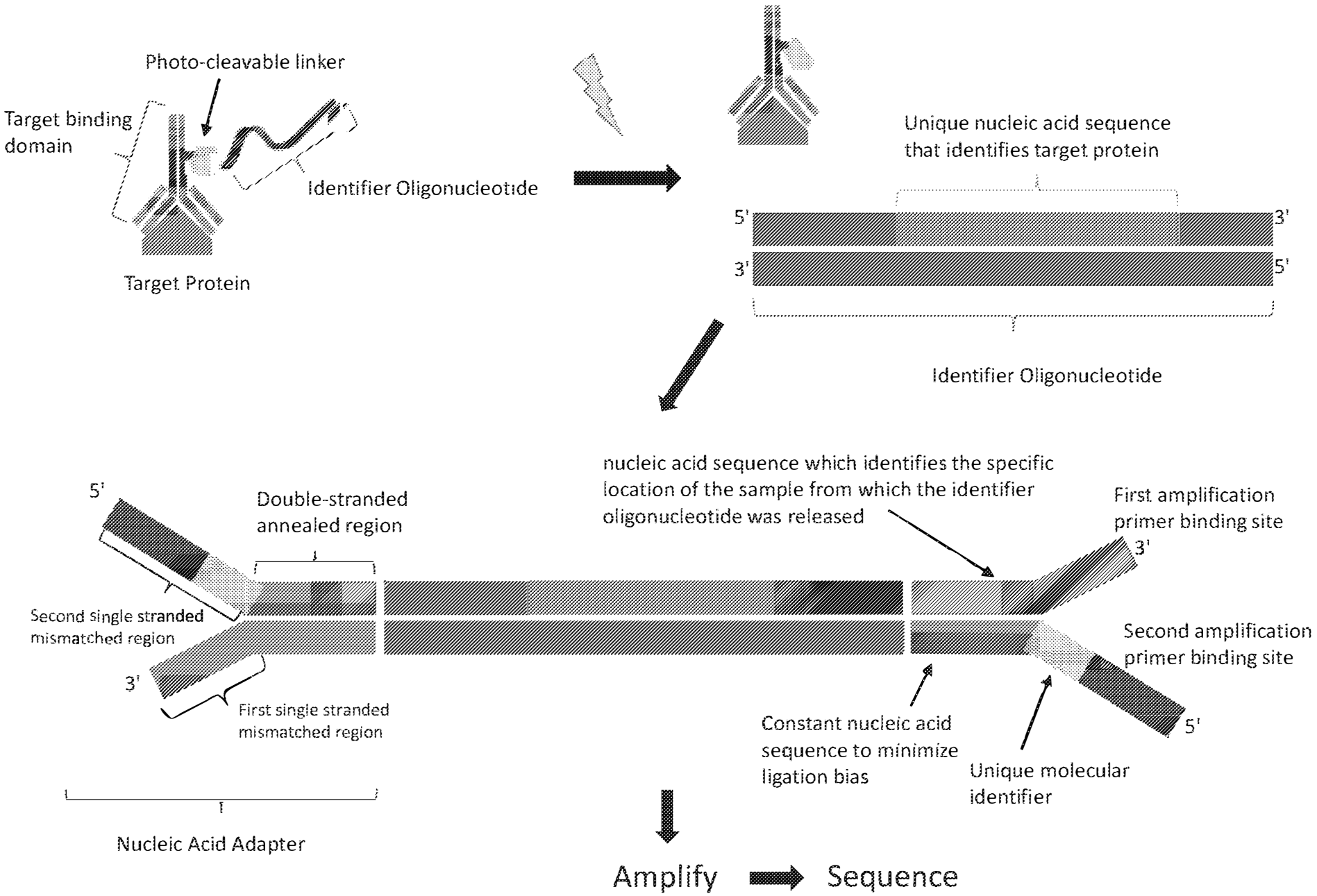


FIG. 1

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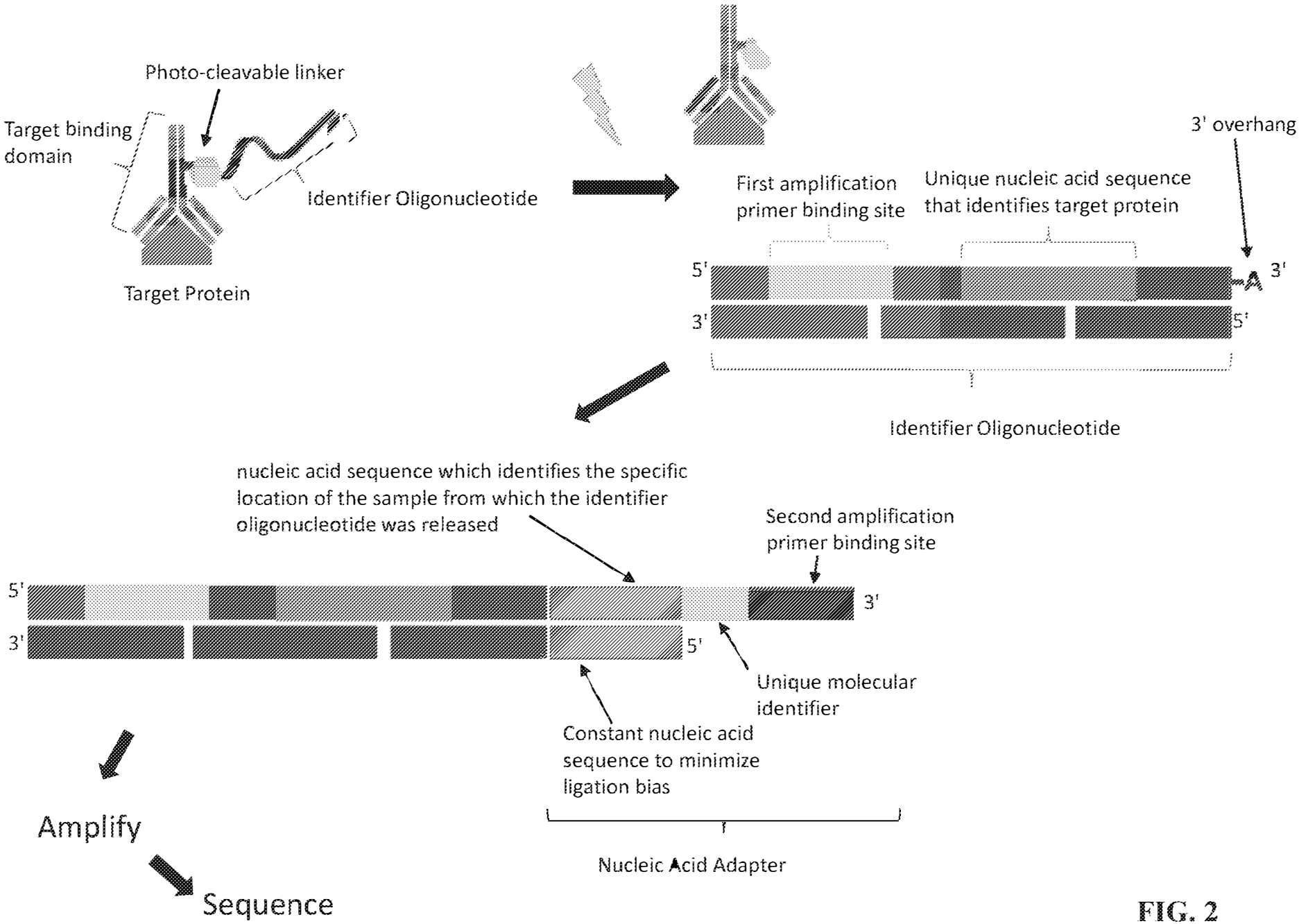

Photo-cleavable linker
Target binding domain
Identifier Oligonucleotide
Target Protein
3' overhang
First amplification primer binding site
Unique nucleic acid sequence that identifies target protein
5'
3'
A
3'
5'
Identifier Oligonucleotide
nucleic acid sequence which identifies the specific location of the sample from which the identifier oligonucleotide was released
Second amplification primer binding site
5'
3'
3'
5'
Unique molecular identifier
Constant nucleic acid sequence to minimize ligation bias
Nucleic Acid Adapter
Amplify
Sequence

FIG. 2

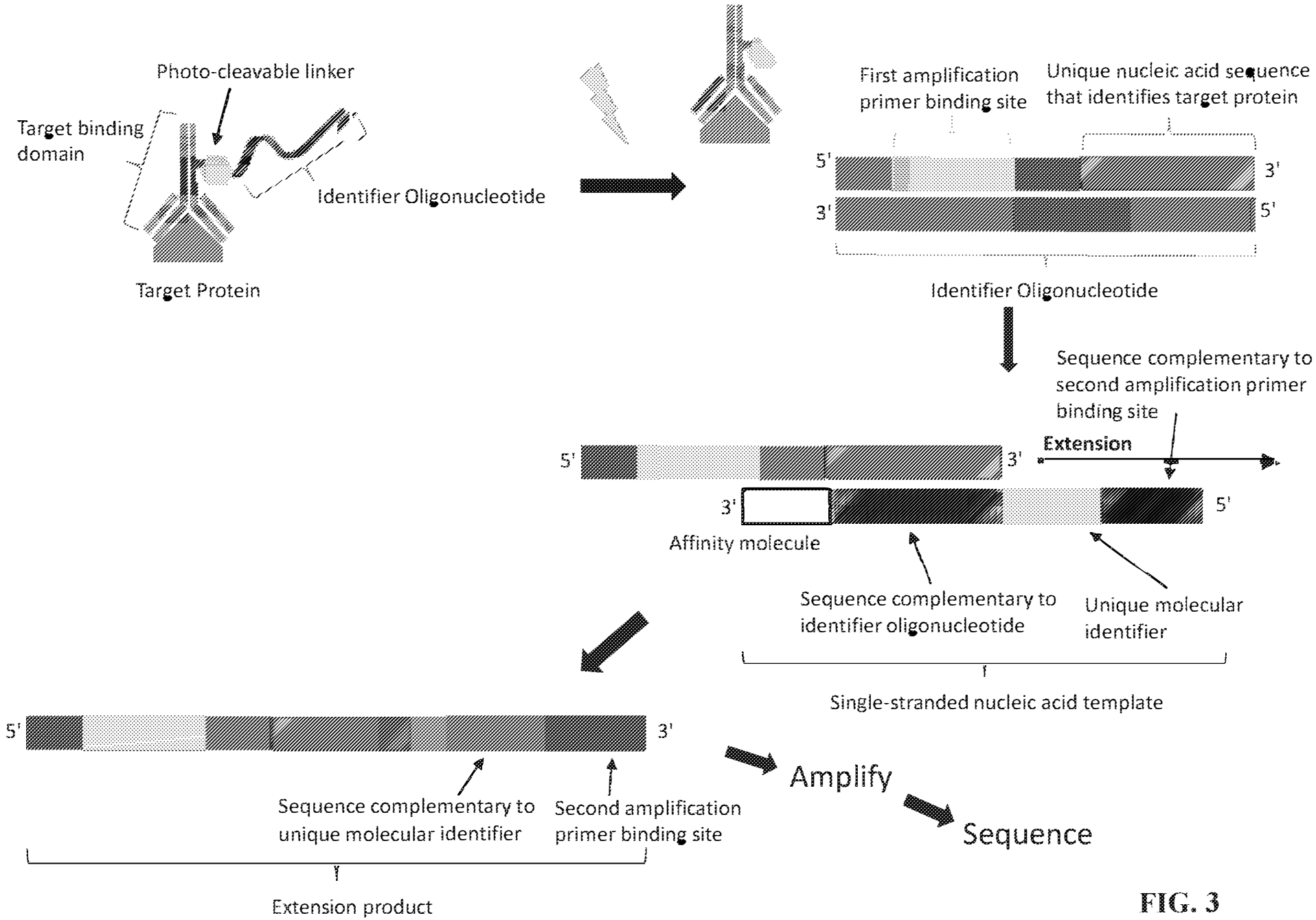


FIG. 3

NanoString EX2011.130

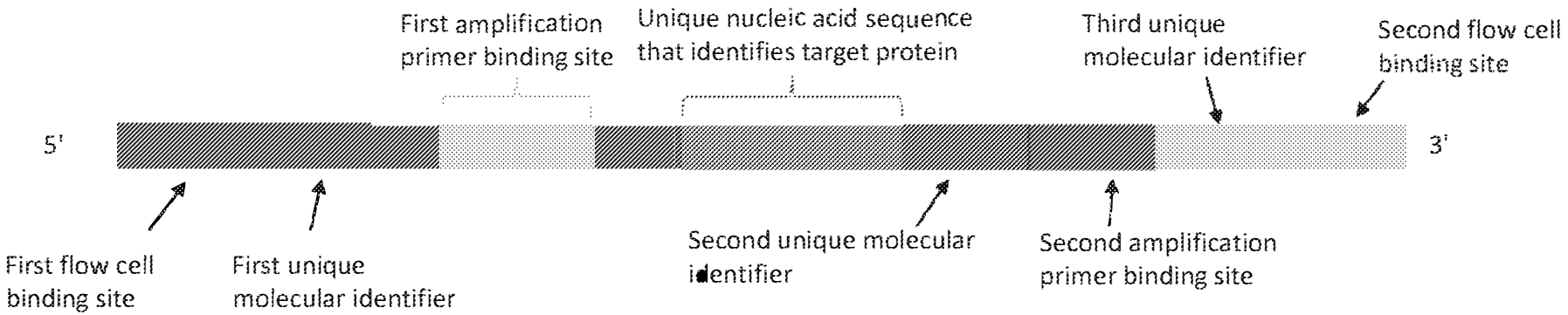


FIG. 4

NanoString EX2011.131

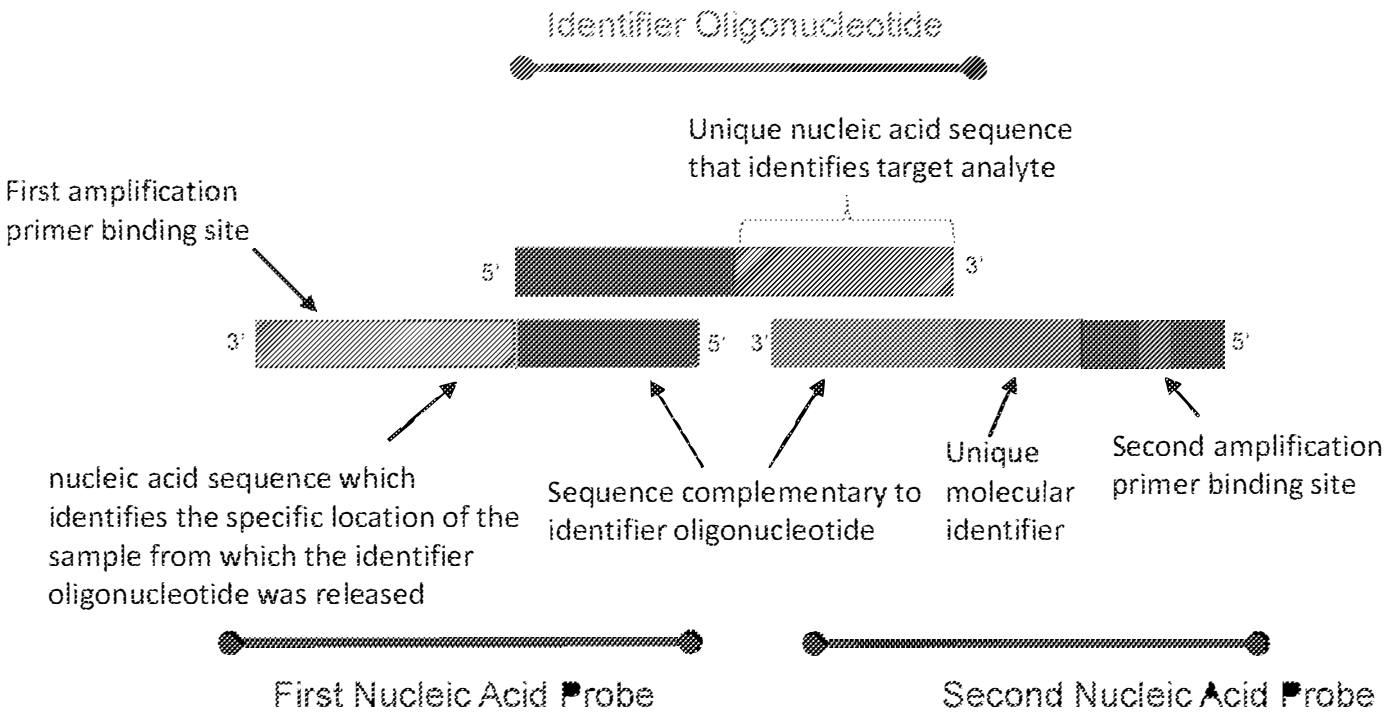


FIG. 5

NanoString EX2011.132

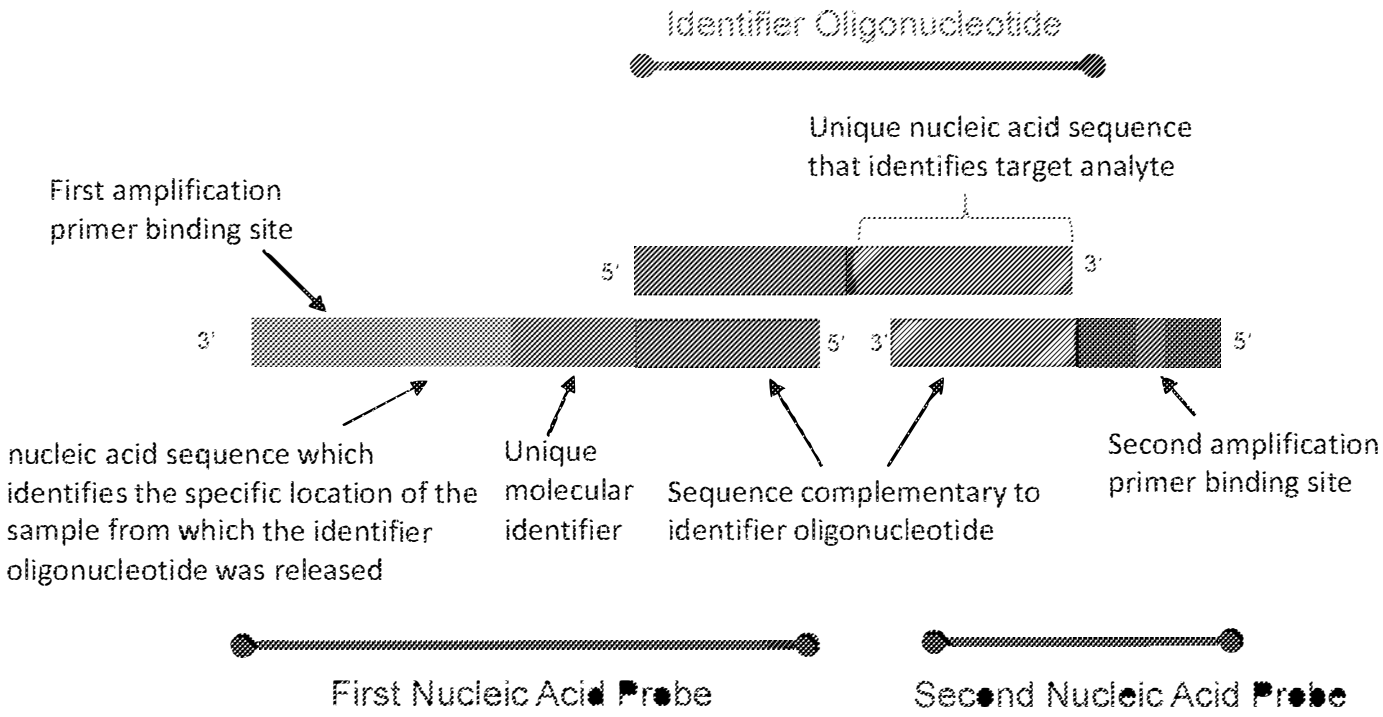


FIG. 6

NanoString EX2011.133

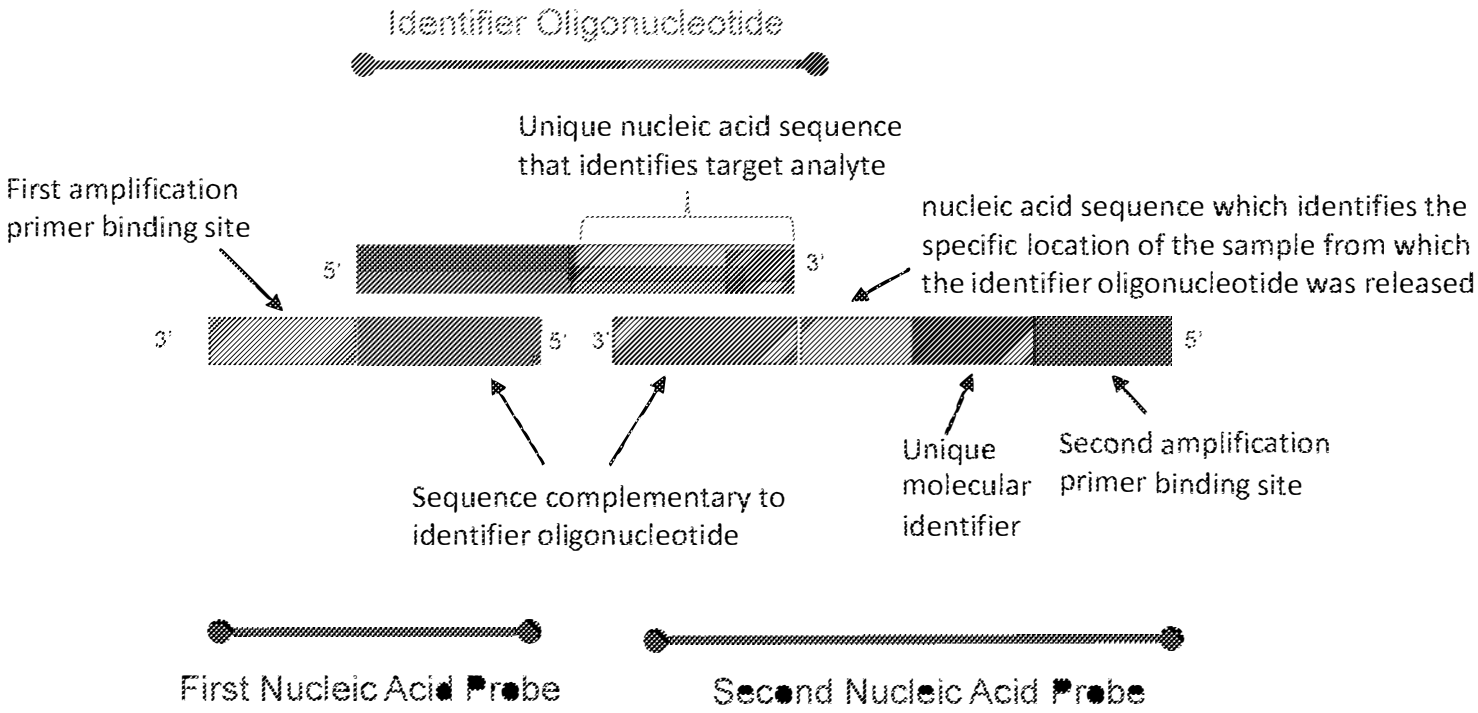


FIG. 7

NanoString EX2011.134

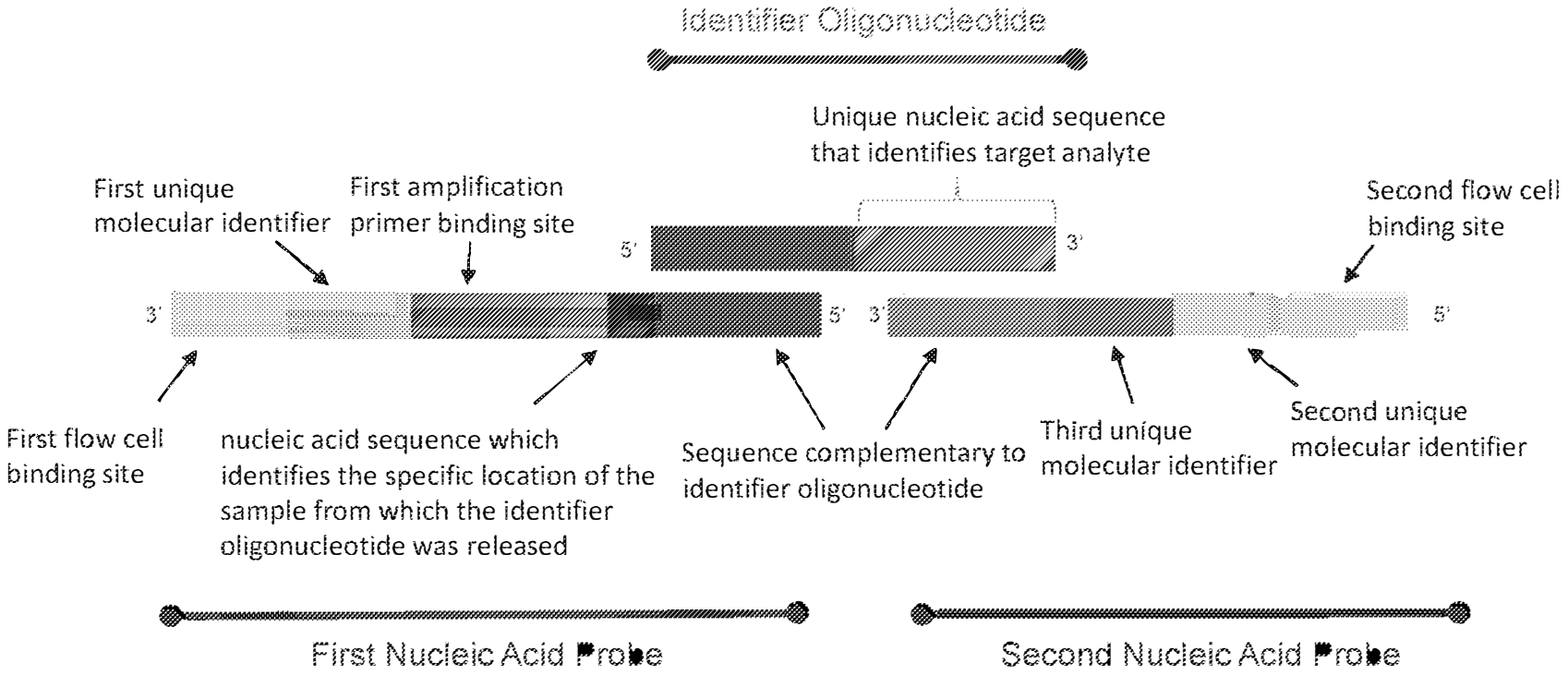


FIG. 8

NanoString EX2011.135

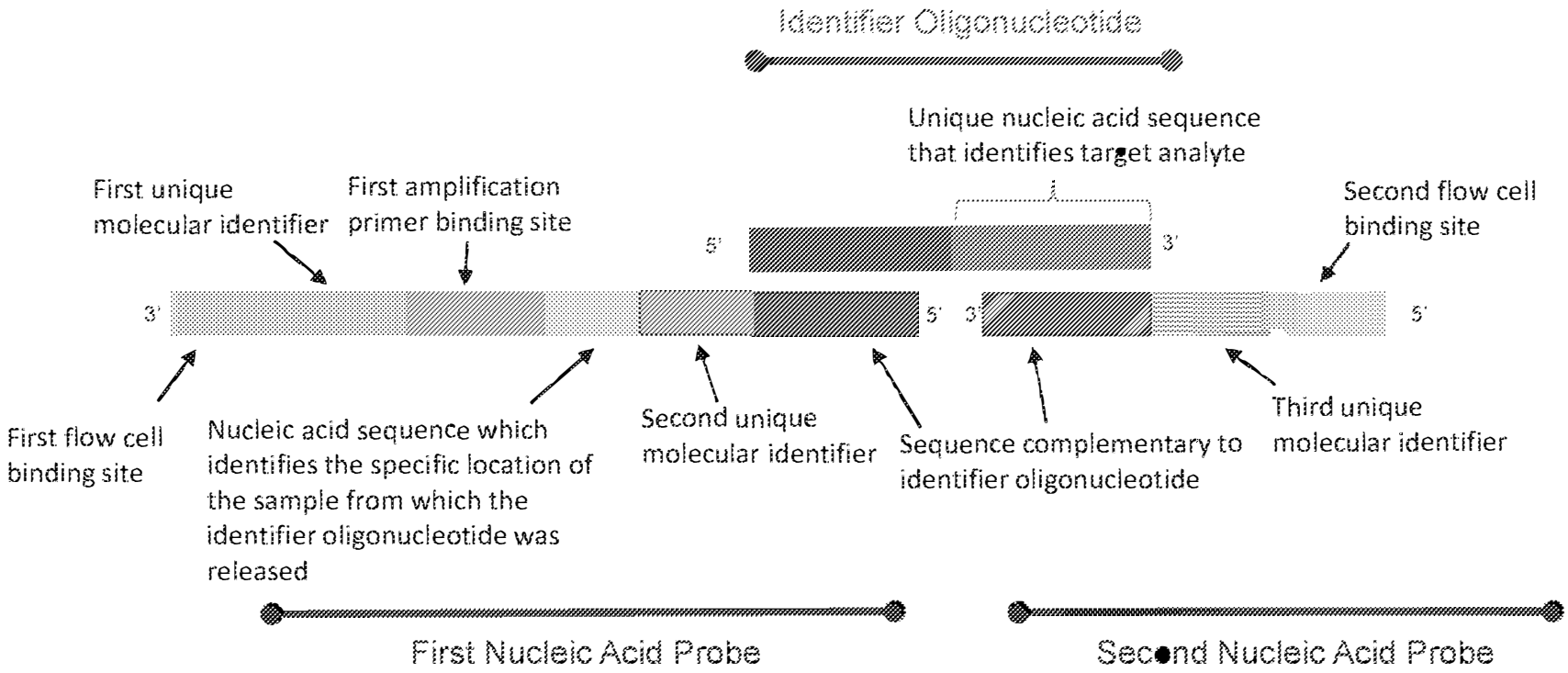


FIG. 9

NanoString EX2011.136

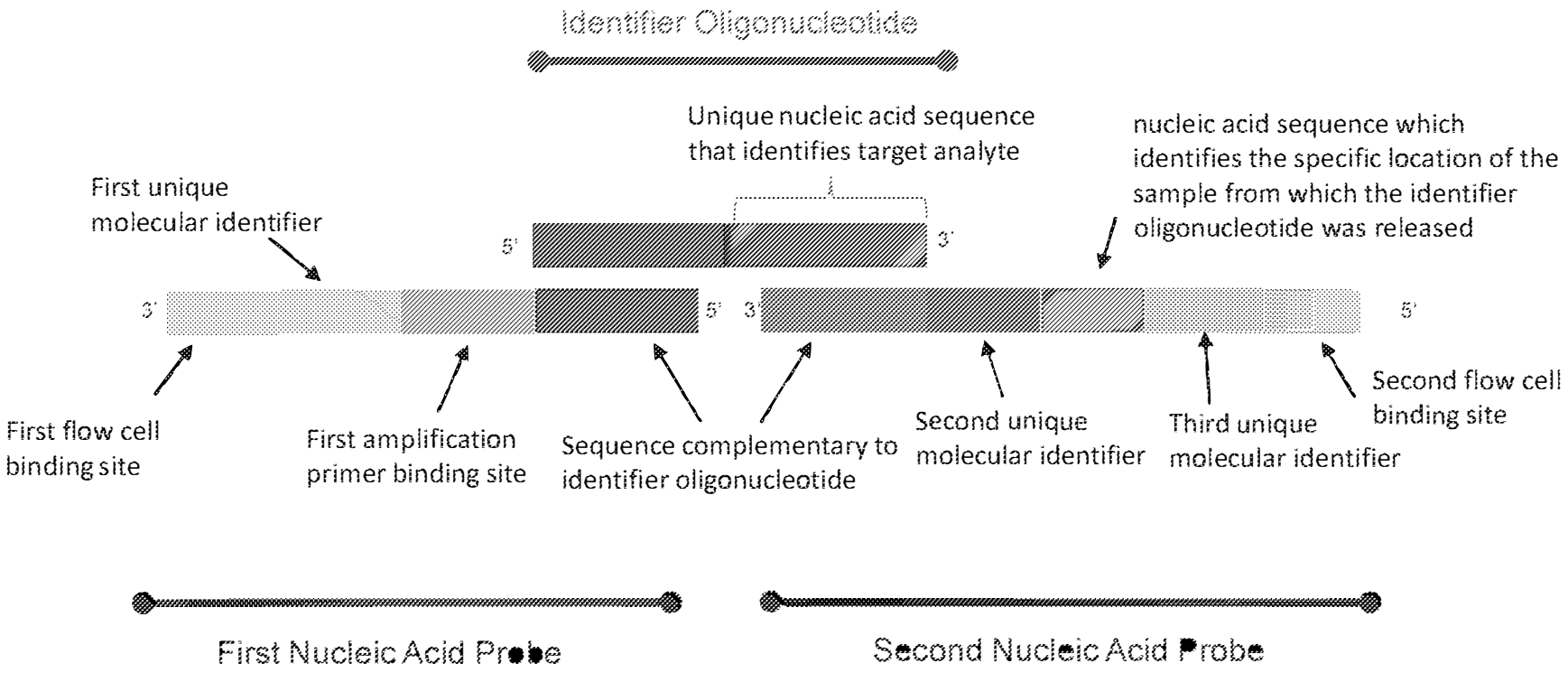


FIG. 10

NanoString EX2011.137

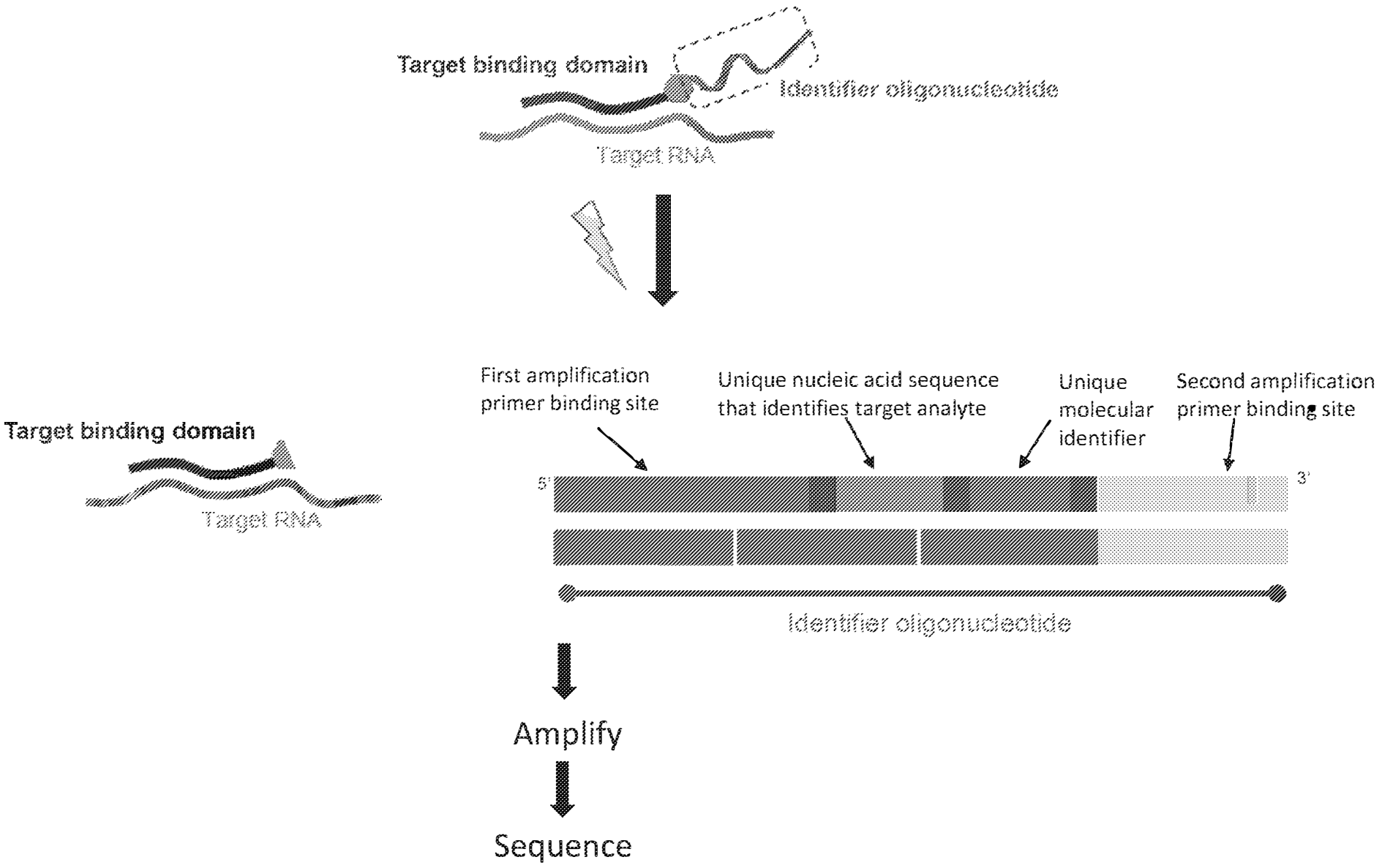


FIG. 11

NanoString EX2011.138

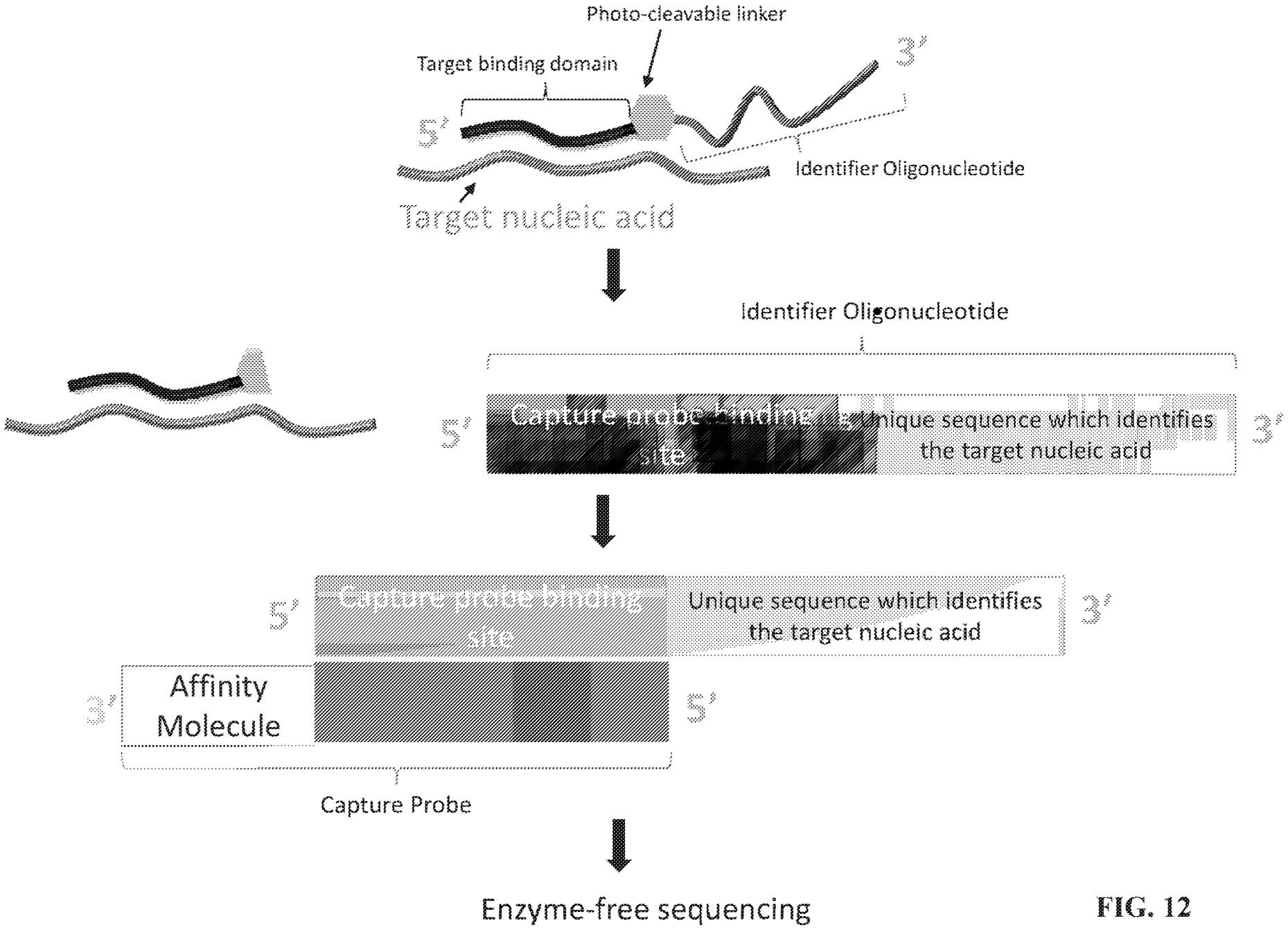


FIG. 12

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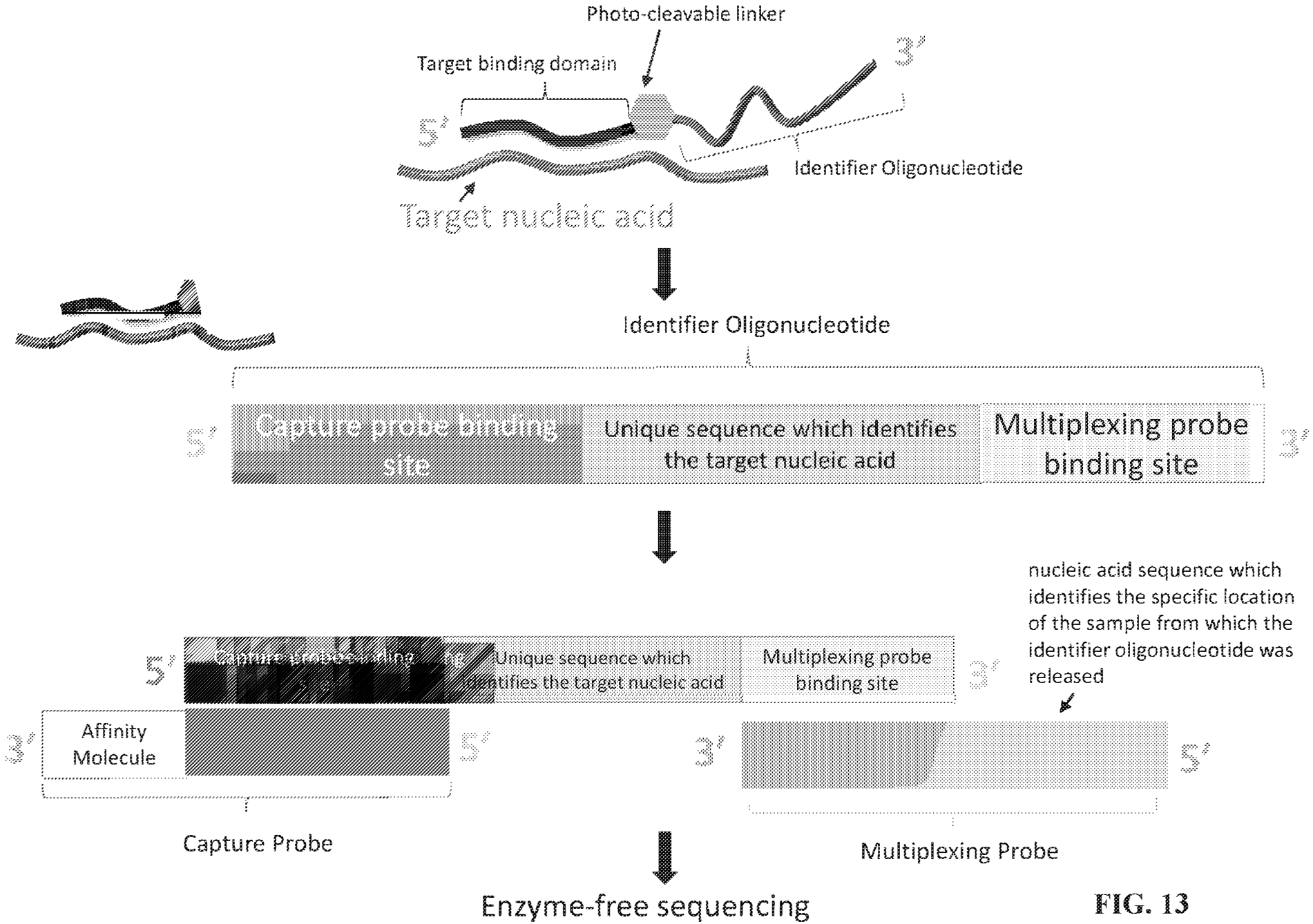


FIG. 13

NanoString EX2011.140

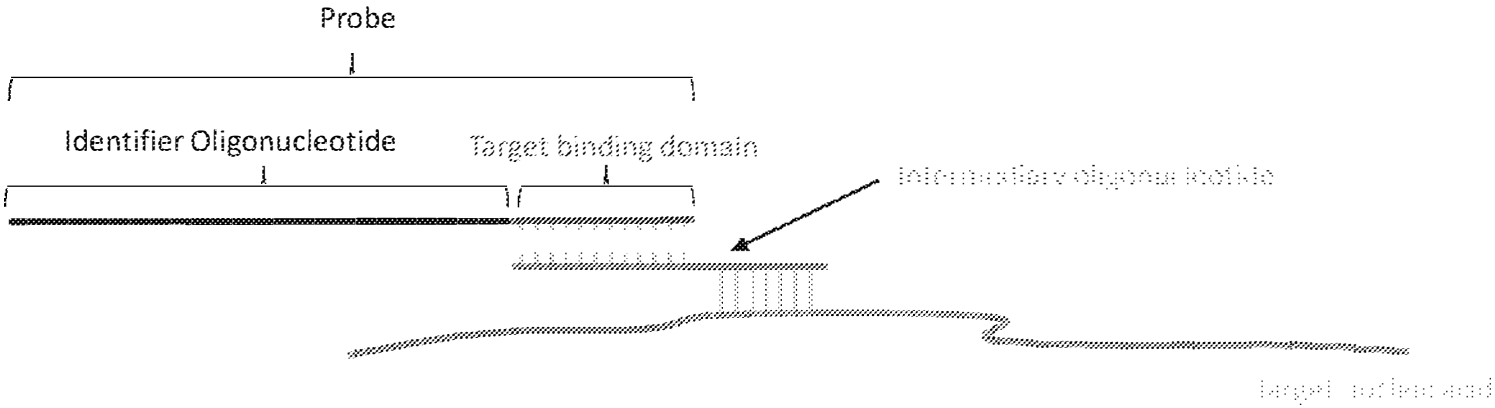


FIG. 14

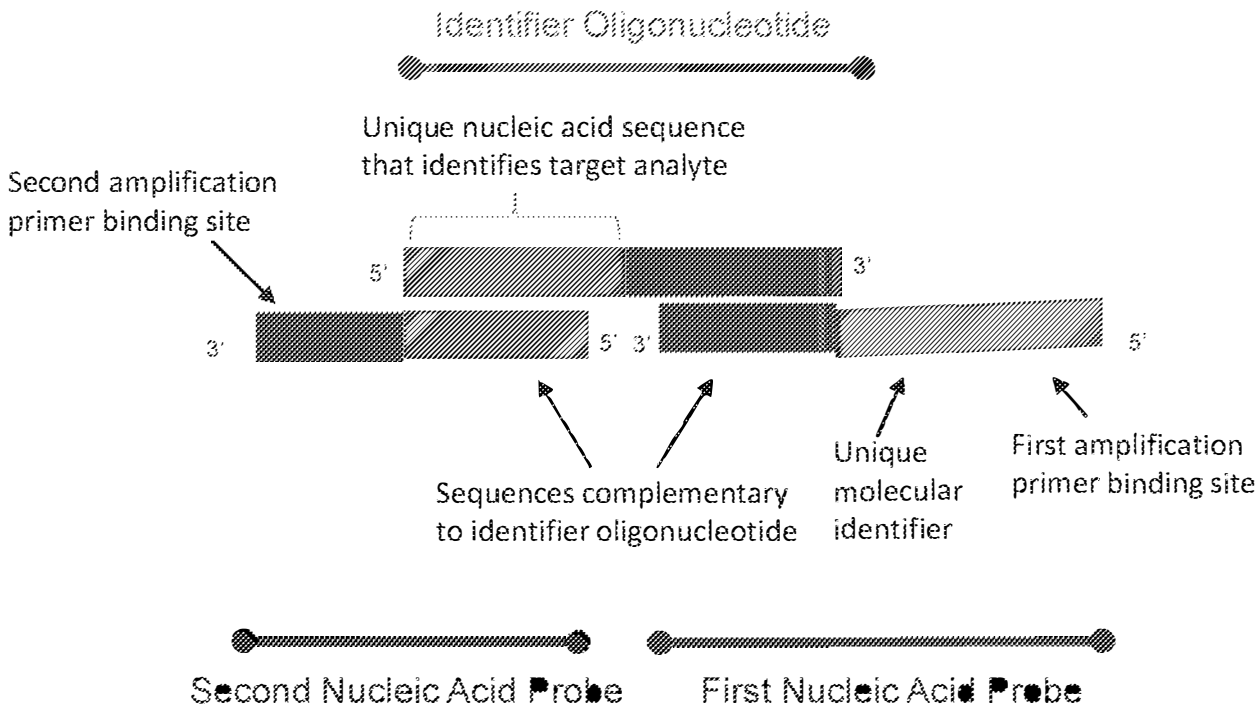


FIG. 15

NanoString EX2011.142

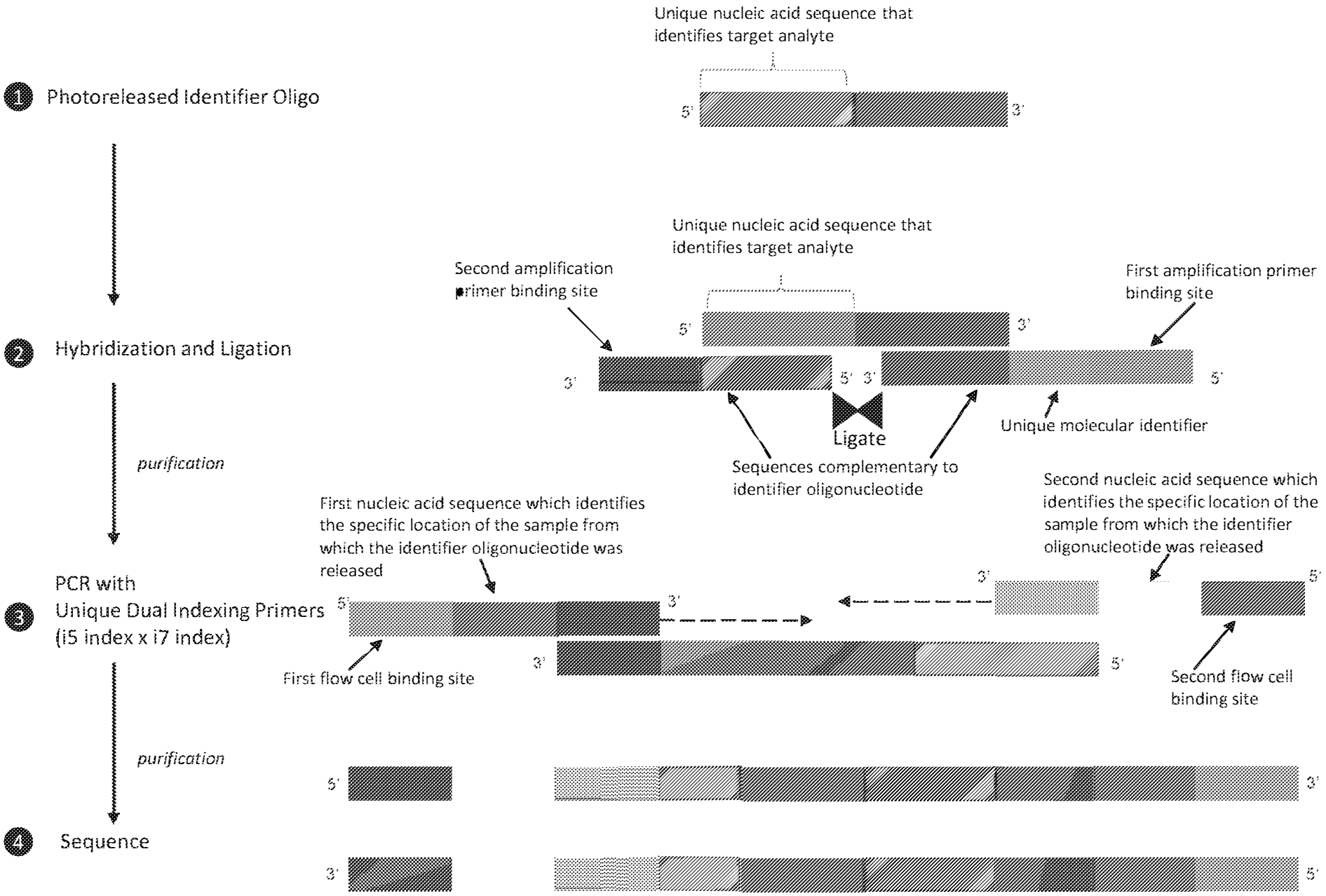


FIG. 16

NanoString EX2011.143

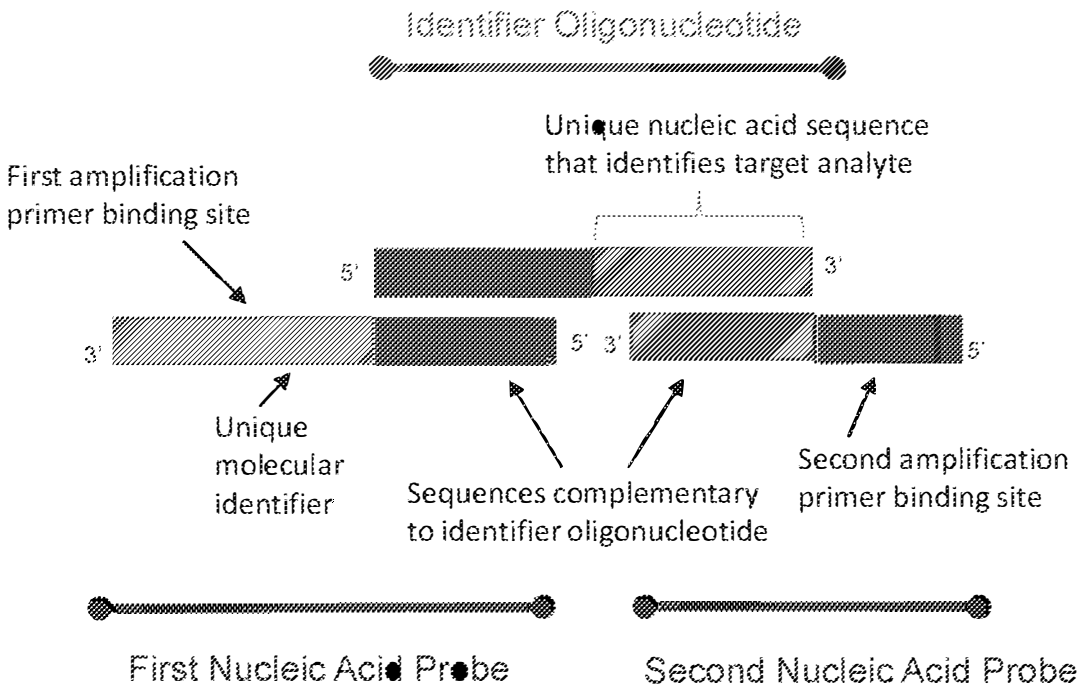


FIG. 17

NanoString EX2011.144

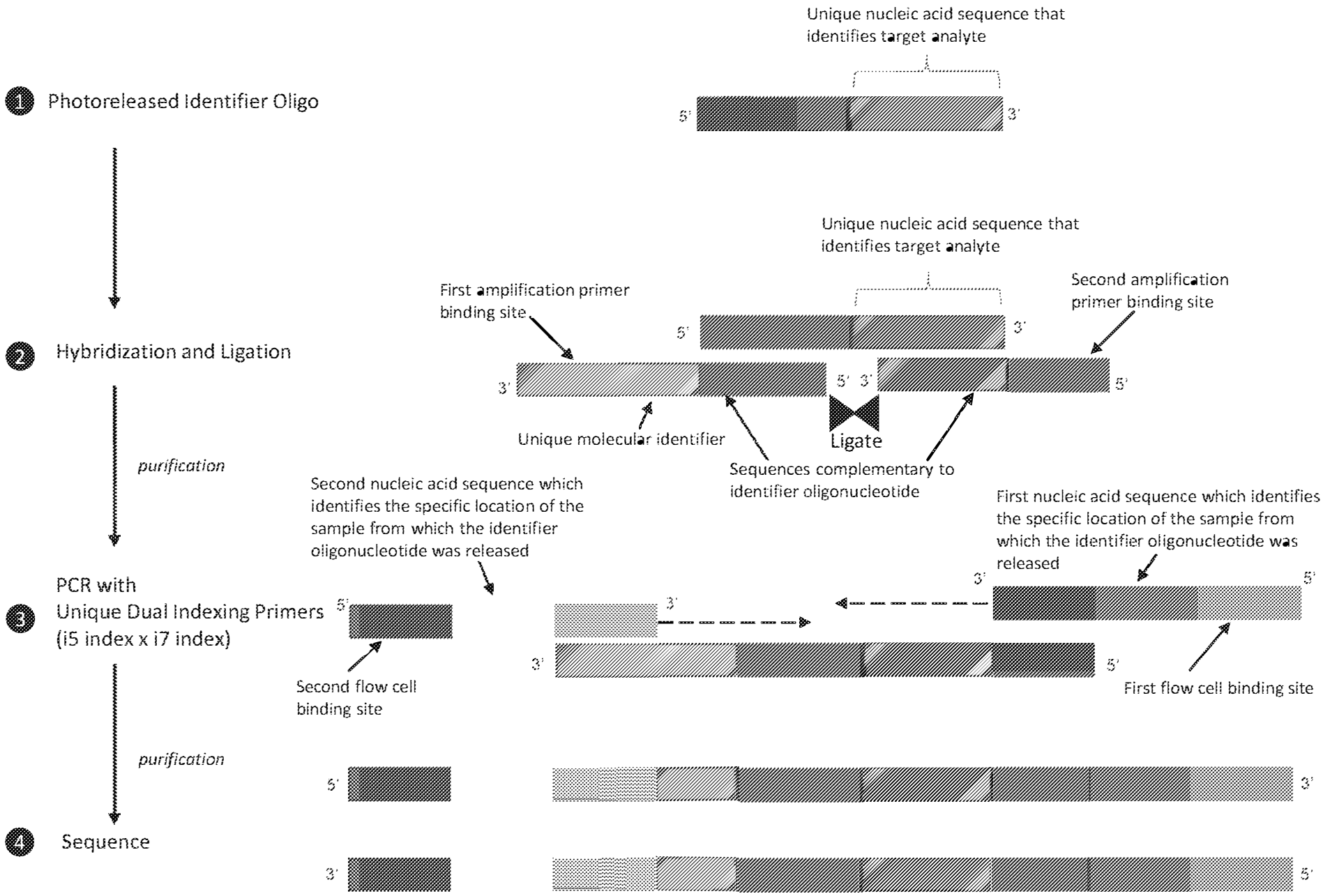


FIG. 18

NanoString EX2011.145

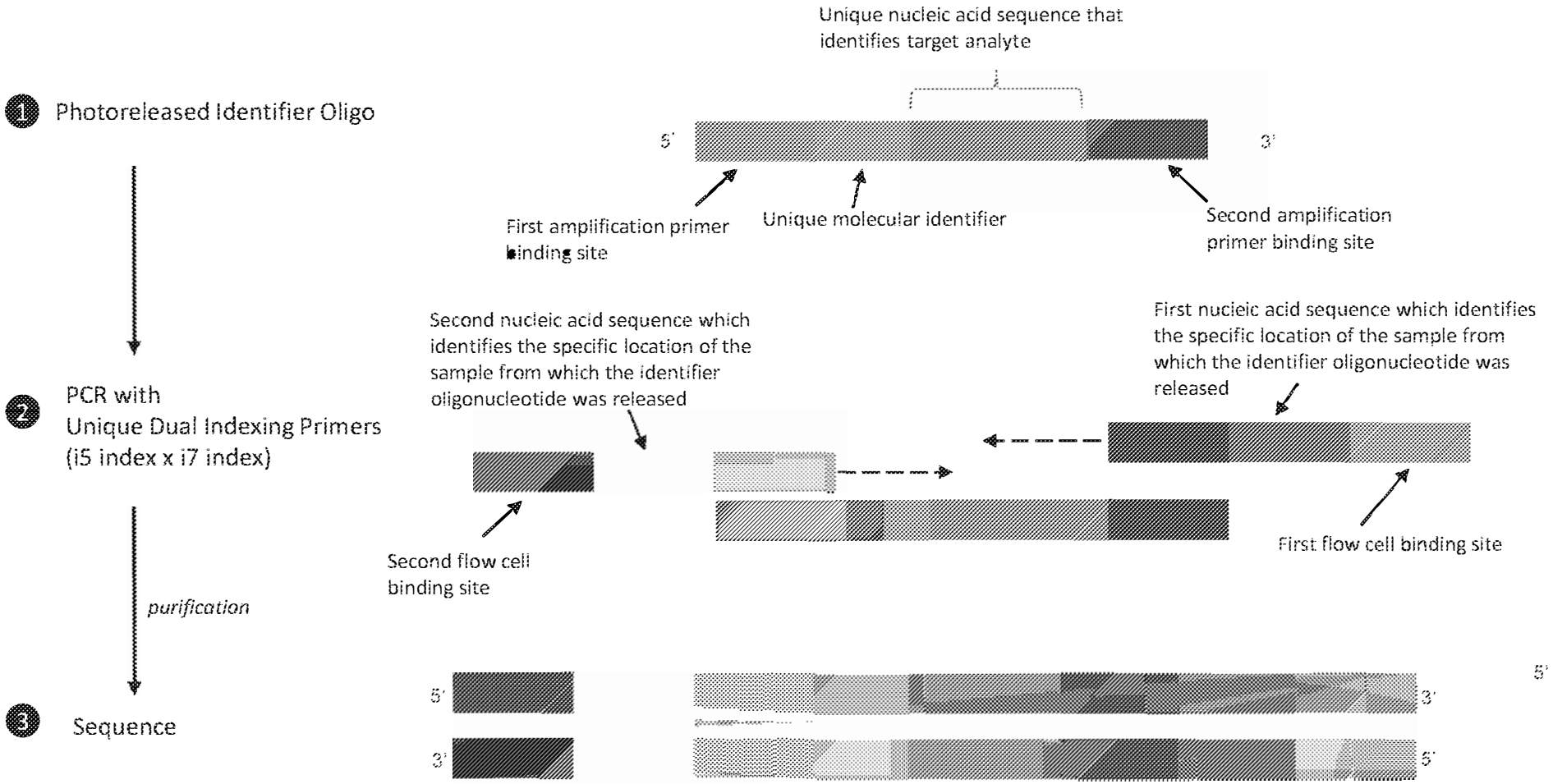


FIG. 19

NanoString EX2011.146

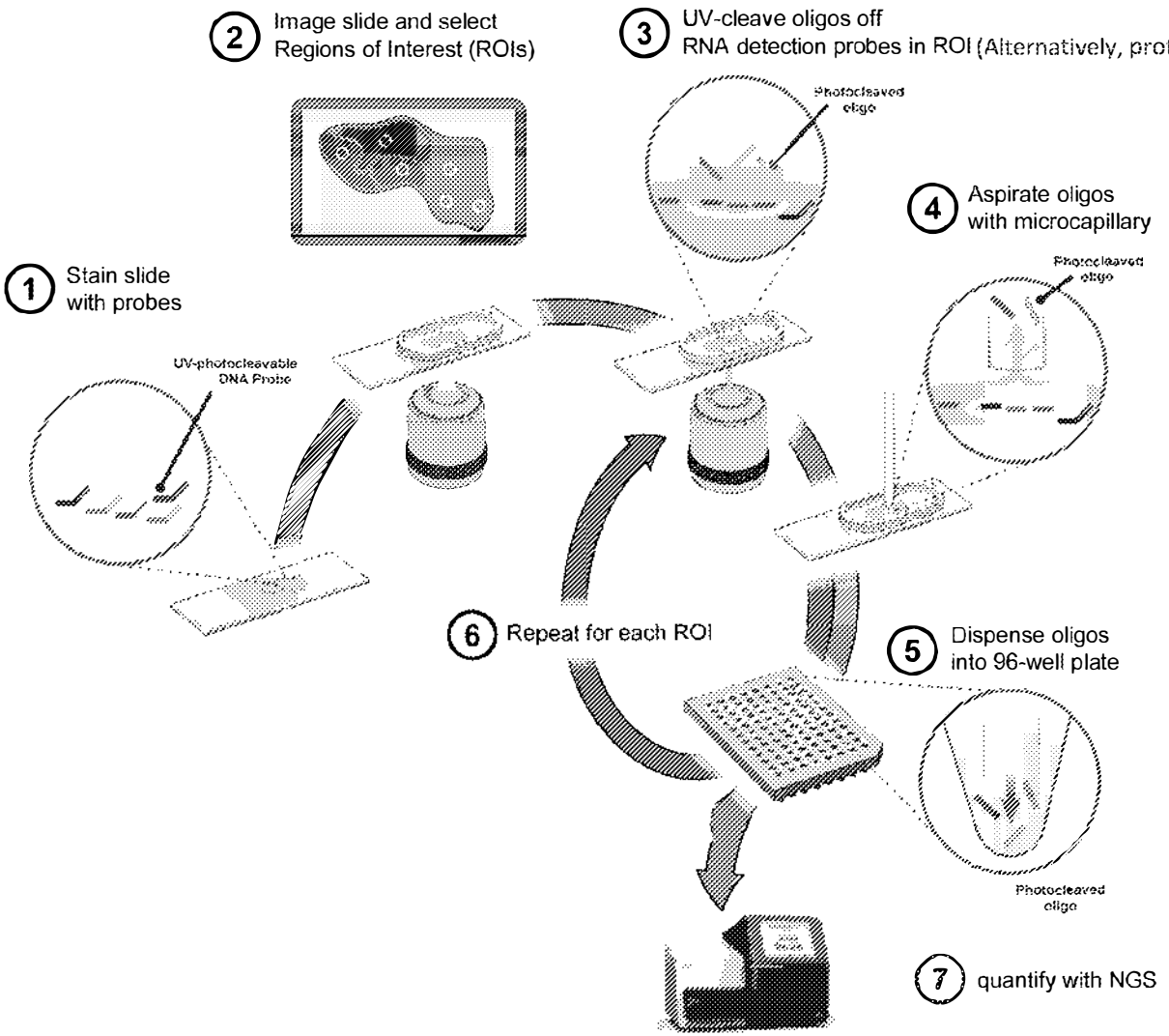

2 Image slide and select Regions of Interest (ROIs)
3 UV-cleave oligos off RNA detection probes in ROI (Alternatively, protein detection or DNA detection probes)
Photocleaved oligo
4 Aspirate oligos with microcapillary
1 Stain slide with probes
UV-photocleavable DNA Probe
6 Repeat for each ROI
5 Dispense oligos into 96-well plate
Photocleaved oligo
7 quantify with NGS

FIG. 20

Protein Immuno-oncology Panel (30-plex)
AKT
B7-H3
Bcl-2
Beta-2-microglobulin
Beta-Catenin
CD14
CD19
CD3
CD4
CD44
CD45
CD45RO
CD56
CD68
CD8A
FOXP3
GZMB
Histone H3
[illegible]
CD20
P-AKT
PanCK
PD1
PD-L1
S6
STAT3
P-STAT3
VISTA
IgG Rabbit isotype control
IgG Mouse isotype control

FIG. 21A

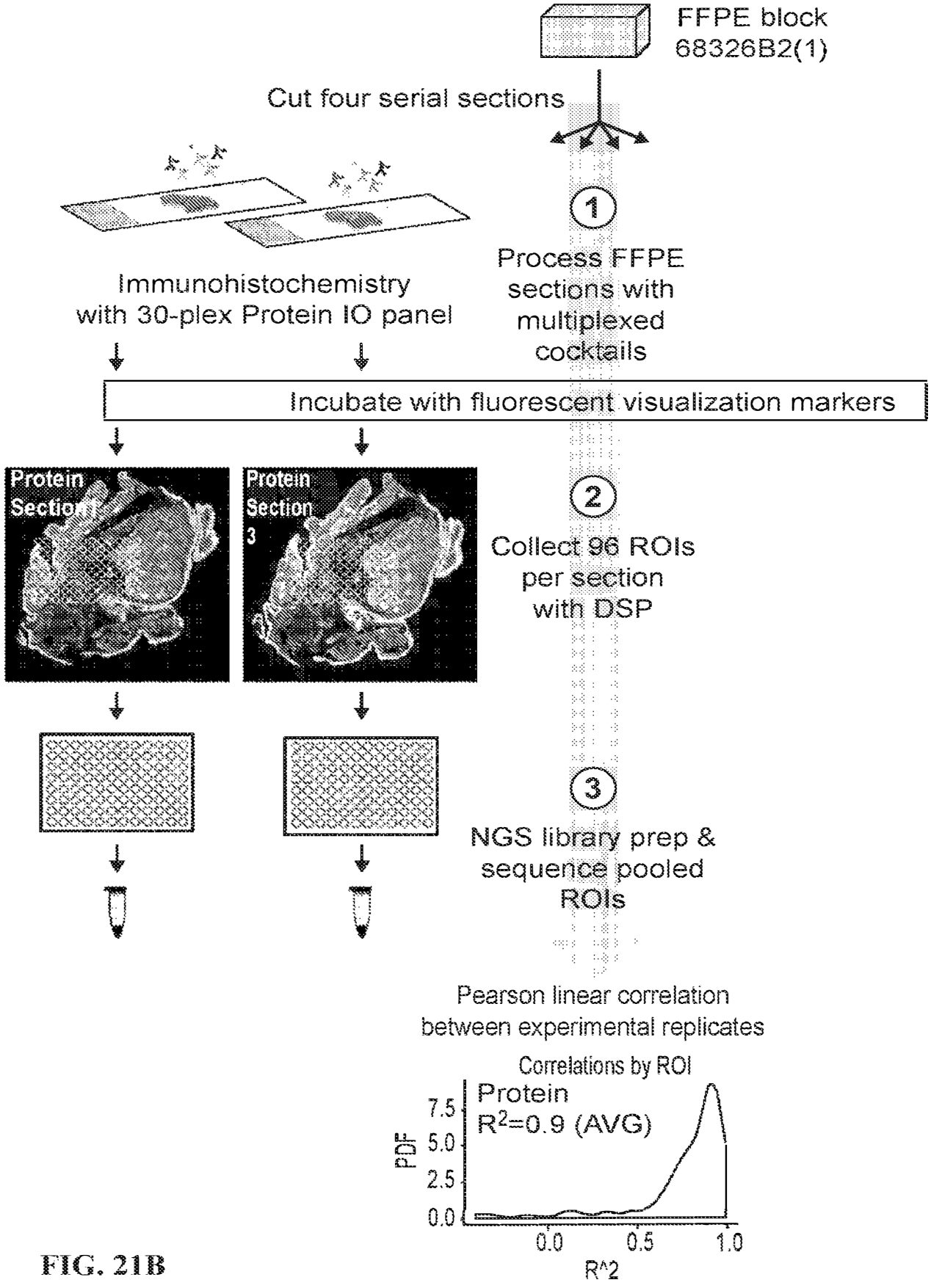

FFPE block
68326B2(1)
Cut four serial sections
1
Process FFPE sections with multiplexed cocktails
Immunohistochemistry with 30-plex Protein IO panel
Incubate with fluorescent visualization markers
Protein Section 5
Protein Section 3
2
Collect 96 ROIs per section with DSP
3
NGS library prep & sequence pooled ROIs
Pearson linear correlation between experimental replicates
Correlations by ROI
Protein
R2=0.9 (AVG)
PDF
7.5
5.0
2.5
0.0
0.0
0.5
1.0
R^2

FIG. 21B

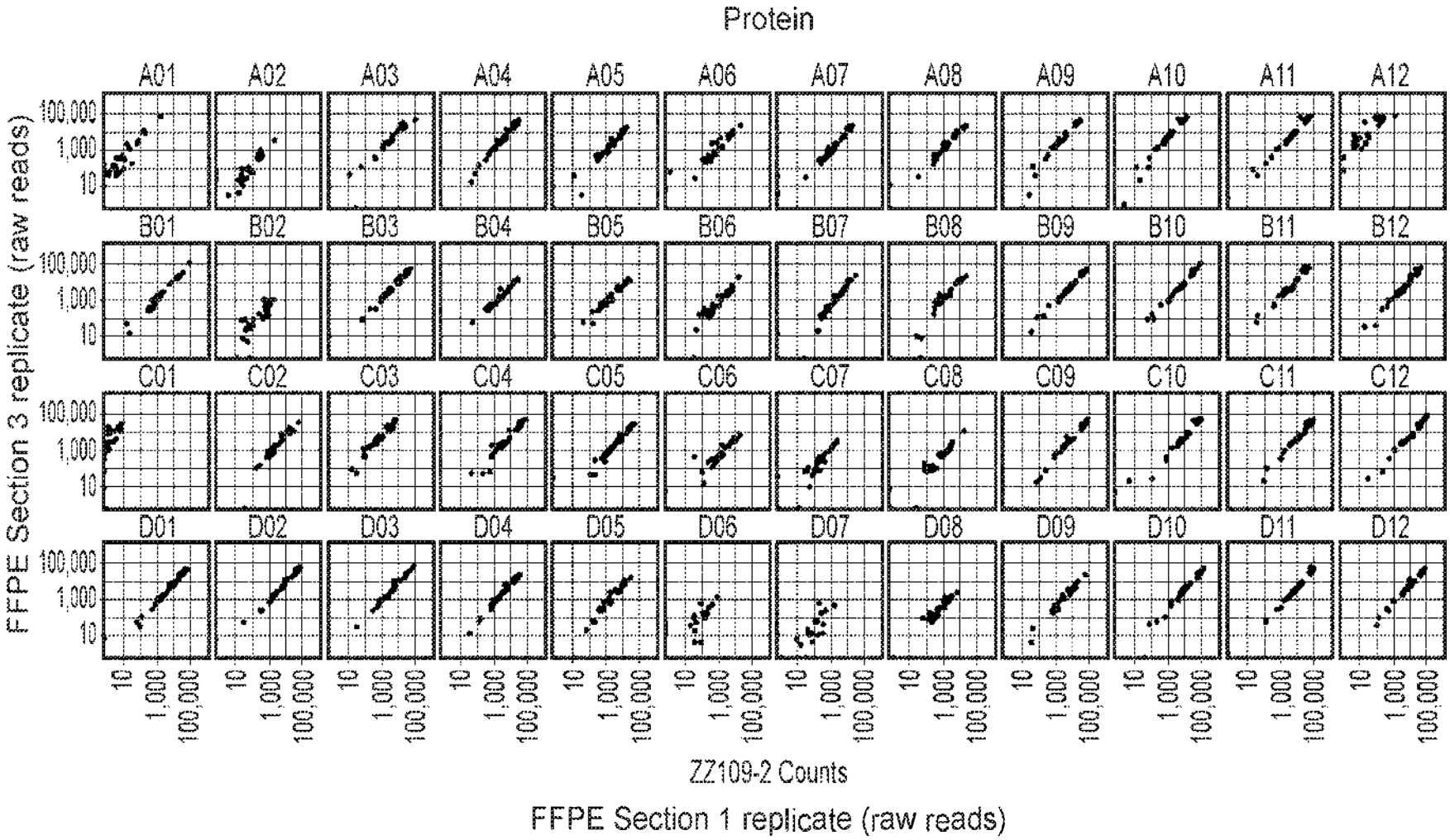


FIG. 21C

NanoString EX2011.150

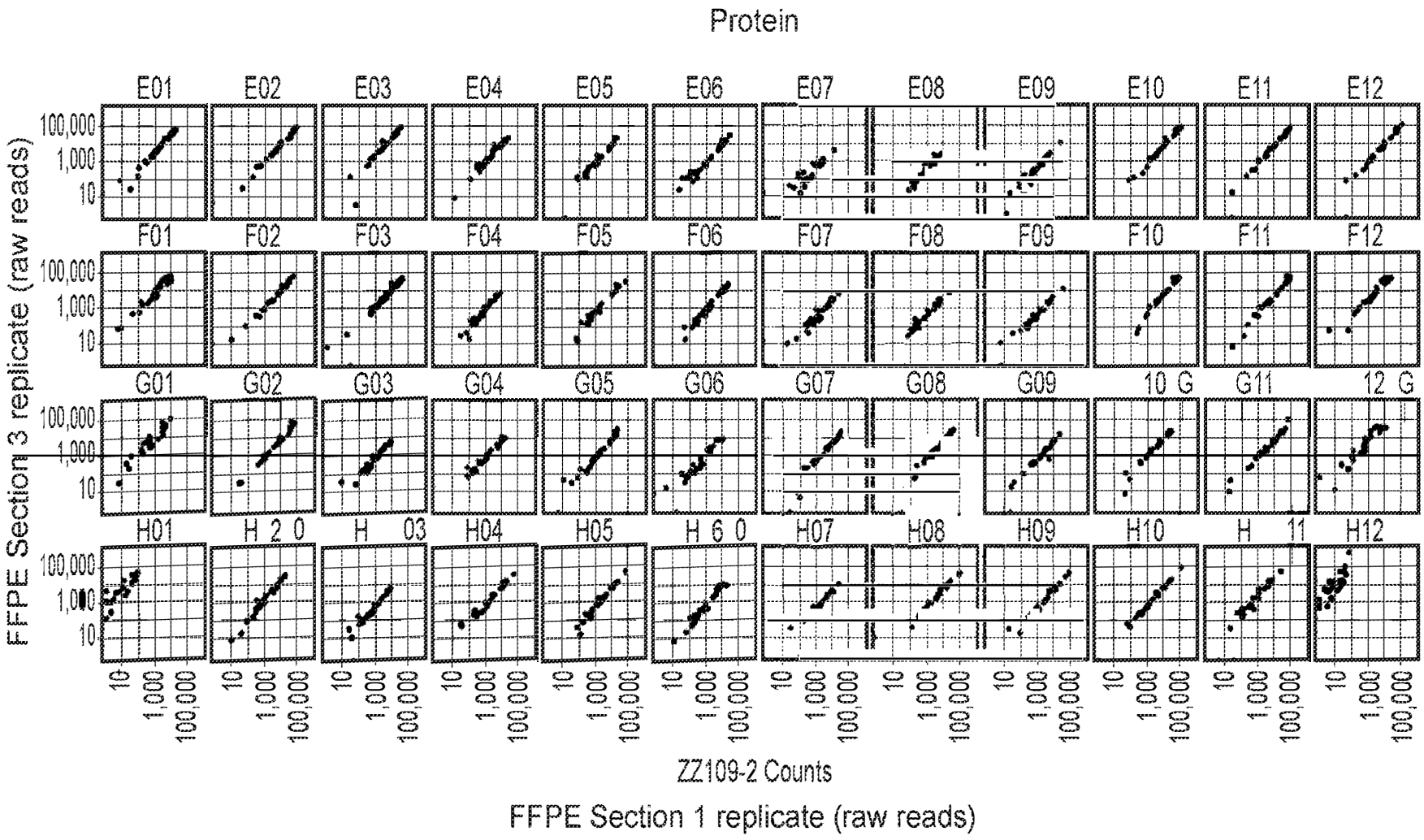

Protein
E01
E02
E03
E04
E05
E06
E07
E08
E09
E10
E11
E12
F01
F02
F03
F04
F05
F06
F07
F08
F09
F10
F11
F12
G01
G02
G03
G04
G05
G06
G07
G08
G09
10 G
G11
12 G
H01
H 2 0
H 03
H04
H05
H 6 0
H07
H08
H09
H10
H 11
H12
100,000
1,000
10
FFPE Section 3 replicate (raw reads)
ZZ109-2 Counts
FFPE Section 1 replicate (raw reads)

FIG. 21D

RNA Immuno-oncology Panel (20-plex)
CD3E
CD3G
CD4
CD20
CD40
CD45
CD74
CD79A
CTLA4
GAPDH
KRT13
PD1
PSA
RPS6
Negative probe 1
Negative probe 2
Negetive probe 3
Negative probe 4
Negative probe 5
Negative probe 6

FIG. 22A

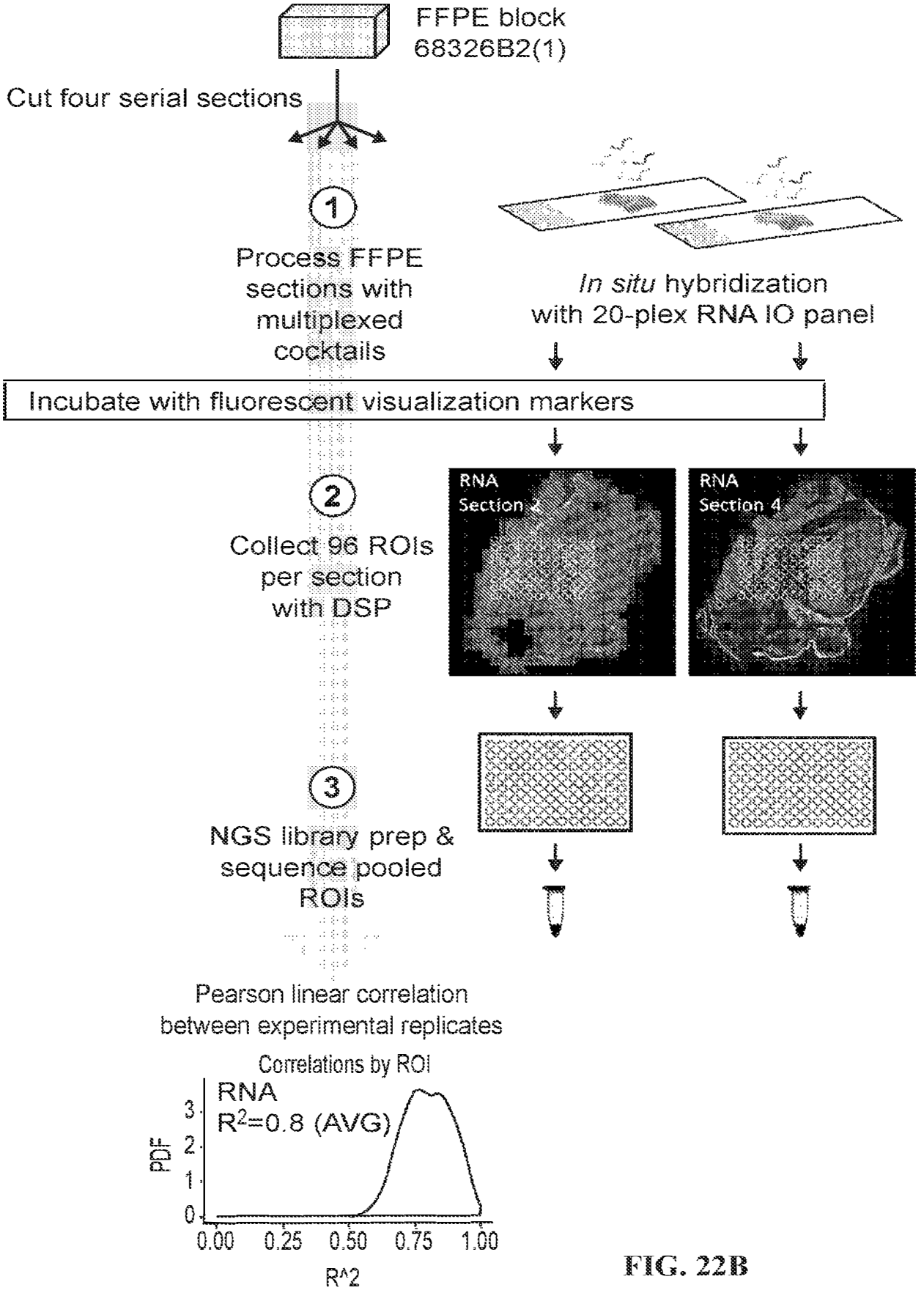

FFPE block
68326B2(1)
Cut four serial sections
1
Process FFPE sections with multiplexed cocktails
In situ hybridization with 20-plex RNA IO panel
Incubate with fluorescent visualization markers
RNA Section 3
RNA Section 4
2
Collect 96 ROIs per section with DSP
3
NGS library prep & sequence pooled ROIs
Pearson linear correlation between experimental replicates
Correlations by ROI
RNA
R2=0.8 (AVG)
PDF
0
1
2
3
0.00
0.25
0.50
0.75
1.00
R^2

FIG. 22B

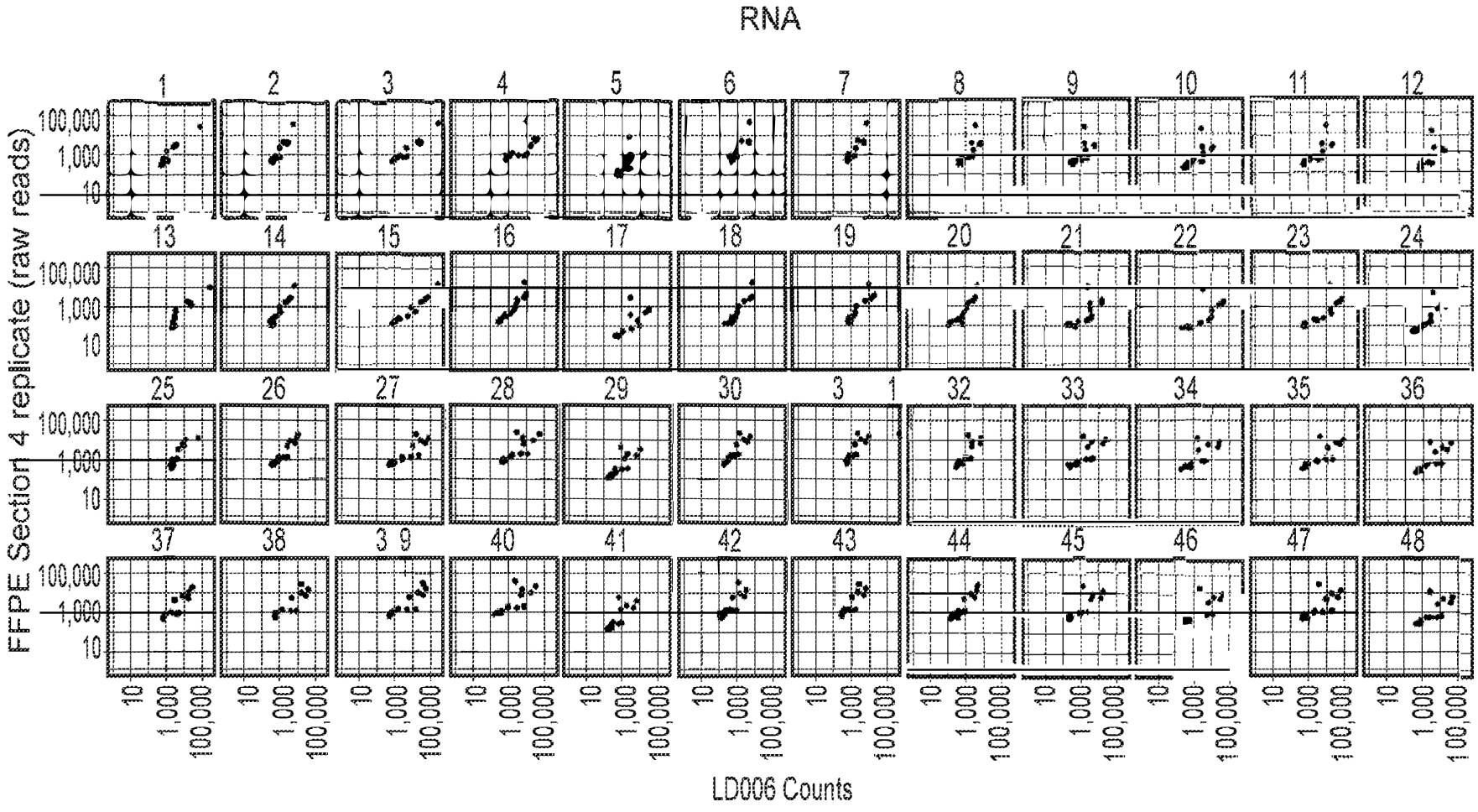


FIG. 22C

NanoString EX2011.154

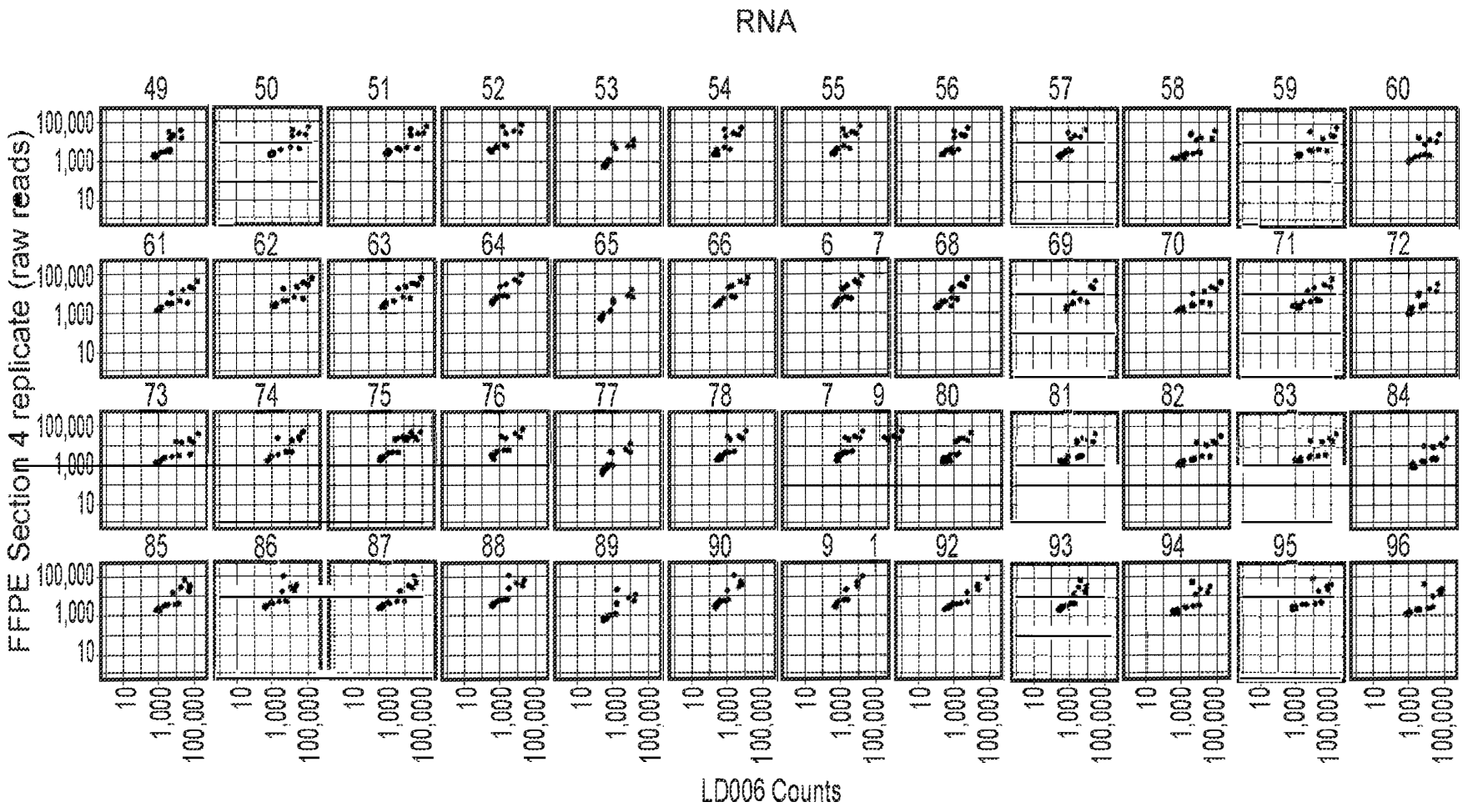


FIG. 22D

NanoString EX2011.155

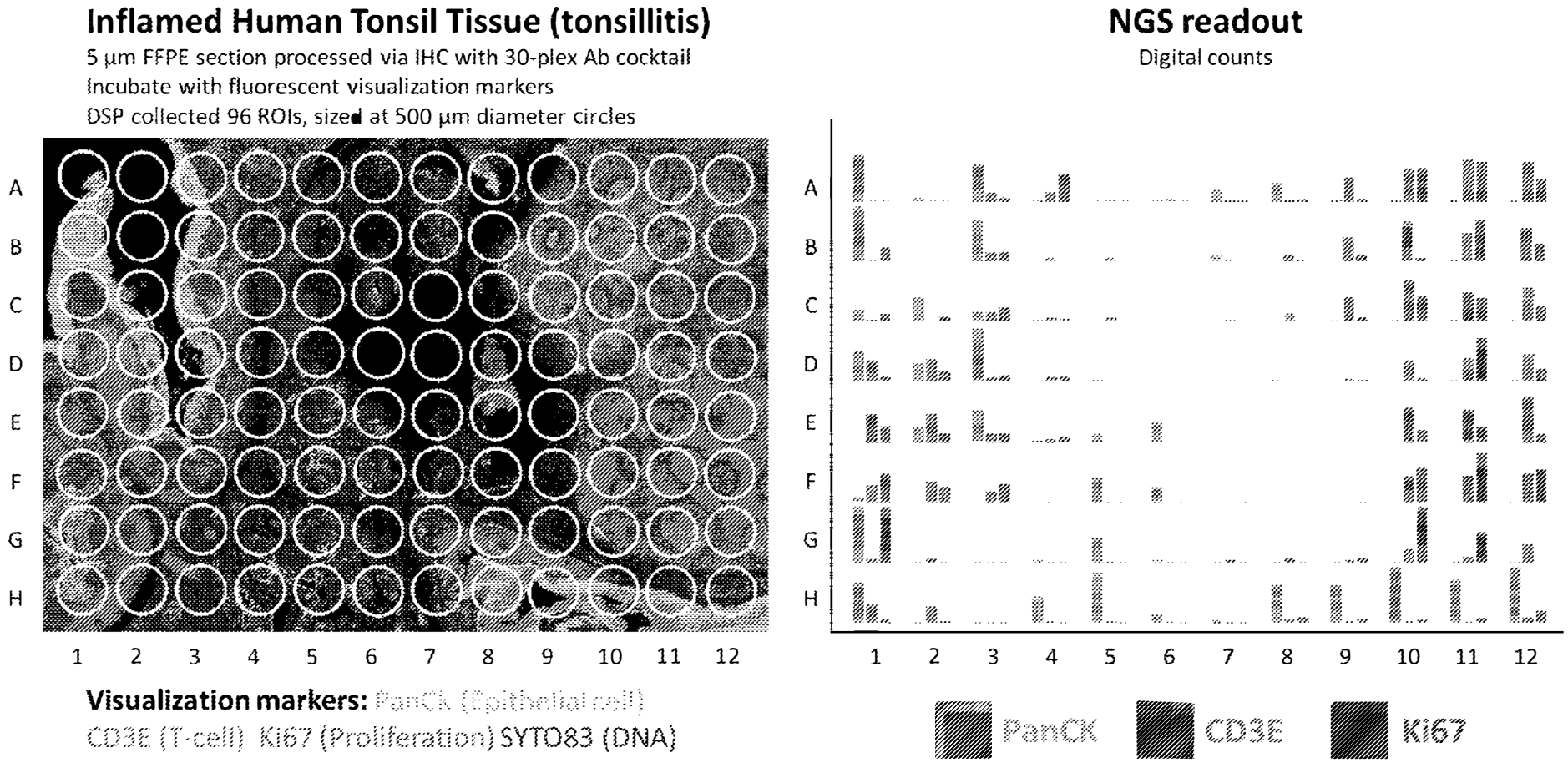

Inflamed Human Tonsil Tissue (tonsillitis)
5 µm FFPE section processed via IHC with 30-plex Ab cocktail
Incubate with fluorescent visualization markers
DSP collected 96 ROIs, sized at 500 µm diameter circles
A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12
Visualization markers: PanCk (Epithelial cell)
CD3E (T-cell) Ki67 (Proliferation) SYTO83 (DNA)
NGS readout
Digital counts
A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12
PanCK
CD3E
Ki67

FIG. 23

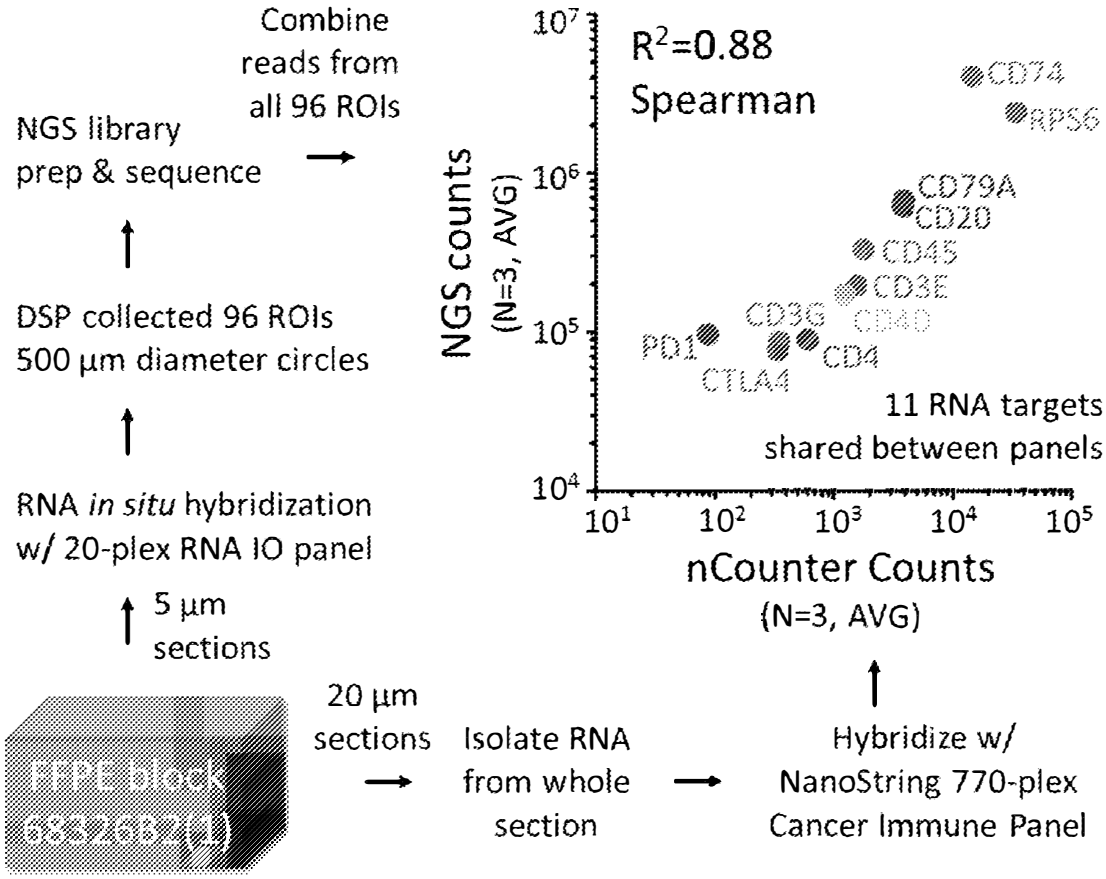
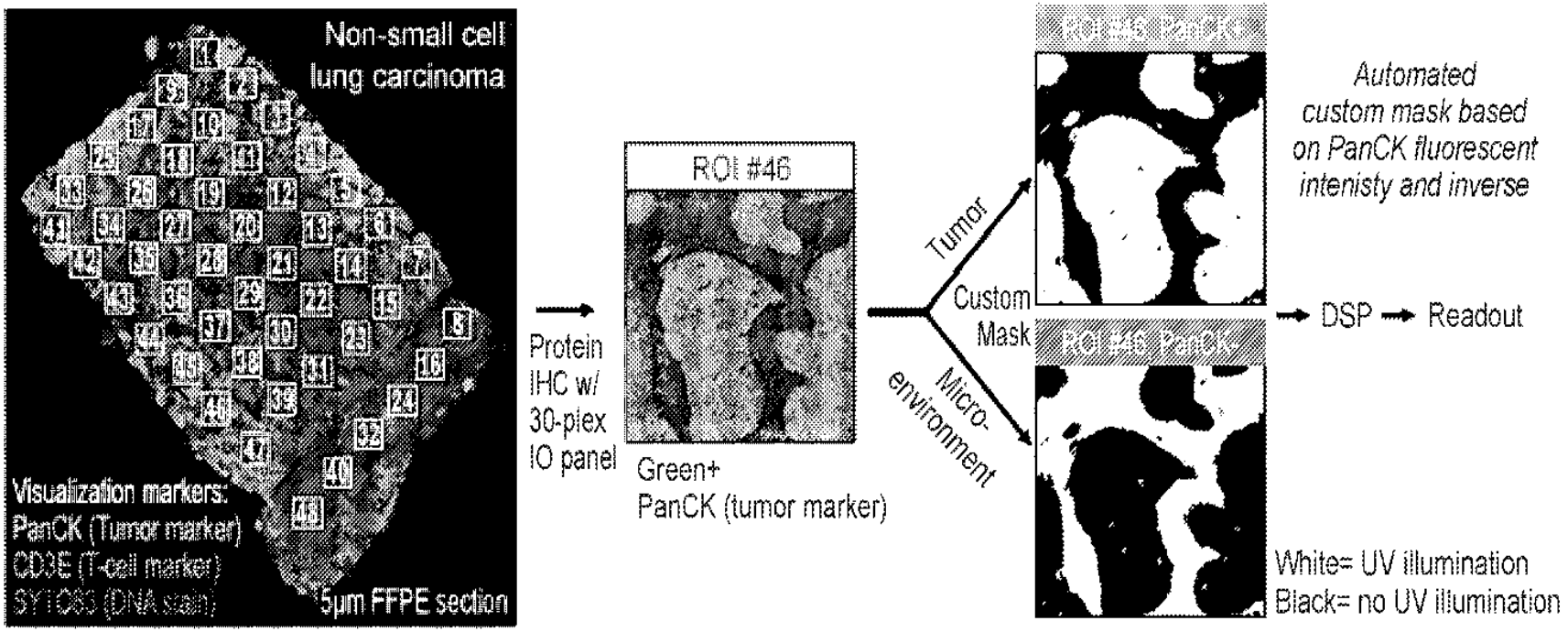


FIG. 24

NanoString EX2011.157


Non-small cell lung carcinoma
Visualization markers:
PanCK (Tumor marker)
CD3E (T-cell marker)
SYTO83 (DNA stain)
5µm FFPE section
Protein IHC w/ 30-piex IO panel
ROI #46
Green+ PanCK (tumor marker)
Tumor
Custom Mask
Micro-environment
ROI #46: PanCK+
ROI #46: PanCK-
Automated custom mask based on PanCK fluorescent intenisty and inverse
DSP
Readout
White= UV illumination
Black= no UV illumination

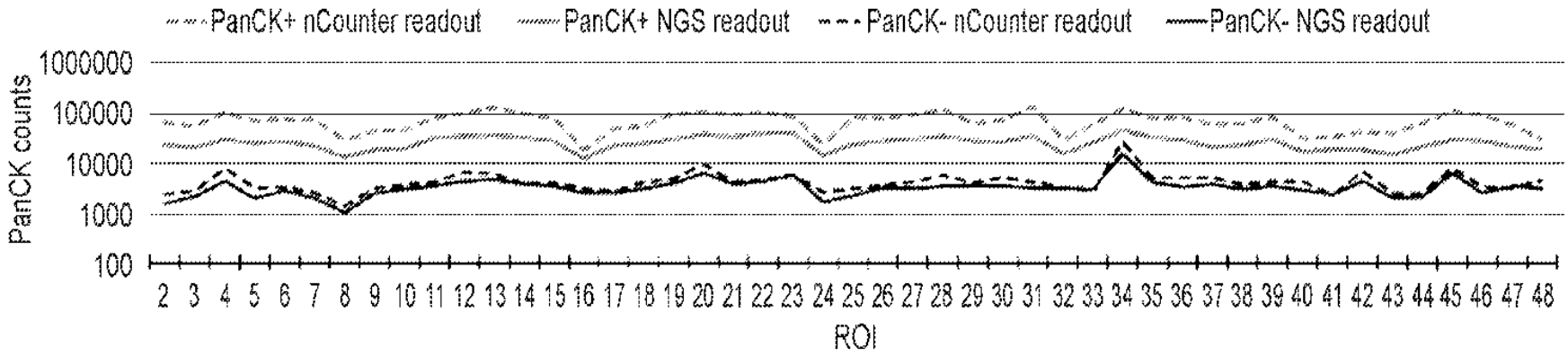
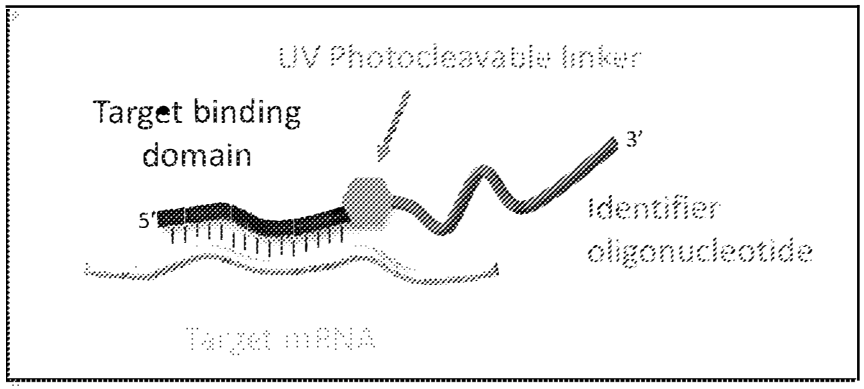

PanCK+ nCounter readout
PanCK+ NGS readout
PanCK- nCounter readout
PanCK- NGS readout
PanCK counts
1000000
100000
10000
1000
100
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
ROI

FIG. 25



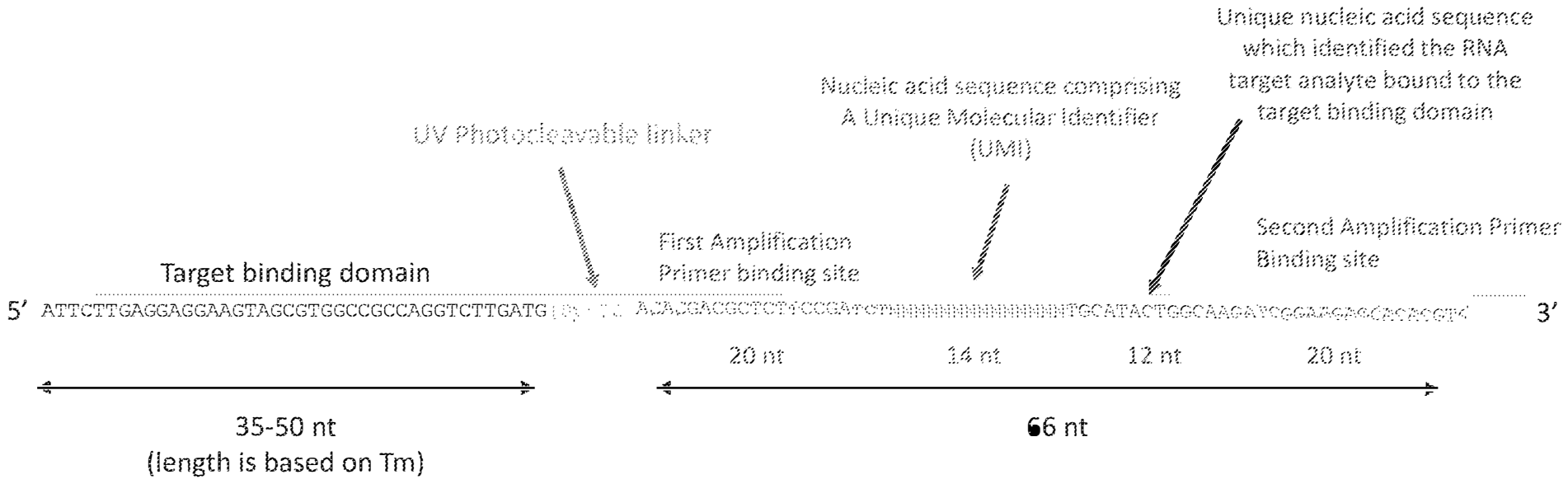


FIG. 26

NanoString EX2011.159

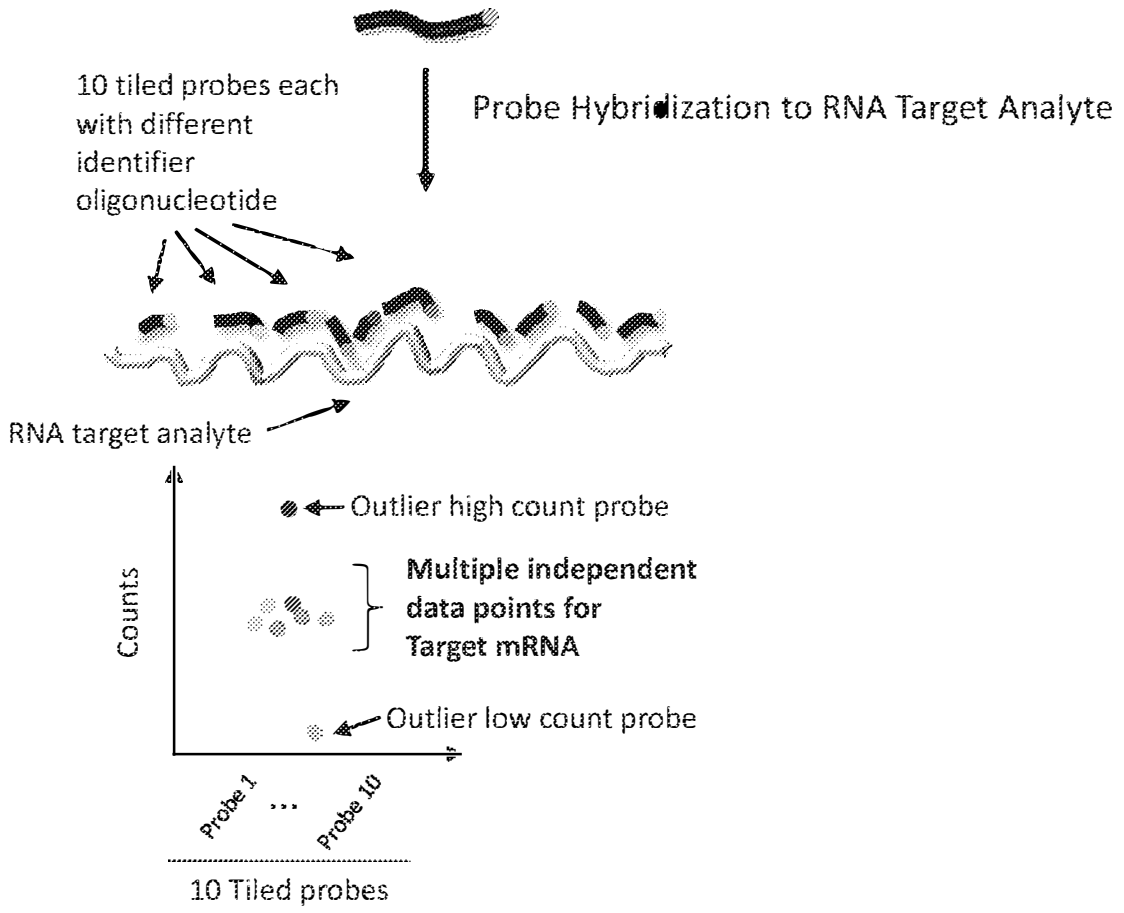


FIG. 27

NanoString EX2011.160

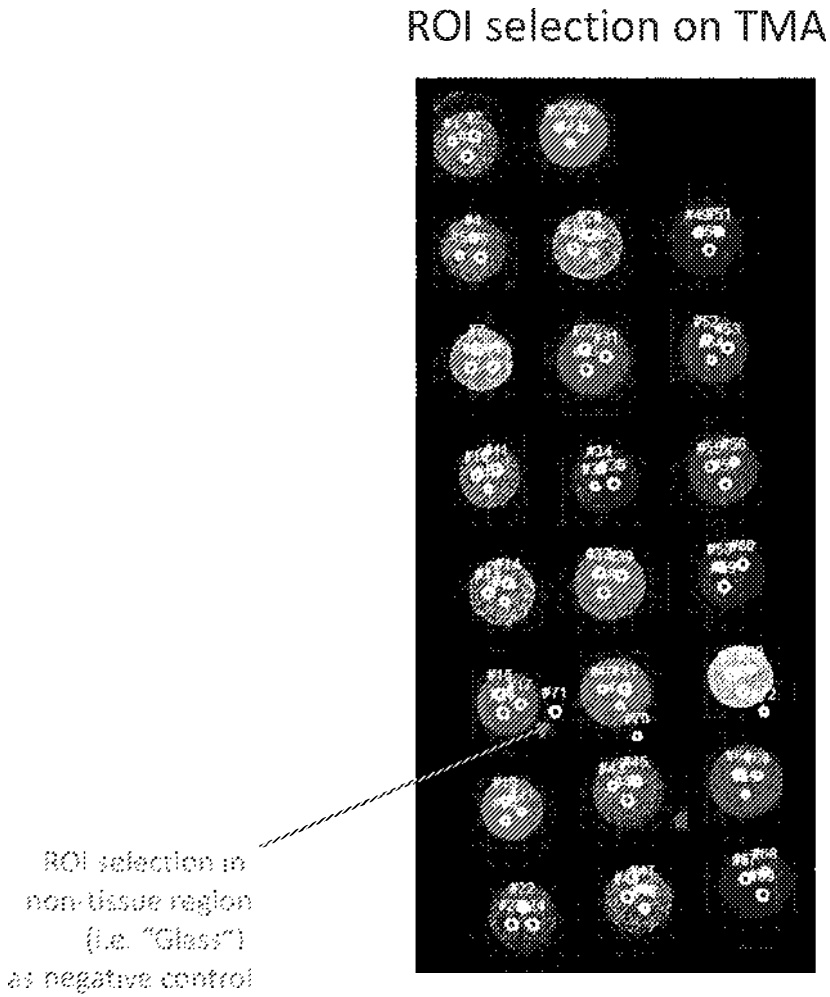


FIG. 28

NanoString EX2011.161

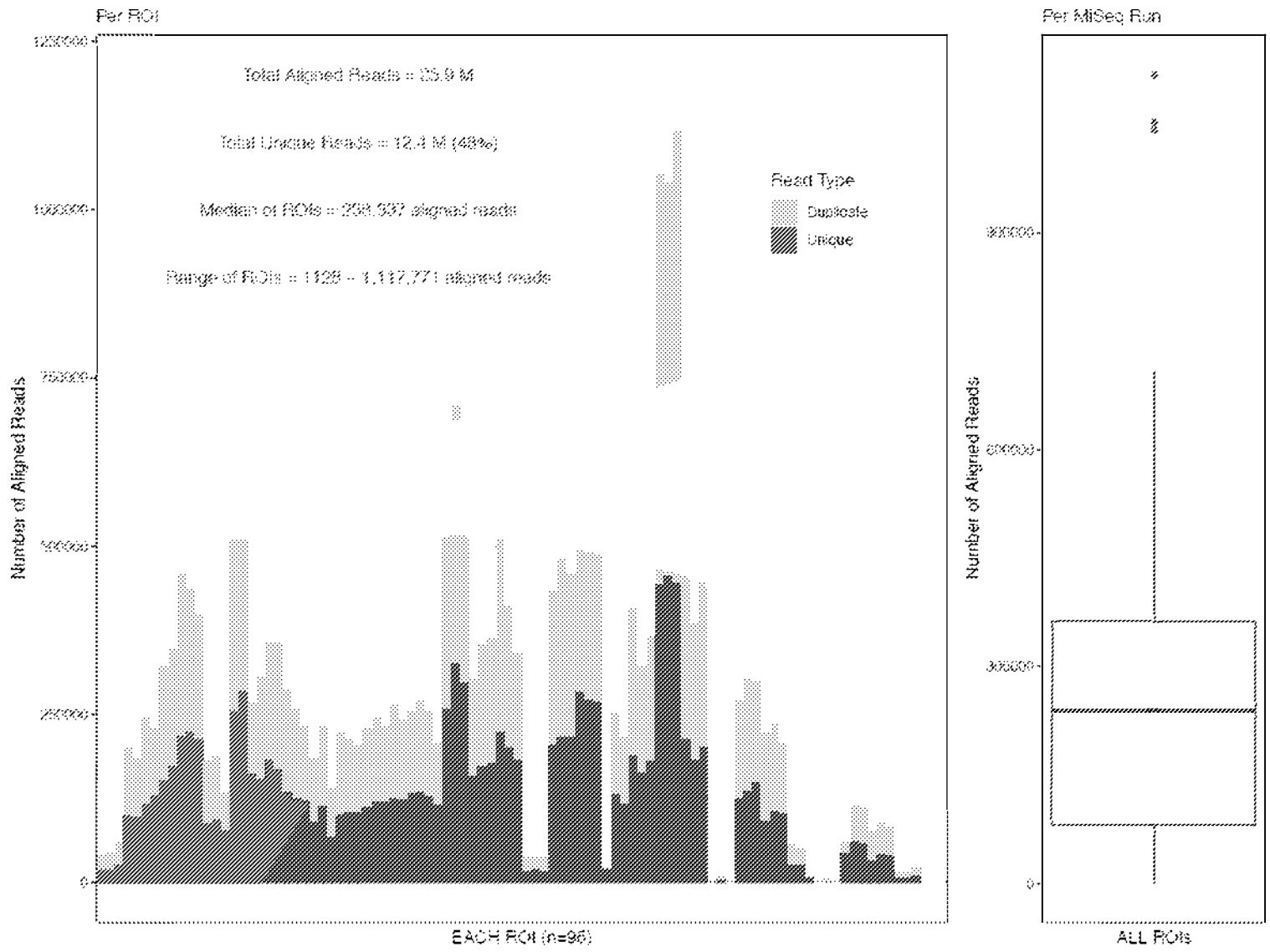


FIG. 29

NanoString EX2011.162

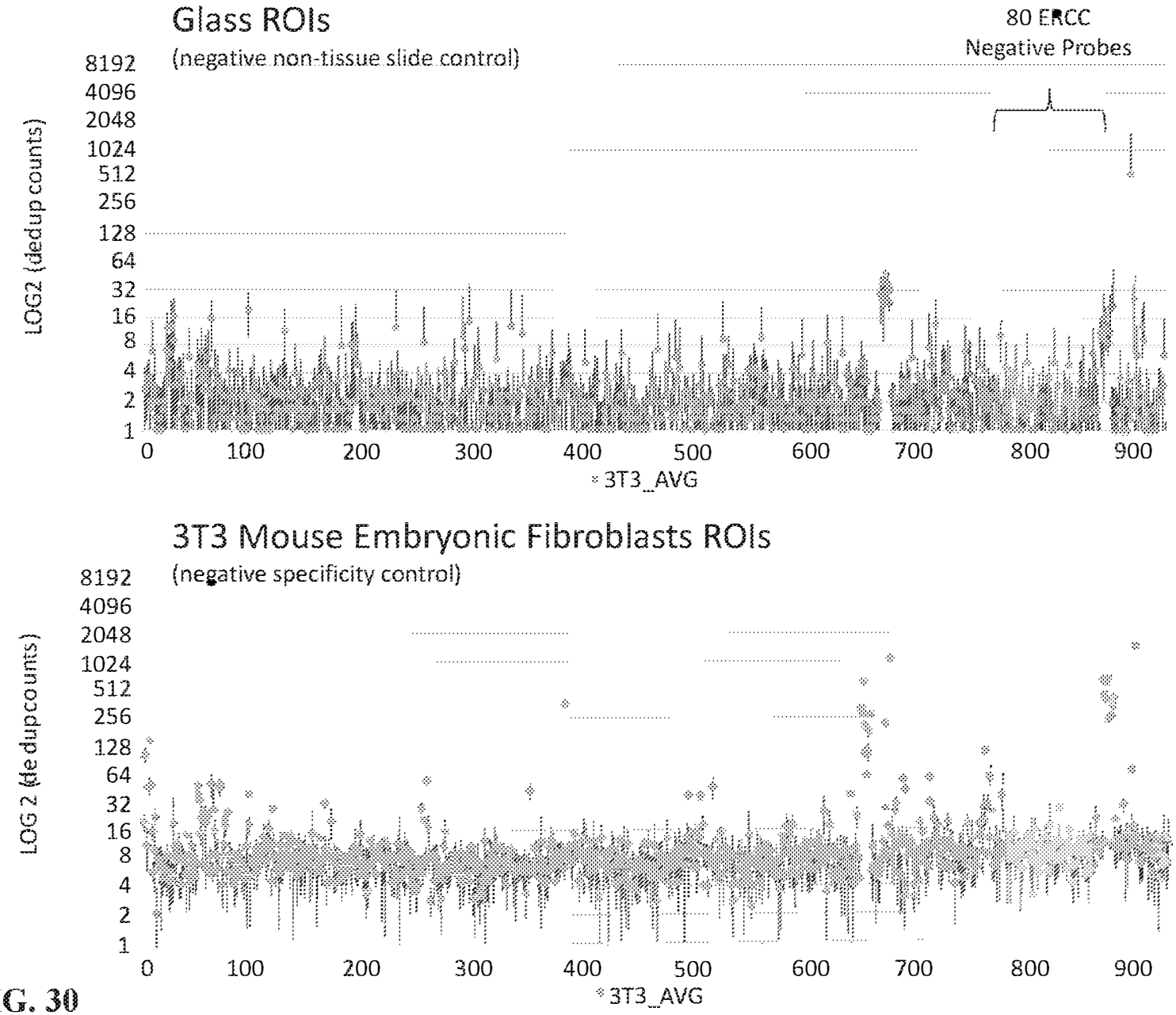

Glass ROIs
(negative non-tissue slide control)
80 ERCC
Negative Probes
LOG2 (dedup counts)
8192
4096
2048
1024
512
256
128
64
32
16
8
4
2
1
0
100
200
300
400
500
600
700
800
900
3T3_AVG
3T3 Mouse Embryonic Fibroblasts ROIs
(negative specificity control)
LOG 2 (de dup counts)
8192
4096
2048
1024
512
256
128
64
32
16
8
4
2
1
0
100
200
300
400
500
600
700
800
900
3T3_AVG

FIG. 30

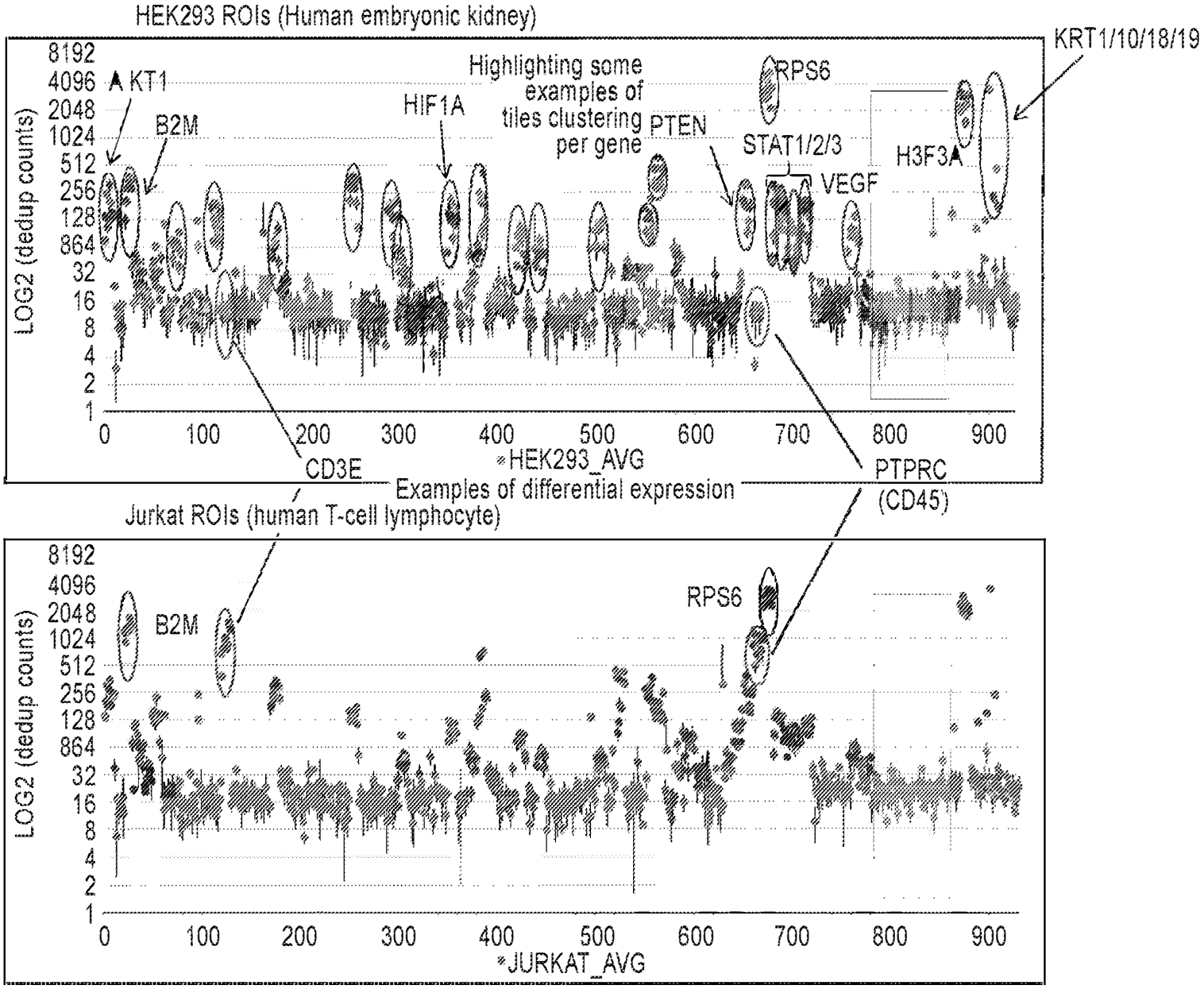

HEK293 ROIs (Human embryonic kidney)
LOG2 (dedup counts)
8192
4096
2048
1024
512
256
128
64
32
16
8
4
2
1
0
100
200
300
400
500
600
700
800
900
KT1
B2M
HIF1A
Highlighting some examples of tiles clustering per gene
PTEN
RPS6
STAT1/2/3
VEGF
H3F3A
KRT1/10/18/19
CD3E
HEK293_AVG
Examples of differential expression
PTPRC (CD45)
Jurkat ROIs (human T-cell lymphocyte)
B2M
RPS6
JURKAT_AVG

FIG. 31

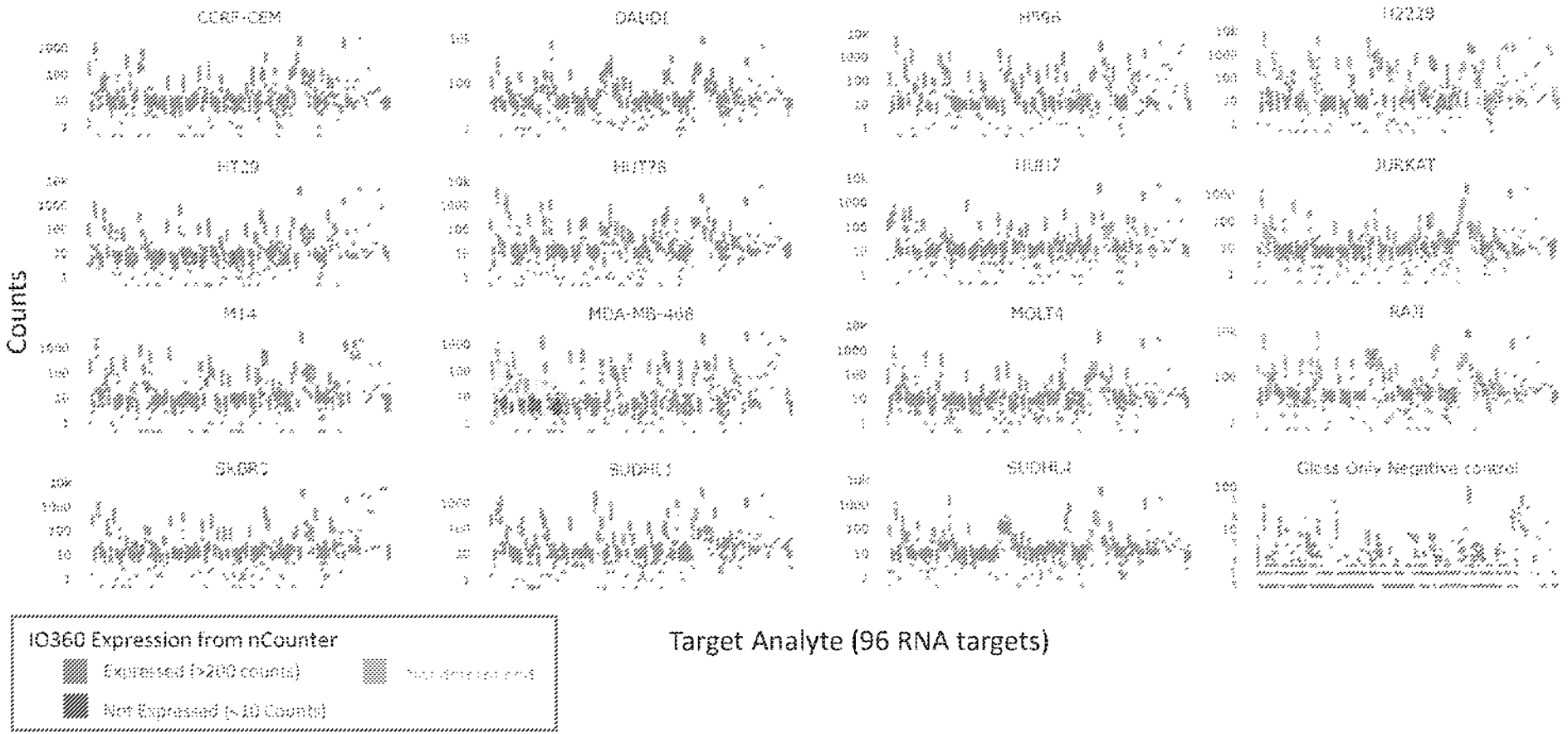


FIG. 32

NanoString EX2011.165

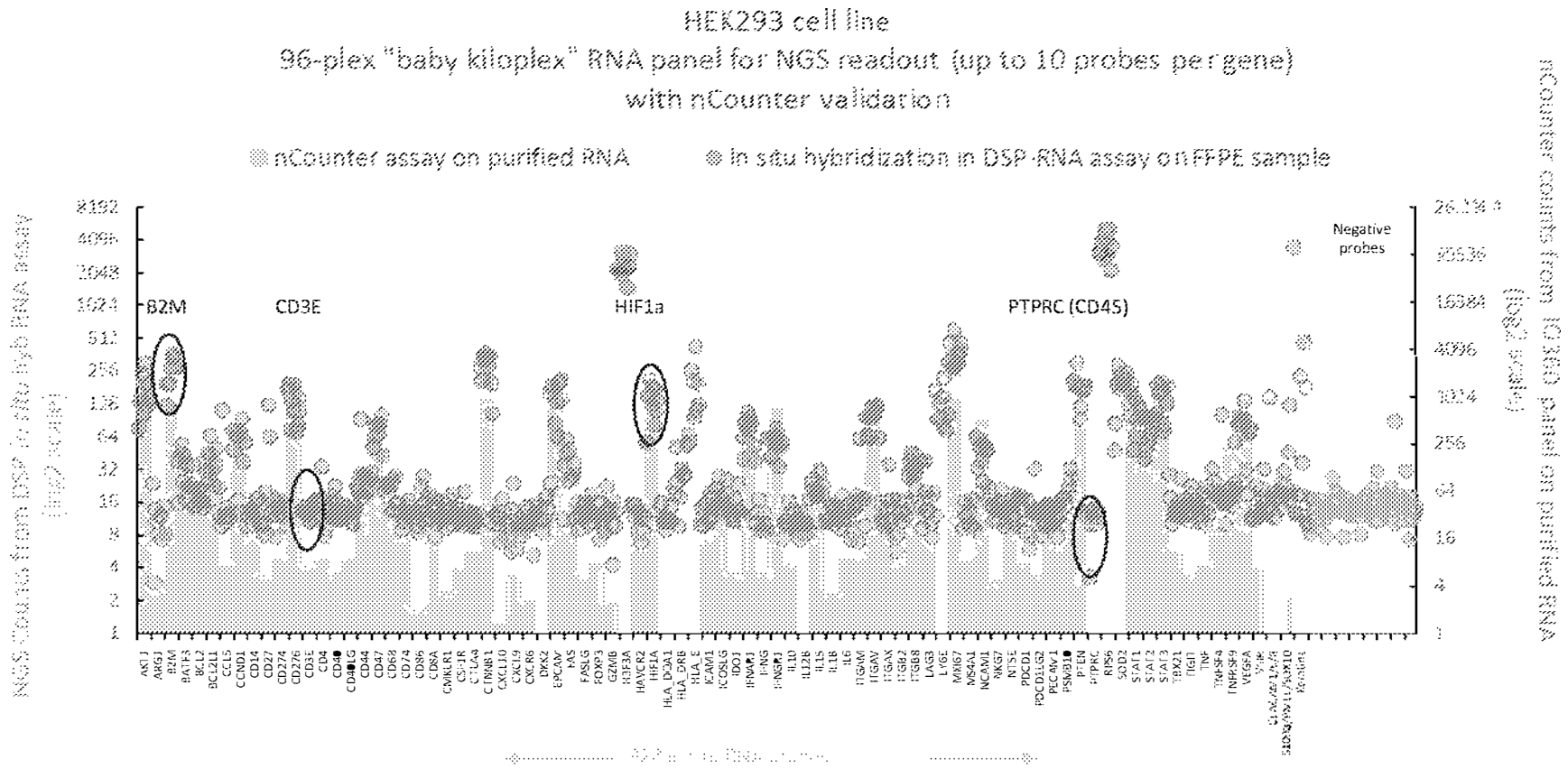


FIG. 33

NanoString EX2011.166

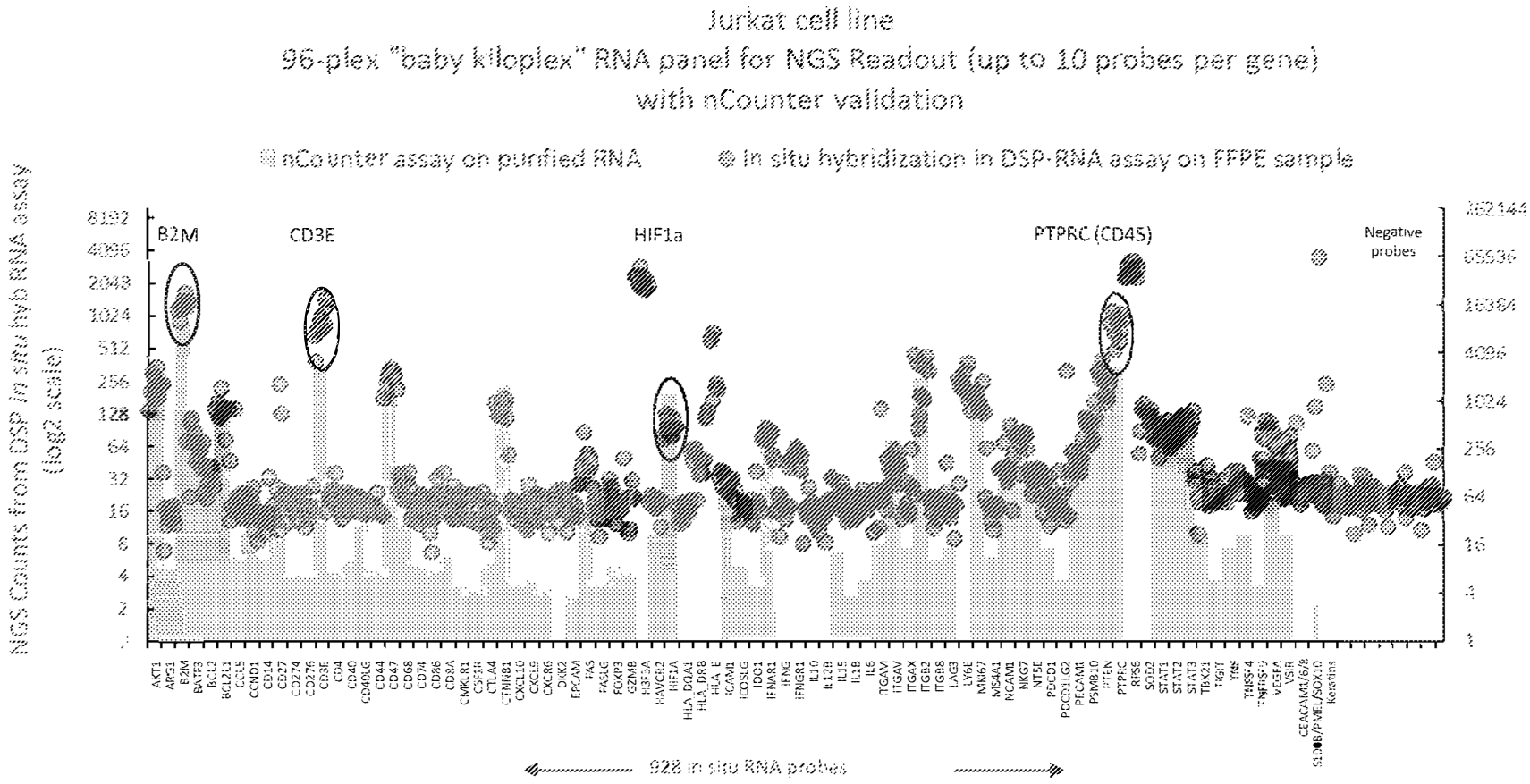


FIG. 34

NanoString EX2011.167

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

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.

Secrecy Order 37 CFR 5.2:

☐ 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
	Joseph	M.	BEECHEM	

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

City	State/Province	Country of Residence
Eugene	OR	US

Mailing Address of Inventor:

Address 1	46 Ridgeline Drive
Address 2	
City	Eugene
State/Province	OR
Postal Code	97405
Country	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Dae		KIM	

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

City	State/Province	Country of Residence
Bellevue	WA	US

Mailing Address of Inventor:

Address 1	16729 SE 63rd Place
Address 2	
City	Bellevue
State/Province	WA
Postal Code	98006
Country	US

Inventor 3 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Margaret		HOANG	

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	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

City	Seattle	State/Province	WA	Country of Residence	US

Mailing Address of Inventor:

Address 1	1111 South Nevada Street		
Address 2			
City	Seattle	State/Province	WA
Postal Code	98108	Country	US

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Mark		GREGORY	

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

City	Boise	State/Province	ID	Country of Residence	US

Mailing Address of Inventor:

Address 1	2147 S. Tollgate Place		
Address 2			
City	Boise	State/Province	ID
Postal Code	83709	Country	US

Inventor 5 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Erin		PIAZZA	

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

City	Edmonds	State/Province	WA	Country of Residence	US

Mailing Address of Inventor:

Address 1	15314 57th Pl W		
Address 2			
City	Edmonds	State/Province	WA
Postal Code	98026	Country	US

Inventor 6 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Denise		ZHOU	

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

City	Seattle	State/Province	WA	Country of Residence	US

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

Mailing Address of Inventor:

Address 1	826 24th Ave S		
Address 2			
City	Seattle	**State/Province**	WA
Postal Code	98144	**Country** i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	141429		
Email Address	zIPPatentDocketingMailboxUS@Cooley.com	Add Email	Remove Email

Application Information:

Title of the Invention	CHEMICAL COMPOSITIONS AND USES THEREOF		
Attorney Docket Number	NATE-037C01US 321329-2719	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	40	**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.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

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)
Customer Number	141429		

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	16/272487	2019-02-11
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
16/272487	Claims benefit of provisional	62/771212	2018-11-26
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
16/272487	Claims benefit of provisional	62/629180	2018-02-12

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

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).

Remove

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

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

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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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

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.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

NanoString EX2011.173

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

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: NanoString Technologies, Inc.

Mailing Address Information For Applicant:

Address 1	530 Fairview Avenue North		
Address 2	Suite 2000		
City	Seattle	**State/Province**	WA
Country	US	Postal Code	98109
Phone Number		Fax Number	
Email Address			

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

Assignee Information including Non-Applicant Assignee 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.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

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.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

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

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1			
Address 2			
City		**State/Province**	
Country i		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

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

Signature	/ Matthew Pavao /			Date (YYYY-MM-DD)	2021-09-16
First Name	Matthew	Last Name	Pavao	Registration Number	50,572

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

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	NATE-037C01US 321329-2719
	Application Number	
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF	

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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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 records.

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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).

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 CooperationTreaty.

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.

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Sequence Listing was accepted.
See attached Validation Report.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Zhang, Yizhu (ASRC)
Timestamp: [year=2021; month=9; day=17; hr=11; min=0; sec=31; ms=436; ]
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Validated By CRFValidator v 1.0.5

Application No: 17476707 Version No: 1.0

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Total Errors: 1
No. of SeqIDs Defined: 175
Actual SeqID Count: 175

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Kim, Dae
Hoang, Margaret
Gregory, Mark
Piazza, Erin
Zhou, Denise

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<210> 43
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ttagaggtga ggaggcgaag ata 83

<210> 57
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<400> 58
caagcagaag acggcatacg agataggtca ctgtgactgg agttcagacg tgtgctcttc

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 17/476,707

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))	19 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 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).						420
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	2240

APPLICATION AS AMENDED - PART II

		(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($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	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	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	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.

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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
17/476,707	09/16/2021	1634	2240	NATE-037C01US 321329-2719	19	1

CONFIRMATION NO. 5473

FILING RECEIPT

141429
Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

OC000000128537902

Date Mailed: 09/22/2021

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.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Joseph M. BEECHEM, Eugene, OR;
Dae KIM, Bellevue, WA;
Margaret HOANG, Seattle, WA;
Mark GREGORY, Boise, ID;
Erin PIAZZA, Edmonds, WA;
Denise ZHOU, Seattle, WA;

Applicant(s)

NanoString Technologies, Inc., Seattle, WA;

Power of Attorney: The patent practitioners associated with Customer Number 141429

Domestic Priority data as claimed by applicant

This application is a CON of 16/272,487 02/11/2019
which claims benefit of 62/771,212 11/26/2018
and claims benefit of 62/629,180 02/12/2018

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

NanoString EX2011.201

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

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The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 17/476,707**
Projected Publication Date: 12/30/2021
Non-Publication Request: No
Early Publication Request: No
Title

CHEMICAL COMPOSITIONS AND USES THEREOF

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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countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Complete if Known	
Application Number	17/476,707
Filing Date	09-16-2021
First Named Inventor	Joseph M. BEECHEM
Art Unit	1634
Examiner Name	Not Yet Assigned
Attorney Docket Number	NATE-037/C01US 321329-2719

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	5,320,814	06-14-1994	Walt et al.	
	2.	5,543,838	08-06-1996	Hosier et al.	
	3.	5,635,352	06-03-1997	Urdea et al.	
	4.	6,100,030	08-08-2000	McCasky Feazel et al.	
	5.	6,207,392	03-27-2001	Weiss et al.	
	6.	6,429,027	08-06-2002	Chee et al.	
	7.	6,596,257	07-22-2003	Bryan	
	8.	6,602,661	08-05-2003	Knezevic et al.	
	9.	6,690,470	02-10-2004	Baer et al.	
	10.	6,942,968	09-13-2005	Dickinson et al.	
	11.	7,060,507	06-13-2006	Akeson et al.	
	12.	7,132,519	11-07-2006	Monforte et al.	
	13.	7,214,477	05-08-2007	Emmert-Buck	
	14.	7,255,999	08-14-2007	Singh et al.	
	15.	7,402,399	07-22-2008	Mukherjeei et al.	
	16.	7,569,392	08-04-2009	Levy et al.	
	17.	7,648,828	01-19-2010	Chan-Hui et al.	
	18.	7,728,287	06-01-2010	Felton et al.	
	19.	8,003,312	08-23-2011	Krutzik et al.	
	20.	8,221,972	07-17-2012	Lemaire et al.	
	21.	8,309,306	11-13-2012	Nolan et al.	
	22.	8,349,574	01-08-2013	Bates et al.	

Examiner Signature:		Date Considered	

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. 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 2 hours 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, 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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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Complete if Known	
Application Number	17/476,707
Filing Date	09-16-2021
First Named Inventor	Joseph M. BEECHEM
Art Unit	1634
Examiner Name	Not Yet Assigned
Attorney Docket Number	NATE-037/C01US 321329-2719

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	23.	8,362,415	01-29-2013	Felton et al.	
	24.	8,486,623	07-16-2013	Monforte et al.	
	25.	8,637,650	01-28-2014	Cherkasov et al.	
	26.	8,753,824	06-17-2014	Papin et al.	
	27.	8,865,414	10-21-2014	Hennig et al.	
	28.	8,906,700	12-09-2014	Lim et al.	
	29.	9,046,477	06-02-2015	Emedcoles et al.	
	30.	9,228,948	01-05-2016	Emedcoles et al.	
	31.	9,297,762	03-29-2016	Emedcoles et al.	
	32.	9,304,084	04-05-2016	Emedcoles et al.	
	33.	9,376,678	06-28-2016	Gormley et al.	
	34.	10,501,777	12-10-2019	Beechem et al.	
	35.	10,640,816	05-05-2020	Beechem et al.	
	36.	2001/0002315	05-31-2001	Schultz et al.	
	37.	2001/0007775	07-12-2001	Seul et al.	
	38.	2001/0023078	09-20-2001	Bawendi et al.	
	39.	2001/0029049	10-11-2001	Walt et al.	
	40.	2001/0034034	10-25-2001	Bruchez et al.	
	41.	2001/0053334	12-20-2001	Chen et al.	
	42.	2002/0028457	03-07-2002	Empedocles et al.	
	43.	2002/0034737	03-21-2002	Drmanac	
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Complete if Known	
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First Named Inventor	Joseph M. BEECHEM
Art Unit	1634
Examiner Name	Not Yet Assigned
Attorney Docket Number	NATE-037/C01US 321329-2719

U.S. PATENT DOCUMENTS

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	45.	2002/0039728	04-04-2002	Kain et al.	
	46.	2002/0051971	05-02-2002	Stuelpnagel et al.	
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	84.	2014/0121117	05-01-2014	Tanner	
	85.	2014/0371088	12-18-2014	Webster	
	86.	2015/0080233	03-19-2015	Bendall et al.	
	87.	2015/0099650	04-09-2015	Sood et al.	
	88.	2015/0132763	05-14-2015	Amorese et al.	

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	90.	2015/0287578	10-08-2015	Bendall et al.	
	91.	2015/0329852	11-19-2015	Nolan	
	92.	2015/0368697	12-24-2015	Samusik et al.	
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	95.	2017/0016053	01-19-2017	Beechem et al.	
	96.	2017/0275669	09-28-2017	Weissleder et al.	
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	101.	JP 2008-542783 A	11-27-2008	Universite des Sciences et Technologie		corresponds to WO 2007/000669 A2
	102.	WO 97/14028 A2	04-17-1997	Luminex Corporation		
	103.	WO 97/07245 A1	02-27-1997	Rabani, Ely		
	104.	WO 99/18434 A1	04-15-1999	Trustees of Tufts College		

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	105.	WO 00/73777 A1	12-07-2000	Bioarray Solutions, LLC		
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	118.	WO 2018/091676 A1	05-24-2018	Spatial Transcriptomics AB		

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	129.	"Digital spatial profiling platform allows for spatially-resolved, high-plex quantification of mRNA distribution and abundance on FFPE and fresh frozen tissue sections," Poster #3434, Power Point Presentation, AACR Annual Meeting, April 14-18, 2018, 1 page.	
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	143.	WERNER, J. H. et al., "Current status of DNA sequencing by single molecule detection, Proc. SPIE 3602, Advances in Fluorescence Sensing Technology IV, pp. 355-366 (1999).	
	144.	ZINCHUK, V. et al., "Quantitative Colocalization Analysis of Multicolor Confocal Immunofluorescence Microscopy Images: Pushing Pixels to Explore Biological Phenomena," The Japan Society of Histochemistry and Cytochemistry, 40(4):101-111 (2007).	
	145.	ZOLLINGER, D. et al., "Abstract 3434: Digital spatial profiling platform allows for spatially resolved, high-plex quantification of mRNA distribution and abundance on FFPE and fresh frozen tissue sections," AACR Annual Meeting, April 14-18, 2018, 2 pages.	

Examiner Signature:		Date Considered	

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. 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 2 hours 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, 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 (1-800-786-9199) and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	43923108
Application Number:	17476707
International Application Number:	
Confirmation Number:	5473
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Customer Number:	141429
Filer:	Robert E. Powers/Sylvana Householder
Filer Authorized By:	Robert E. Powers
Attorney Docket Number:	NATE-037C01US 321329-2719
Receipt Date:	01-OCT-2021
Filing Date:	16-SEP-2021
Time Stamp:	09:54:41
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	Transmittal Letter	NATE-037-C01US_321329-2719_IDSTransmittal.pdf	111906 e21040e9f7ba7e32ffdec49d99206a8e279d5dd7	no	3

Warnings:

NanoString EX2011.214

Information:					
2	Information Disclosure Statement (IDS) Form (SB08)	NATE-037-C01US_321329-2719_sb08.pdf	319150 2e357062c39577d54eee50c699b887468749e208	no	9
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Non Patent Literature	DefinitionOfAbundance.pdf	18585 d182444e9b2ddb426be8f430915af21740fa1760	no	1
Warnings:					
Information:					
4	Non Patent Literature	StrategiesForDetecting.pdf	226710 fae7fe1528a6ea6471315d51418ce3e598c71fac	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	676351		

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.

Docket No.: NATE-037/C01US 321329-2719
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Joseph M. BEECHEM	Confirmation No.:	5473
Application No.:	17/476,707	Group Art Unit:	1634
Filed:	September 16, 2021	Examiner:	Not Yet Assigned

For: CHEMICAL COMPOSITIONS AND USES THEREOF

Via EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.56, 1.97, AND 1.98

In accordance with the duty of disclosure set forth in 37 C.F.R. §1.56, Applicant hereby submits the following information in conformance with 37 C.F.R. §§1.97 and 1.98. It is respectfully requested that the information be expressly considered during the prosecution of this application, and the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

[X] Pursuant to 37 C.F.R. §1.98, a copy of non-US patent document Cite No. 127 and Cite No. 140 cited on the attached Form used in lieu of PTO/SB/08 is enclosed.

[X] No copies of the remaining foreign patent, foreign patent application, or non-patent literature publications listed on the attached Form used in lieu of PTO/SB/08 are being provided pursuant to 37 C.F.R. §1.98(d) because the publications were previously cited by or submitted to the Office in prior Application Serial No. 16/272,487 to which the above-identified application claims priority under 35 U.S.C. §120.

[X] No copies of any U.S. patents or U.S. patent application publications listed on the attached Form used in lieu of PTO/SB/08 are being provided pursuant to 37 C.F.R. §1.98.

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[] Publication(s) listed on the attached Form used in lieu of PTO/SB/08 were cited in a foreign search or examination report corresponding to application serial no. and mailed on .

[] Enclosed is a copy of a non-English publication(s) _____ Pursuant to §609 of the M.P.E.P., Applicant submits the attached foreign search or examination report, which cites such non-English language publication(s).

[] Enclosed is a copy of a non-English publication(s) _____ English language publication _____ (copy enclosed) claims priority from this non-English publication.

[] Enclosed is an explanation of non-English publication(s) _____ for which an English translation is not available.

[] Enclosed is an English translation of non-English publication(s) _____ cited on the attached Form used in lieu of PTO/SB/08.

[] Enclosed is an English language Abstract of non-English publication(s) _____ cited on the attached Form used in lieu of PTO/SB/08.

[] Enclosed is a copy of pending patent Application No. _____.

[X] Applicant would like to draw the Examiner's attention to the following co-pending U.S. applications:

U.S. Application No. 16/272,487, filed February 11, 2019
U.S. Application No. 16/596,587, filed October 8, 2019
U.S. Application No. 16/596,596, filed October 8, 2019
U.S. Application No. 17/476,712, filed September 16, 2021

[X] In accordance with **37 C.F.R. §1.97(b)**, no additional fee for submission of this Information Disclosure Statement is required, as it is filed within any one of the following time periods:

[] within three months from the filing date of this national application other than a CPA under 37 C.F.R. § 1.53(d);

[] within three months from the date of entry of the national stage as set forth in 37 C.F.R. §1.491 in this international application;

[X] before the mailing date of a first Office action on the merits; or

[] before the mailing of a first Office action after the filing of a request for continued examination under 37 C.F.R. § 1.114.

In accordance with 37 C.F.R. § 1.97(g), this Information Disclosure Statement shall not be construed as to mean that a search has been made.

In accordance with 37 C.F.R. § 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be material to patentability as defined by 37 C.F.R § 1.56(b).

Remarks

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Form used in lieu of PTO/SB/08 to the undersigned.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 50-1283, under Order No. NATE-037/C01US 321329-2719 from which the undersigned is authorized to draw.

Dated: October 1, 2021

Respectfully submitted,
COOLEY LLP

COOLEY LLP
ATTN: IP Docketing Department
1299 Pennsylvania Avenue NW, Suite 700
Washington, DC 20004

Tel: (617) 937-2300
Fax: (617) 937-2400

By: /Robert E. Powers/
Robert E. Powers
Reg. No. 77,802
Matthew Pavao
Reg. No. 50,572

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037C01US 321329-2719	5473

141429 7590 12/03/2021

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

EXAMINER	
CENTRAL, DOCKET	

ART UNIT	PAPER NUMBER
OPAP	

NOTIFICATION DATE	DELIVERY MODE
12/03/2021	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):

zIPPatentDocketingMailboxUS@cooley.com

NanoString EX2011.219

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 17/476,707	**Applicant(s)** BEECHEM et al.	
	Examiner ANGELA E WALKER	**Art Unit** OPET	**AIA (FITF) Status** Yes

1. THE REQUEST FILED 16 September 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 ANGELA WALKER at (571) 272-1058. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/IRVIN DINGLE/ Lead Paralegal Specialist, OPET	

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037C01US 321329-2719

CONFIRMATION NO. 5473

141429
Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

PUBLICATION NOTICE



Title:CHEMICAL COMPOSITIONS AND USES THEREOF

Publication No.US-2021-0403998-A1
Publication Date:12/30/2021

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.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

NanoString EX2011.221

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037C01US 321329-2719	5473

141429 7590 01/19/2022

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

EXAMINER	
LU, FRANK WEI MIN	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
01/19/2022	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):

zIPPatentDocketingMailboxUS@cooley.com

NanoString EX2011.222

Office Action Summary	Application No. 17/476,707	Applicant(s) BEECHEM et al.	
	Examiner FRANK W LU	Art Unit 1634	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 _____.

☐ 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) 1-19 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) 1-19 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 9/16/2021 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 10/1/2021.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

NanoString EX2011.223

DETAILED ACTION

Drawings

1. Some words in Figures 26, 29, 32, and 33 cannot be recognized. Applicant is required to submit new Figures 26, 29, 32, and 33. No new matter may be introduced in the required drawing. Each drawing sheet submitted after the filing date of an application must be labeled in the top margin as either "Replacement Sheet" or "New Sheet" pursuant to 37 CFR 1.121(d).

Claim Objections

2. Claim 1 is objected to because of the following informalities: (1) "at least one target analyte" should be "at least one target nucleic acid" in view of step a); and (2) "in the first and the at least second location from the tissue sample" in step f) should be "in the first location and the at least second location from the tissue sample".

3. Claim 2 is objected to because of the following informality: "contacting the tissue sample with probes in step (a)" should be "said contacting the tissue sample with probes in step (a)".

4. Claim 9 is objected to because of the following informality: "performing the extension reaction of step (d) and step (e) comprises" should be "step (d) and step (e) comprise".

5. Claim 10 or 11 is objected to because of the following informality: "the sequencing" should be "the sequencing step".

Appropriate correction is required.

Claim Rejections - 35 USC § 112

6. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

> (a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to

make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. Scope of Enablement

Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for performing step a) of claim 1, does not reasonably provide enablement for spatially detecting at least one target analyte in a tissue sample using the methods recited in claims 1-19. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

Factors to be considered in determining whether a disclosure meets the enablement requirement of 35 USC 112, first paragraph, have been described by the court in *In re Wands*, 8 USPQ2d 1400 (CA FC 1988). *Wands* states at page 1404,

"Factors to be considered in determining whether a disclosure would require undue experimentation have been summarized by the board in Ex parte Forman. They include (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims."

The Nature of the invention

The claims are drawn to a method for spatially detecting at least one target analyte in a tissue sample. The invention is a class of invention which the CAFC has characterized as "the

unpredictable arts such as chemistry and biology." *Mycogen Plant Sci., Inc. v. Monsanto Co.,* 243 F.3d 1316, 1330 (Fed. Cir. 2001).

The Breadth of The Claims

Claims 1-19 encompass a method for spatially detecting at least one target analyte in a tissue sample comprising: a) contacting the tissue sample with probes comprising a target binding domain that hybridizes to the at least one target analyte; b) collecting the probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample; c) collecting the probes, or portions thereof, bound to the at least one target analyte in an at least second location of the tissue sample; d) performing an extension reaction to form extension products comprising the probes, or portions thereof, collected in step (b) and at least one nucleic acid sequence that identifies the first location of the tissue sample; e) performing an extension reaction to form extension products comprising the probes, or portions thereof, collected in step (c) and at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and f) identifying the extension products produced in step (d) and step (e) by sequencing, thereby spatially detecting the at least one target analyte in the first and the at least second location of the tissue sample.

Working Examples

The specification provides working examples (see pages 28-42 of US 2021/0403998 A1, which is US Publication of this instant case) for: (1) Example 1-Two-Ended Adapter Ligation Method for 96 Multiplexed Samples; (2) Example Two-One-Ended Adapter Ligation Method for 96 Multiplexed Samples; (3) Example 3-Templated-Primer Extension Method for 96

Multiplexed Samples; (4) Example Four-Long Probe Hybridization Method for 96 Multiplexed Samples; (5) Example Five-Short Probe Hybridization Method for 96 Multiplexed Samples; (6) Example Six-Direct PCR Method for 96 Multiplexed Samples; (7) Example Seven-Spatially Detecting Target Analytes in a FFPE Sample; (8) Example Eight-Spatially Detecting Target Proteins in a Fluorescently Stained FFPE Sample; (9) Example Nine-Spatially Detecting Target RNAs in a FFPE Sample; (10) Example Ten-Spatially Detecting Target RNAs in Specific Sub-Regions of an ROI; and (11) Example Eleven-96-Plex Human Immuno-Oncology Panel. However, the specification provides no working example for spatially detecting at least one target analyte in a tissue sample using the methods recited in claims 1-19.

The Amount of Direction or Guidance Provided and The State of The Prior Art

Although the specification provides working examples (see pages 28-42 of US 2021/0403998 A1, which is US Publication of this instant case) for: (1) Example 1-Two-Ended Adapter Ligation Method for 96 Multiplexed Samples; (2) Example Two-One-Ended Adapter Ligation Method for 96 Multiplexed Samples; (3) Example 3-Templated-Primer Extension Method for 96 Multiplexed Samples; (4) Example Four-Long Probe Hybridization Method for 96 Multiplexed Samples; (5) Example Five-Short Probe Hybridization Method for 96 Multiplexed Samples; (6) Example Six-Direct PCR Method for 96 Multiplexed Samples; (7) Example Seven-Spatially Detecting Target Analytes in a FFPE Sample; (8) Example Eight-Spatially Detecting Target Proteins in a Fluorescently Stained FFPE Sample; (9) Example Nine-Spatially Detecting Target RNAs in a FFPE Sample; (10) Example Ten-Spatially Detecting Target RNAs in Specific Sub-Regions of an ROI; and (11) Example Eleven-96-Plex Human Immuno-Oncology Panel. However, the specification provides a guidance to show that at least

one target analyte in a tissue sample can be spatially detected using the methods recited in claims 1-19. Furthermore, there is no experimental condition and/or experimental data in the specification to support the claimed invention. During the process of the prior art search, the examiner has not found any prior art which is related to spatially detect at least one target analyte in a tissue sample using the methods recited in claims 1-19.

Level of Skill in The Art, The Unpredictability of The Art, and The Quantity of Experimentation Necessary

While the relative skill in the art is very high (the Ph.D. degree with laboratory experience), there is no predictability whether at least one target analyte in a tissue sample can be spatially detected using the methods recited in claims 1-19.

First, since the specification teaches that "[T]he present disclosure provides a method for spatially detecting at least one target analyte in at least one cell from a tissue sample comprising: (1) contacting at least one target analyte in at least one cell in a tissue sample with at least one probe comprising a target binding domain and an identifier oligonucleotide, wherein the identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain; (2) providing a force to a location of the tissue sample sufficient to release the identifier oligonucleotide; (3) collecting the released identifier oligonucleotide; (4) hybridizing to the released identifier oligonucleotide a single stranded nucleic acid template, wherein the nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, a nucleic acid sequence

comprising a unique molecular identifier, a nucleic acid sequence complementary to a second amplification primer binding site and optionally, an affinity molecule; (5) extending the identifier oligonucleotide of step (4) to form an extension product complementary to the single stranded nucleic acid template, wherein the extension product comprises the identifier oligonucleotide, the nucleic acid sequence complementary to the nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide was released, the nucleic acid sequence complementary to the unique molecular identifier and the second amplification primer binding site; (6) amplifying the extension product produced in step (5); and (7) Identifying the released identifier oligonucleotide by sequencing the amplified products produced in step (6), thereby spatially detecting the at least one target analyte in the at least one cell in a tissue sample" (see paragraph [0022] of US 2021/0403998 A1, which is US Publication of this instant case), the specification clearly indicate that at least one probe comprises a target binding domain and an identifier oligonucleotide wherein the identifier oligonucleotide comprises a first amplification primer binding site and a unique nucleic acid sequence which identifies the target analyte bound to the target binding domain and a single stranded nucleic acid template comprises a region complementary to the unique nucleic acid sequence of the identifier oligonucleotide, a nucleic acid sequence which identifies the specific location of the tissue sample from which the identifier oligonucleotide is released. However, **the scope of claim 1 is much broader than the scope of the teachings in the specification because claim 1 does not indicate that in extension products in step c), where at least one nucleic acid sequence that identifies the first location of the tissue sample comes from and in extension products in step d), where at least one nucleic acid sequence that identifies the at least second location of the tissue sample comes from.** Since claim 1 does not require that a

first probe from the probes only specifically hybridizes to a first nucleic acid from the least one target analyte in the first location of the tissue sample, the at least one nucleic acid sequence that identifies the first location of the tissue sample only specifically hybridizes the first probe, a second probe from the probes only specifically hybridizes to a second nucleic acid from the least one target analyte in the second location of the tissue sample, and the at least one nucleic acid sequence that identifies the second location of the tissue sample only specifically hybridizes the second probe, if each of the probes can hybridize to both the first nucleic acid from the least one target analyte in the first location of the tissue sample and the second nucleic acid from the least one target analyte in the second location of the tissue sample, the at least one nucleic acid sequence that identifies the first location of the tissue sample does not hybridize the first probe and the at least one nucleic acid sequence that identifies the second location of the tissue sample does not hybridize the second probe, it is predictable how the at least one nucleic acid sequence that identifies the first location of the tissue sample can be used to identify the first location of the tissue sample and how the at least one nucleic acid sequence that identifies the second location of the tissue sample can be used to identify the second location of the tissue sample such that the at least one target analyte in the first location and the at least second location of the tissue sample cannot be spatially detected by identifying the extension products produced in step (d) and step (e) by sequencing. Furthermore, since it is known that macromolecules such as proteins, polysaccharides, and nucleic acids are simply too large and too charged or polar to pass through biological membranes (see “Large Molecules Cross Membranes via Vesicles”) and claim 1 does not require to permeabilize the cells of the tissue sample, if the cells of the tissue sample are not permeabilized, it is unpredictable how probes comprising a target binding domain

can enter the cells of the tissue sample and hybridize to the nucleic acids of the at least one target analyte.

Second, since claim 8 does not require to contact the probes or portions thereof with the plurality of primers immobilized on the microscope slide, without contacting the probes or portions thereof with the plurality of primers immobilized on the microscope slide, it is unpredictable how the solid-phase amplification can be carried out in association with the microscope slide using the plurality of primers immobilized on the microscope slide as recited in claim 9.

Third, since extension products in step c) of claim 1 comprises the probes, or portions thereof, collected in step (b) and at least one nucleic acid sequence that identifies the first location of the tissue sample, without indicating where at least one unique molecular identifier comes from, it is unpredictable how the extension products of step (d) in claim 12 can further comprise at least one unique molecular identifier. Furthermore, since extension products in step d) of claim 1 comprises the probes, or portions thereof, collected in step (c) and at least one nucleic acid sequence that identifies the at least second location of the tissue sample, without indicating where at least one unique molecular identifier comes from, it is unpredictable how the extension products of step (e) in claim 13 can further comprise at least one unique molecular identifier.

Fourth, since extension products in step c) of claim 1 comprises the probes, or portions thereof, collected in step (b) and at least one nucleic acid sequence that identifies the first location of the tissue sample, without indicating where at least one amplification primer binding site comes from, it is unpredictable how the extension products of step (d) in claim 15 can further comprise at least one amplification primer binding site. Furthermore, since extension products in

step d) of claim 1 comprises the probes, or portions thereof, collected in step (c) and at least one nucleic acid sequence that identifies the at least second location of the tissue sample, without indicating where at least one amplification primer binding site comes from, it is unpredictable how the extension products of step (e) in claim 16 can further comprise at least one amplification primer binding site.

Case law has established that "(t)o be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation'." *In re Wright* 990 F.2d 1557, 1561. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970) it was determined that "[T]he scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art". The amount of guidance needed to enable the invention is related to the amount of knowledge in the art as well as the predictability in the art. Furthermore, the Court in *Genentech Inc. v Novo Nordisk* 42 USPQ2d 1001 held that "[I]t is the specification, not the knowledge of one skilled in the art that must supply the novel aspects of the invention in order to constitute adequate enablement".

In view of above discussions, the skilled artisan will have no way to predict the experimental results. Accordingly, it is concluded that undue experimentation is required to make the invention as it is claimed. The undue experimentation at least includes to test whether at least one target analyte in a tissue sample can be spatially detected using the methods recited in claims 1-19.

Conclusion

In the instant case, as discussed above, the level of unpredictability in the art is high, the specification provides one with no guidance that leads one to claimed methods. One of skill in the art cannot readily anticipate the effect of a change within the subject matter to which the claimed invention pertains. Thus given the broad claims in an art whose nature is identified as unpredictable, the unpredictability of that art, the large quantity of research required to define these unpredictable variables, the lack of guidance provided in the specification, the absence of any working example related to claimed invention and the no teaching in the prior art balanced only against the high skill level in the art, it is the position of the examiner that it would require undue experimentation for one of skill in the art to perform the method of the claim as broadly written.

8. The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

> The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

9. Claims 1-19 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention.

10. Claim 1 is rejected as vague and indefinite in view of step f) because it is unclear that the extension products produced in step (d) and step (e) are identified by sequencing what. Please clarify.

11. Claim 2 is rejected as vague and indefinite because it is unclear that at least two probes are from the probes in claim 1 or not since the claim does not indicate the relationship between at the least two probes and the probes in claim 1. Please clarify.

Conclusion

12. No claim is allowed.

13. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center. The faxing of such papers must conform with the notices published in the Official Gazette, 1096 OG 30 (November 15, 1988), 1156 OG 61 (November 16, 1993), and 1157 OG 94 (December 28, 1993)(See 37 CAR § 1.6(d)). The CM Fax Center number is (571)273-8300.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Frank Lu, Ph.D., whose telephone number is (571)272-0746. The examiner can normally be reached on Monday-Friday from 9 A.M. to 5 P.M.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen, can be reached on (571)272-0731.

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). If you would

like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/FRANK W LU/
Primary Examiner, Art Unit 1634
January 12, 2022

Notice of References Cited	Application/Control No. 17/476,707	Applicant(s)/Patent Under Reexamination BEECHEM et al.	
	Examiner FRANK W LU	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A					
	B					
	C					
	D					
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FOREIGN PATENT DOCUMENTS

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	N					
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	P					
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	S					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	"Large Molecules Cross Membranes via Vesicles". Printed on 1/10/2022.
	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.

NanoString EX2011.236

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/476,707	BEECHEM et al.
	Examiner	Art Unit
	FRANK W LU	1634

CPC - Searched*		
Symbol	Date	Examiner
See attached file	01/10/2022	/FL/

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
See attached file	01/10/2022	/FL/

US Classification - Searched*			
Class	Subclass	Date	Examiner
435	6.1, 6.11. 6.12, 91.1, 91.2, 91.51, 283.1, 287.1, 287.2	01/10/2022	/FL/
436	94, 501	01/10/2022	/FL/
536	23.1, 24.3, 24.33, 25.3	01/10/2022	/FL/

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
STN and EAST (see attached files) and Inventor Searches	01/10/2022	/FL/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/FRANK W LU/ Primary Examiner, Art Unit 1634	January 10, 2022

NanoString EX2011.237

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     FILE 'MEDLINE, CAPLUS, BIOSIS, SCISEARCH, EMBASE' ENTERED AT 23:40:49 ON
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L1     14188678 S TISSUE OR SLIDE
L2     10462493 S HYBRIDIZE OR HYBRIDIZING OR HYBRIDIZATION OR BIND OR BINDING
L3      2600630 S COLLECT OR COLLECTING OR COLLECTION
L4        38397 S FIRST LOCATION OR FIRST SITE OR FIRST POSITION
L5        40378 S S SECOND LOCATION OR SECOND  SITE OR SECOND POSITION
L6      2658196 S AMPLIFY OR AMPLIFYING OR AMPLIFICATION OR EXTENSION OR EXTENS
L7      2177173 S SEQUENCING
L8            0 S L1 AND 2 AND L3 AND L4 AND L5 AND L6 AND L7
L9            2 S L1 AND L2 AND L3 AND L4 AND L5 AND L6
L10           2 DUP REM L9 (0 DUPLICATES REMOVED)
L11           2 S L1 AND L2 AND L3 AND L4 AND L5
L12    18215847 S LOCATION OR SITE OR POSITION
L13          37 S L1 AND L2 AND L3 AND L6 AND L7 AND L12
L14          29 DUP REM L13 (8 DUPLICATES REMOVED)
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	12	(tissue or slide) same (hybridize or hybridizing or hybridization or bind or binding) same (collect or collecting or collection) same first near3 (site or location) same second near3 (site or location) same (amplify or amplifying or amplification or extension or extense or extensing) same sequencing	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/01/10 23:34
L2	15	(tissue or slide) same (hybridize or hybridizing or hybridization or bind or binding) same (collect or collecting or collection) same first near3 (site or location) same second near3 (site or location) same (amplify or amplifying or amplification or extension or extense or extensing)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/01/10 23:37
L3	15	2 AND ((C12Q1/6806 OR C12Q2563/179 OR C12Q2565/514 OR C12Q1/6869 OR C12Q2525/191 OR C12Q2525/161 OR C12Q2521/501 OR C12Q1/6841 OR C12Q1/6876 OR C12Q2523/101 OR C12Q2531/113 OR C12Q1/6804 OR C12Q1/6855 OR C12Q1/6818 OR C12Q1/6848 OR C12Q1/6851 OR C12Q1/6858 OR C12Q1/686 OR C12Q2525/15 OR C12Q2545/107 OR C12Q2563/159 OR C12Q2600/16 OR C12Q1/6816 OR C12Q2523/319 OR C12Q2537/143 OR C12Q2525/204 OR C12Q2535/122 OR C12Q2563/185 OR C12Q2600/156 OR C12Q2600/166 OR C12Q1/24 OR C12Q1/6834 OR C12Q1/6874 OR C12Q1/6886 OR C12Q2600/106 OR C12Q2600/154 OR C12Q2600/158 OR C12Q2600/172 OR C12N15/1093 OR C12N15/1065 OR C12N15/1089 OR C12N15/113 OR C12N15/10 OR C12N15/1082 OR C40B40/06 OR B01L2300/0654 OR B01L2200/16 OR B01L2300/0627 OR B01L2300/0816 OR B01L2300/0819 OR B01L2300/0822 OR B01L2300/0851 OR B01L2300/0887 OR B01L2300/123 OR B01L2300/168 OR B01L2400/0406 OR B01L3/502707 OR B01L3/502715 OR	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/01/10 23:39

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[] Enclosed is a copy of pending patent Application No. _____.

[X] Applicant would like to draw the Examiner's attention to the following co-pending U.S. applications:

U.S. Application No. 16/272,487, filed February 11, 2019
U.S. Application No. 16/596,587, filed October 8, 2019
U.S. Application No. 16/596,596, filed October 8, 2019
U.S. Application No. 17/476,712, filed September 16, 2021

/FRANK W LU/

01/10/2022

[X] In accordance with **37 C.F.R. §1.97(b)**, no additional fee for submission of this Information Disclosure Statement is required, as it is filed within any one of the following time periods:

[] within three months from the filing date of this national application other than a CPA under 37 C.F.R. § 1.53(d);

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Complete if Known	
Application Number	17/476,707
Filing Date	09-16-2021
First Named Inventor	Joseph M. BEECHEM
Art Unit	1634
Examiner Name	Not Yet Assigned
Attorney Docket Number	NATE-037/C01US 321329-2719

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	5,320,814	06-14-1994	Walt et al.	
	2.	5,543,838	08-06-1996	Hosier et al.	
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Examiner Signature:	/FRANK W LU/	Date Considered	01/10/2022

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	23.	8,362,415	01-29-2013	Felton et al.	
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	43.	2002/0034737	03-21-2002	Drmanac	
	44.	2002/0034827	03-21-2002	Singh et al.	

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Filing Date	09-16-2021
First Named Inventor	Joseph M. BEECHEM
Art Unit	1634
Examiner Name	Not Yet Assigned
Attorney Docket Number	NATE-037/C01US 321329-2719

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	66.	2008/0118934	05-22-2008	Gerdes et al.	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Examiner Signature:	/FRANK W LU/	Date Considered	01/10/2022

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. 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 2 hours 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, 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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257095743 v1

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /F.W.L/

PTO/SB/06 (09-11)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 17/476,707	Filing Date 09/16/2021	☐ 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	04/19/2022	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 18	Minus	** 20	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 1	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	0

		(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".	/YOLANDA CHADWICK/
*** 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.**

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DocCode – SCORE

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Application Number: 17476707 Document Date: 04/19/2022

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Form Revision Date: March 1, 2019

Docket No.: NATE-037/C01US 321329-2719
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Joseph M. BEECHEM	Confirmation No.:	5473
Application No.:	17/476,707	Group Art Unit:	1634
Filed:	September 16, 2021	Examiner:	F. W. Lu

For: CHEMICAL COMPOSITIONS AND USES THEREOF

VIA EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT/RESPONSE TO OFFICE ACTION

This paper is filed in response to the Non-final Office Action mailed January 19, 2022. This paper is timely filed on or before April 19, 2022. Applicant submits the following Amendments and/or Remarks, and respectfully requests reconsideration of the application in view thereof.

Any extensions of time necessary to prevent abandonment of this application are hereby petitioned for under 37 C.F.R. §1.136(a), and any additional fees required (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 50-1283.

Amendments to the Claims begin on page 2 of this paper.
Amendments to the Drawings appear on page 6 of this paper.
Remarks/Arguments begin on page 7 of this paper.

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AMENDMENTS TO THE CLAIMS

Set forth below in ascending order, with status identifiers, is a complete listing of all claims currently under examination. Changes to any amended claims are indicated by [[double brackets]], ~~strikethrough~~ and/or underlining. This listing also reflects any cancellation and/or addition of claims.

1. (Currently Amended) A method for spatially detecting at least one target analyte in a tissue sample comprising:

a) contacting the tissue sample with a plurality of probes, wherein each of the probes ~~comprising~~comprise a target binding ~~domain~~domain that ~~hybridizes~~binds to the at least one target analyte, wherein the tissue sample has been treated to facilitate binding of the probes to the target analyte;

b) collecting the probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the probes, or portions thereof, from the first location of the tissue sample;

c) collecting the probes, or portions thereof, bound to the at least one target analyte in ~~an at least~~a second location of the tissue sample under conditions that release the probes, or portions thereof, from the second location of the tissue sample;

d) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the probes, or portions thereof, collected in step (b), thereby ~~to form~~forming a first plurality of extension products that ~~comprising~~comprise the probes, or portions thereof, collected in step (b) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;

e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the probes, or portions thereof, collected in step (c), thereby ~~to form~~forming a second plurality extension products that ~~comprising~~comprise the probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and

f) identifying the first plurality of extension products and the second plurality of extension products ~~produced in step (d) and step (e)~~ by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location and the ~~at least~~ second location of the tissue sample.

2. (Currently Amended) The method of claim 1, wherein ~~contacting the tissue sample with probes in step (a) comprises contacting the at least one target analyte with~~at least two probes in the plurality of probes from step (a) bind to the same target analyte, thereby tiling the probes on the ~~at least one~~ target analyte.

3. (Original) The method of claim 2, wherein step (a) further comprises ligating the at least two probes.

4. (Original) The method of claim 1, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.

5. (Canceled)

6. (Original) The method of claim 1, wherein the tissue sample is immobilized onto a microscope slide.

7. (Original) The method of claim 6, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.

8. (Currently Amended) The method of claim 7, wherein ~~the primers in~~ the plurality of primers [[are]]is immobilized on the microscope slide at their 5' ends.

9. (Currently Amended) The method of claim 8, wherein ~~performing the extension reaction~~each of step (d) and step (e) ~~comprises~~comprise performing a solid-phase amplification

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reaction, wherein the solid-phase amplification reaction is carried out ~~in association with~~on the microscope slide using the plurality of primers immobilized on the microscope slide.

10. (Currently Amended) The method of claim 1, wherein the sequencing step is next generation sequencing.

11. (Currently Amended) The method of claim 1, ~~wherein the sequencing comprises~~further comprising amplifying a library using the ~~extension products~~first plurality of extension products and the second plurality of extension products as templates.

12. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (d) further comprise~~at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier.

13. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (e) further comprise~~ at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier.

14. (Original) The method of claim 2, wherein one of the at least two probes comprises an amplification primer binding site.

15. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (d) further comprise~~ at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site.

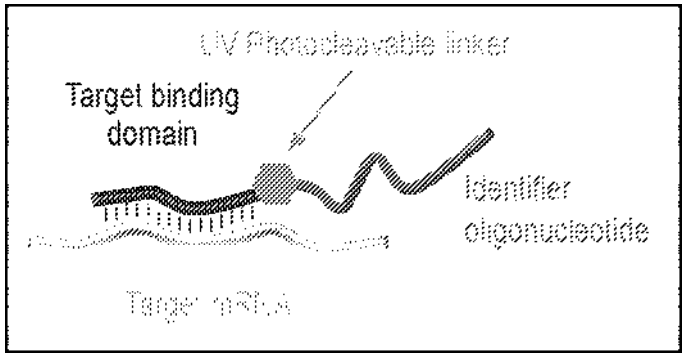
16. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (e) further comprise~~ at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one amplification primer binding site.

17. (Original) The method of claim 1, wherein the target binding domains comprise a single-stranded nucleic acid molecule.

18. (Original) The method of claim 1, wherein steps (b) and (c) are performed simultaneously.

19. (Original) The method of claim 1, wherein steps (d) and (e) are performed simultaneously.

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UV Photocleavable linker
Target binding domain
Identifier oligonucleotide
Target mRNA

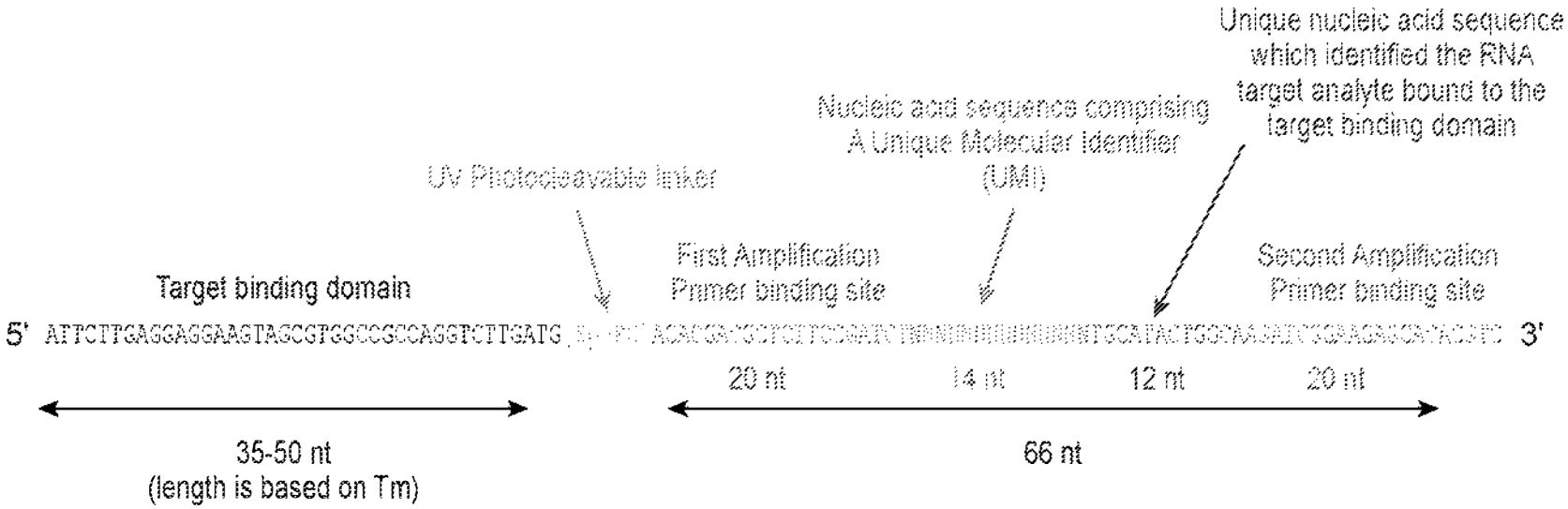

Unique nucleic acid sequence which identified the RNA target analyte bound to the target binding domain
Nucleic acid sequence comprising A Unique Molecular Identifier (UMI)
UV Photocleavable linker
First Amplification Primer binding site
Second Amplification Primer binding site
Target binding domain
5'
3'
20 nt
14 nt
12 nt
20 nt
35-50 nt
(length is based on Tm)
66 nt

FIG. 26

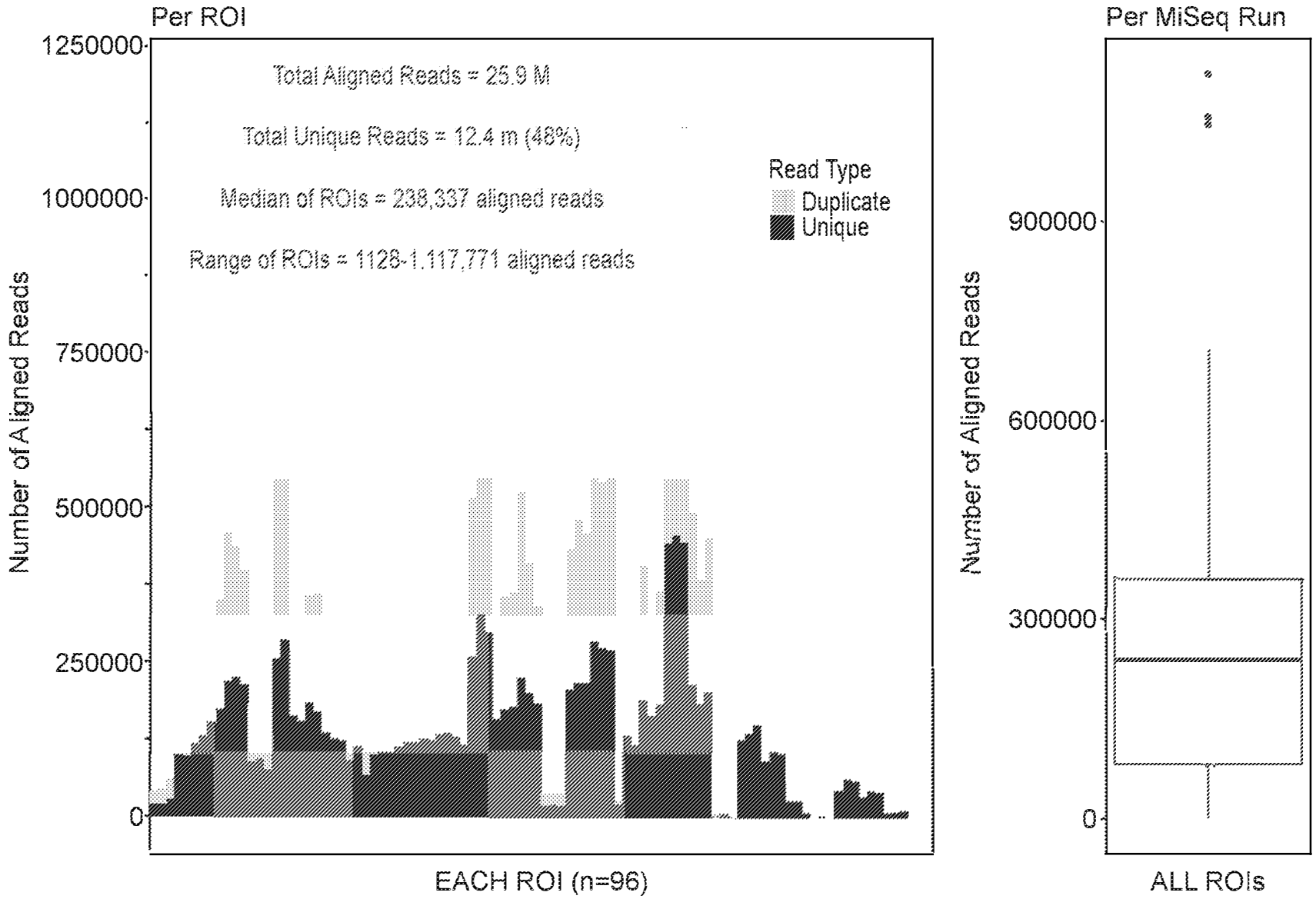


FIG. 29

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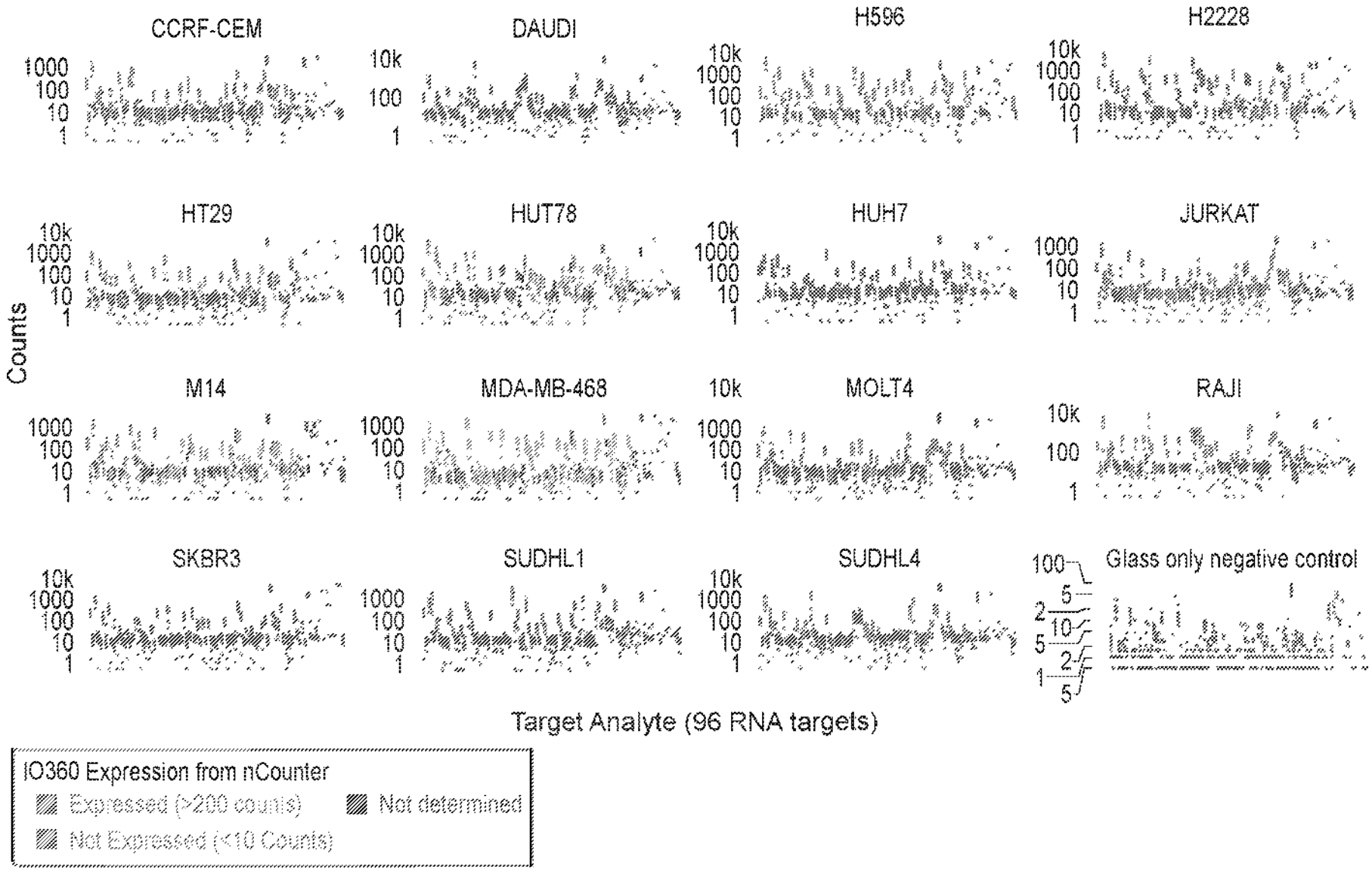

CCRF-CEM
DAUDI
H596
H2228
HT29
HUT78
HUH7
JURKAT
M14
MDA-MB-468
MOLT4
RAJI
SKBR3
SUDHL1
SUDHL4
Glass only negative control
Counts
Target Analyte (96 RNA targets)
IO360 Expression from nCounter
Expressed (>200 counts)
Not determined
Not Expressed (<10 Counts)

FIG. 32

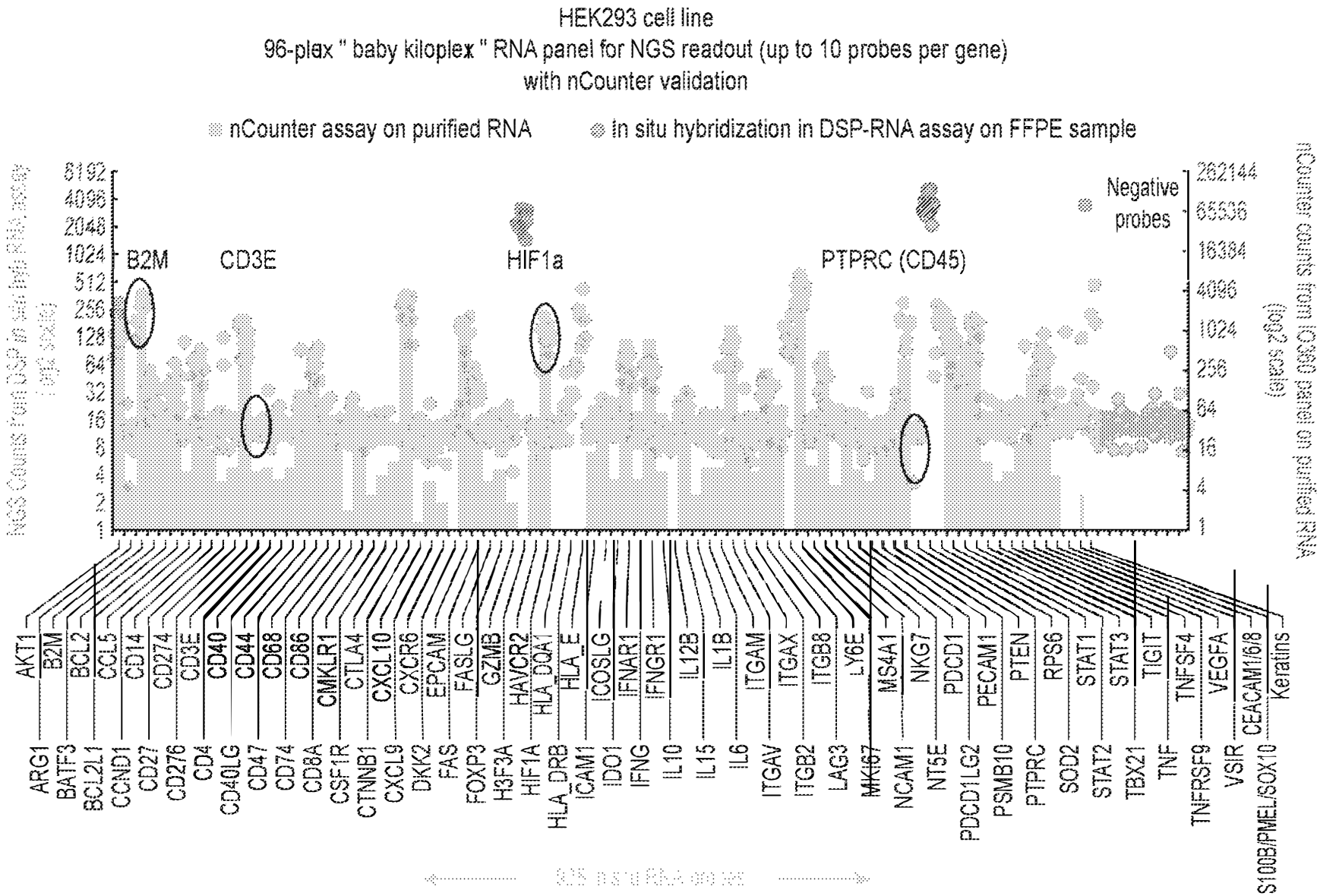

HEK293 cell line
96-plex " baby kiloplex " RNA panel for NGS readout (up to 10 probes per gene)
with nCounter validation
nCounter assay on purified RNA
in situ hybridization in DSP-RNA assay on FFPE sample
B2M
CD3E
HIF1a
PTPRC (CD45)
Negative probes
AKT1
B2M
BCL2
CCL5
CD14
CD274
CD3E
CD40
CD44
CD68
CD86
CMKLR1
CTLA4
CXCL10
CXCR6
EPCAM
FASLG
GZMB
HAVCR2
HLA_DQA1
HLA_E
ICOSLG
IFNAR1
IFNGR1
IL12B
IL1B
ITGAM
ITGAX
ITGB8
LY6E
MS4A1
NKG7
PDCD1
PECAM1
PTEN
RPS6
STAT1
STAT3
TIGIT
TNFSF4
VEGFA
CEACAM1/6/8
Keratins
ARG1
BATF3
BCL2L1
CCND1
CD27
CD276
CD4
CD40LG
CD47
CD74
CD8A
CSF1R
CTNNB1
CXCL9
DKK2
FAS
FOXP3
H3F3A
HIF1A
HLA_DRB
ICAM1
IDO1
IFNG
IL10
IL15
IL6
ITGAV
ITGB2
LAG3
MKI67
NCAM1
NT5E
PDCD1LG2
PSMB10
PTPRC
SOD2
STAT2
TBX21
TNF
TNFRSF9
VSIR
S100B/PMEL/SOX10

FIG. 33

Docket No.: NATE-037/C01US 321329-2719
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Joseph M. BEECHEM	Confirmation No.:	5473
Application No.:	17/476,707	Group Art Unit:	1634
Filed:	September 16, 2021	Examiner:	F. W. Lu

For: CHEMICAL COMPOSITIONS AND USES THEREOF

Via EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT
UNDER 37 C.F.R. §§ 1.56, 1.97, AND 1.98

In accordance with the duty of disclosure set forth in 37 C.F.R. §1.56, Applicant hereby submits the following information in conformance with 37 C.F.R. §§1.97 and 1.98. It is respectfully requested that the information be expressly considered during the prosecution of this application, and the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

[X] Pursuant to 37 C.F.R. §1.98, a copy of each non-US patent document cited on the attached Form used in lieu of PTO/SB/08 is enclosed.

[] No copies of the foreign patent, foreign patent application, or non-patent literature publications listed on the attached Form used in lieu of PTO/SB/08 are being provided pursuant to 37 C.F.R. §1.98(d) because the publications were previously cited by or submitted to the Office in prior Application Serial No(s). ____ to which the above-identified application claims priority under 35 U.S.C. §120.

[X] No copies of any U.S. patents or U.S. patent application publications listed on the attached Form used in lieu of PTO/SB/08 are being provided pursuant to 37 C.F.R. §1.98.

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[] Publication(s) listed on the attached Form used in lieu of PTO/SB/08 were cited in a foreign search or examination report corresponding to application serial no. and mailed on .

[] Enclosed is a copy of a non-English publication(s) _____ Pursuant to §609 of the M.P.E.P., Applicant submits the attached foreign search or examination report, which cites such non-English language publication(s).

[] Enclosed is a copy of a non-English publication(s) _____ English language publication _____ (copy enclosed) claims priority from this non-English publication.

[] Enclosed is an explanation of non-English publication(s) _____ for which an English translation is not available.

[] Enclosed is an English translation of non-English publication(s) _____ cited on the attached Form used in lieu of PTO/SB/08.

[] Enclosed is an English language Abstract of non-English publication(s) _____ cited on the attached Form used in lieu of PTO/SB/08.

[] Enclosed is a copy of pending patent Application No. _____.

[X] Applicant would like to draw the Examiner's attention to the following co-pending U.S. application:

U.S. Application No. 17/705,580, filed March 28, 2022

[X] In accordance with **37 C.F.R. §1.97(c)**, this Information Disclosure Statement is filed after the period specified in 37 C.F.R. § 1.97(b), but before the mailing of any of the following: a final action under 37 C.F.R. §1.113; a notice of allowance under 37 C.F.R. §1.311; or an action that otherwise closes prosecution in this application.

In accordance with 37 C.F.R. §1.97(c) also enclosed is:

[X] Fee under 37 C.F.R. §1.17(p) in the amount of $260.00;

[] Fee under 37 C.F.R. §1.17(p) in the amount of $130.00;

[] Fee under 37 C.F.R. §1.17(p) in the amount of $65.00; or

[] Statement as specified in 37 C.F.R. §1.97(e):

[] Each item of information contained in the Information Disclosure Statement cited herein 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 date of the Information Disclosure Statement; or

[] No item of information contained in the Information Disclosure Statement submitted herewith was cited in any communication from a foreign patent

office in a counterpart foreign application, and, to the knowledge of the undersigned, having made a reasonable inquiry, no item of information contained in the Information Disclosure Statement was known to any individual designated in 37 C.F.R. §1.56(c) more than three months prior to the filing date of the Information Disclosure Statement.

In accordance with 37 C.F.R. § 1.97(g), this Information Disclosure Statement shall not be construed as to mean that a search has been made.

In accordance with 37 C.F.R. § 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be material to patentability as defined by 37 C.F.R § 1.56(b).

Remarks

It is respectfully requested that the Examiner consider the above-noted information and return an initialed copy of the attached Form used in lieu of PTO/SB/08 to the undersigned.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Director is hereby authorized to charge any additional fees which may be required with respect to this communication, or credit any overpayment, to Deposit Account No. 50-1283, under Order No. NATE-037/C01US 321329-2719.

Dated: April 19, 2022

Respectfully submitted,
COOLEY LLP

USPTO CUSTOMER NO. 58249

By: /Robert E. Powers/
Robert E. Powers
Reg. No. 77,802
Matthew Pavao
Reg. No. 50,572

COOLEY LLP
ATTN: IP Docketing Department
1299 Pennsylvania Avenue NW, Suite 700
Washington, DC 20004

Tel: (617) 937-2300
Fax: (617) 937-2400

AMENDMENTS TO THE DRAWINGS

The attached four (4) sheets of drawings are replacement drawing sheets in compliance with 37 C.F.R. 1.84 and 1.121(d) for originally filed Figures 26, 29, 32 and 33. Please replace Figures 26, 29, 32 and 33 filed on September 16, 2021 with the replacement drawing sheets attached hereto. No new matter is added.

APPENDIX A: Replacement Drawing Sheets

NanoString EX2011.265

REMARKS

Upon entry to these amendments, claims 1-4 and 6-19 are pending in the present application. Claim 5 is canceled herein without prejudice or disclaimer. Applicant reserves the right to pursue the subject matter of the canceled claims in one or more continuation applications. Claims 1, 2, 8, 9, 10-13, 15 and 16 are amended herein. Support for the claim amendments can be found throughout the application as originally filed. Amendment and cancelation of certain claims is not to be construed as a dedication to the public of any of the subject matter of the claims as previously presented. No new matter is added.

Drawings

The Office asserts that some words in Figures 26, 29, 32 and 33 cannot be recognized and that Applicant is required to submit new Figures 26, 29, 32 and 33. Applicant herewith submits replacement sheets for Figures 26, 29, 32 and 33, as required by the Office. No new matter has been added. Applicant submits that with submission of the replacement sheets, this objection is moot.

Claim Objections

Claim 1 is objected to because of its recitation of "at least one target analyte" and "in the first and the at least second location from the tissue sample". Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 1 is amended herein to recite "contacting the tissue sample with a plurality of probes, wherein each of the probes comprise a target binding domain that binds to the at least one target analyte" in step (a) and "in the first location and the second location of the tissue sample" in step (f). Applicant submits that these amendments obviate this objection.

Claim 2 is objected to because of the recitation of "contacting the tissue sample with probes in step (a)". Without acquiescing as to the validity of the objection, and solely in an effort to expedite prosecution, claim 2 is amended herein to remove the objected to language. Applicant submits that this amendment obviates this objection.

NanoString EX2011.266

Claim 9 is objected to because of the recitation of "performing the extension reaction of step (d) and step (e) comprises". Without acquiescing as to the validity of the objection, and solely in an effort to expedite prosecution, claim 9 is amended herein per the suggestion of the Office to recite "each of step (d) and step (e) comprise". Applicant submits that this amendment obviates this objection.

Claim 10 is objected to because of the recitation of "the sequencing". Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 10 is amended herein per the suggestion of the Office to recite "the sequencing step". Applicant submits that this amendment obviates this objection.

Claim 11 is objected to because of the recitation of "the sequencing". Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 11 is amended herein to remove the objected-to language. Applicant submits that this amendment obviates this objection.

Withdrawal of the objections to the claims is respectfully requested in view of the amendments described *supra*.

Claim Rejections – 35 U.S.C. §112(a)

Claims 1-19 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for performing step (a) of claim 1, allegedly does not reasonably provide enablement for spatially detecting at least one target analyte in a tissue sample using the methods recited in claims 1-19. Applicant respectfully disagrees and traverses the rejection with respect to the pending claims, as amended herein.

As an initial matter, without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 5 is canceled herein without prejudice or disclaimer. Thus, the rejection, as it applies to the canceled claim, is moot.

The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation.[1] Importantly, a patent need not teach, and preferably omits, what is well

[1] *see* MPEP §2164.01 citing *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988)

known in the art.[2] Furthermore, the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation.[3] In order to make an enablement rejection, the Office has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention.[4] A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.[5]

Applicant submits that the Office has failed to meet their initial burden of establishing a reasonable basis to question the enablement for the claimed invention and that the specification clearly and unambiguously teaches the skilled artisan how to make and use the claimed invention. Moreover, Applicant submits that, guided by the teachings of the specification, the skilled artisan would not be forced to undertake any undue experimentation.

As an initial matter, Applicant submits that the skill level of a person of ordinary skill in the art in the context of the instant application is high, at least that of a PhD in biology, if not higher.

Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 1 is amended herein to recite a method for spatially detecting at least one target analyte in a tissue sample comprising: a) contacting the tissue sample with a plurality of probes, wherein each of the probes comprise a target binding domain that binds to the at least one target analyte, wherein the tissue sample has been treated to facilitate binding of the probes to the target analyte; b) collecting the probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the probes, or

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[2] *see* MPEP §2164.01 citing *In re Buchner,* 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.,* 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied,* 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.,* 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984)

[3] *see* MPEP §2164.01 citing *In re Certain Limited-Charge Cell Culture Microcarriers,* 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom.,Massachusetts Institute of Technologyv.A.B. Fortia,* 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985)

[4] *see* MPEP §2164.04

[5] *Id.*

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portions thereof, from the first location of the tissue sample; c) collecting the probes, or portions thereof, bound to the at least one target analyte in a second location of the tissue sample under conditions that release the probes, or portions thereof, from the second location of the tissue sample; d) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the probes, or portions thereof, collected in step (b), thereby forming a first plurality of extension products that comprise the probes, or portions thereof, collected in step (b) and the at least one nucleic acid sequence that identifies the first location of the tissue sample; e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the probes, or portions thereof, collected in step (c), thereby forming a second plurality extension products that comprise the probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and f) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location and the second location of the tissue sample. For clarity, Applicant submits that claim 1, as amended herein, encompasses methods wherein steps (b), (e) and (f) are repeated at a one or more additional locations of the tissue sample (*e.g.* a third location, a fourth location, a fifth location, etc.).

Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 9 is amended herein to recite wherein each of step (d) and step (e) comprise performing a solid-phase amplification reaction, wherein the solid-phase amplification reaction is carried out on the microscope slide using the plurality of primers immobilized on the microscope slide.

Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 15 is amended herein to recite that the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site and claim 16 is amended herein to recite that the at least one nucleic acid sequence

that identifies the second location of the tissue sample comprises at least one amplification primer binding site.

Applicant submits that it would be well within the purview of a person of ordinary skill in the art (*i.e.* a person with at least a PhD in biology) to practice the methods recited in claims 1-4 and 6-19, as amended herein. Importantly, the specification includes numerous examples that describe the spatial detection of target analytes in a tissue sample comprising the steps of contacting a tissue sample with a plurality of probes comprising a target binding domain; collecting the probes, or portions thereof, bound to target analytes in a first location of a tissue sample; collecting the probes, or portions thereof, bound to target analytes in a second location of a tissue sample; performing an extension reaction that incorporates a nucleic acid sequence that identifies the first location of the tissue sample into each of the probes, or portions thereof, collected from the first location; performing an extension reaction that incorporates a nucleic acid sequence that identifies the second location of the tissue sample into each of the probes, or portions thereof, collected from the second location; and sequencing the products of the extension reactions, thereby spatially detecting the target analyte in the first location and the second location of the tissue sample.

More specifically, Example 7 of the instant specification (*see* paragraphs [00418]-[00425] and Figures 21A-D and 22A-22D) describes the spatial detection of 30 different target proteins and 20 different target RNAs in tissue samples using a method comprising the steps of contacting the sample with probes comprising a target binding domain that binds to one of the 30 different target proteins or 20 different target RNAs, collecting the probes bound to target proteins or RNAs from more than one discrete, location on the tissue sample, performing extension reactions to incorporate into the collected probes nucleic acid sequences that specifically identify the location from which the probe was collected from, and sequencing the extension products, thereby spatially detecting the target proteins and target RNAs. Similarly, Example 8 (*see* paragraphs [00412]-[00413] and Figure 23) describes the spatial detection of 30 target proteins in a tissue sample, Example 9 (*see* paragraphs [00414]-[00415] and Figure 24) describes the spatial detection of 20 target RNAs in a tissue sample, Example 10 (*see* paragraphs [00416]-[00417] and Figure 25) describes the spatial detection of 30 target proteins in a tissue

sample, and Example 11 (*see* paragraphs [00418]-[00425] and Figures 26-34) describes the spatial detection of 96 target RNAs in a tissue sample using the methods recited in the instant claims. That is, the specification provides no less than five specific examples of the instantly claimed methods being successfully used to spatially detect dozens of different target analytes within discrete locations of tissue samples.

Thus, Applicant submits that the skilled artisan, guided both by the teachings of the specification, specifically the examples described *supra*, as well as by common knowledge known in the art, would readily be able to practice the instantly claimed method without any undue experimentation. For at least the reasons described *supra*, Applicant submits that the instant claims comply with the enablement requirement of 35 USC §112(a) or 35 U.S.C. §112 (pre-AIA), first paragraph.

Reconsideration of the claims and withdrawal of the rejection are respectfully requested.

Claim Rejections – 35 U.S.C. § 112(b)

Claims 1-19 are rejected under 35 U.S.C. § 112(b) as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor (or for applications subject to pre-AIA 35 U.S.C. 112, the applicant), regards as the invention. Applicant respectfully disagrees and traverses the rejection with respect to the pending claims, as amended herein.

As an initial matter, without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 5 is canceled herein without prejudice or disclaimer. Thus, the rejection, as it applies to the canceled claim, is moot.

The Office has rejected claim 1 as vague and indefinite in view of step (f) because it is allegedly unclear what is sequenced in step (f) to identify the extension products produced in step (d) and step (e). Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, step (f) of claim 1 is amended herein to recite "identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products". Applicant submits that this amendment obviates this rejection.

The Office has rejected claim 2 as vague and indefinite because it is allegedly unclear whether the two probes recited in claim 2 are the probes from claim 1 or not. Without acquiescing as to the validity of the rejection, and solely in an effort to expedite prosecution, claim 2 is amended herein to recite "wherein at least two probes in the plurality of probes from step (a) bind to the same target analyte, thereby tiling the probes on the target analyte". Applicant submit that these amendments obviate this rejection, and the skilled artisan, informed both from the teachings of the specification and knowledge known in the field, would readily appreciate the metes and bounds of claim 2, as amended herein.

Withdrawal of the rejections to the claims under 35 U.S.C. § 112(b) is respectfully requested in view of the amendments described *supra*.

CONCLUSION

In view of the foregoing, Applicant respectfully submits that no further impediments exist to the allowance of this application and, therefore, requests an indication of allowability. However, the Examiner is requested to call the undersigned if any questions or comments arise. The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283 **referencing Docket No.** NATE-037/C01US 321329-2719**.**

Dated: April 19, 2022

Respectfully submitted,
COOLEY LLP

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Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	9,376,677	06-28-2016	Mir	
	2.	9,644,204	05-09-2017	Hindson et al.	
	3.	9,975,122	05-22-2018	Masquelier et al.	
	4.	10,053,723	08-21-2018	Hindson et al.	
	5.	10,071,377	09-11-2018	Bharadwaj et al.	
	6.	2021/0403999	12-30-2021	Beechem et al.	

FOREIGN PATENT DOCUMENTS

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	7.	WO 2018/026873 A1	02-08-2018	California Institute of Techology		

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. 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 2 hours 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, 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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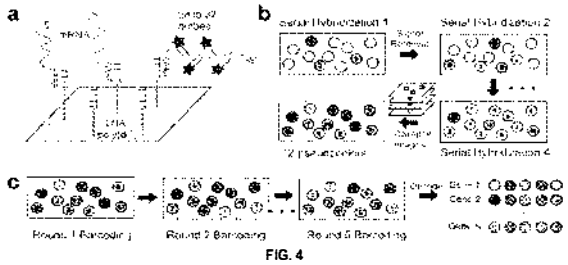


FIG. 4

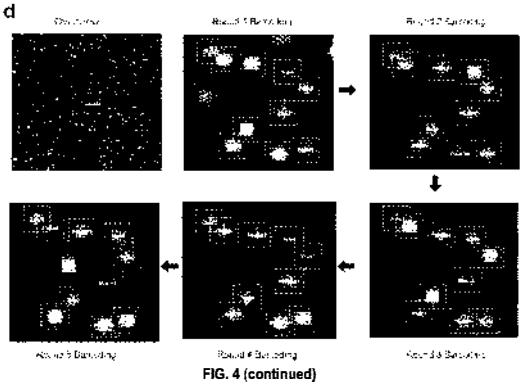


FIG. 4 (continued)

(57) Abstract: The present invention, among other things, provides technologies for detecting and/or quantifying nucleic acids in cells, tissues, organs or organisms. Pre-designed barcodes are associated specific molecular targets through sequential hybridization experiments. A pseudo-color based barcoding scheme is developed to overcome limitations in the previous generation of the technology such as lack of visual signals that can be associated with the probes or small internal within cell when carrying out in situ experiments.. The current method can be applied to both in vitro and in situ analysis. According to the method, each barcoding round comprises multiple serial hybridizations where a small number of colored signals (that are associated with probes) are used in each hybridization experiment within a serial hybridization round. Images from each serial hybridization experiment within the same serial hybridization round are combined to form a composite image for each barcoding round. In each barcoding round, the same set of molecular targets are analyzed. After all barcoding rounds are completed, associated of the barcode with these molecular targets is completed.

[Continued on next page]

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SEQUENTIAL PROBING OF MOLECULAR TARGETS BASED ON PSEUDO-COLOR BARCODES WITH EMBEDDED ERROR CORRECTION MECHANISM

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to United States Patent Application No. 15/225,820, filed on August 1, 2016 and entitled "Multiplex Labeling of Molecules by Sequential Hybridization Barcoding Using Probes With Cleavable Linkers," United States Patent Application No. 15/298,219, filed on October 19, 2016 and entitled "Error Correction of Multiplex Imaging Analysis by Sequential Hybridization," United States Patent Provisional Application No. 62/428,910, filed on December 1, 2016 and entitled "Single Molecule Profiling Through Serial and Barcoded Hybridization," United States Patent Provisional Application No. 62/456,291, filed on February 8, 2017 and entitled "Imaging-based Transcriptomic and Translational Profiling of 1000 Genes with *in vitro* seqFISH," and United States Patent Provisional Application No. 62/523,127, filed on June 21, 2017 and entitled "Transcriptome Profiling of 10,000 mRNAs by RNA SPOTs," each of which is hereby incorporated by reference herein in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Grant No. HD075605 and under Grant No. OD008530 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Transcription profiling of cells are essential for many purposes. Microscopy imaging which can resolve multiple mRNAs in single cells can provide valuable information regarding transcript abundance and localization, which are important for understanding the molecular basis of cell identify and developing treatment for diseases. Molecular profiling such as transcriptomic profiling of biological samples is essential for various purposes. For example, it would allow one to assess gene expression levels to detect and identify abnormal growth states such as cancers. Using nucleic acid detection as an example, current nucleic acid-based assays

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such as qPCR and microarrays have been useful, but they do not reach single molecule sensitivity. Next generation sequencing, on the other hand, involves amplification of the sample and reverse transcription of mRNA which can introduce biases and inaccuracies in quantification. Moreover, sample preparation and sequencing can be time-consuming and economically costly. Despite the fact that imaging has been used for mRNA transcripts quantification, it is limited to a few hundreds of genes. Many scientific questions require thousands of genes or even the whole transcriptome to be quantified.

[0004] What is needed in the are better methods and systems for carrying out imaging based transcriptomic profiling at a single molecule sensitivity with high accuracy in a time efficient manner.

SUMMARY OF THE INVENTION

[0005] The present invention provides certain insights into challenges or defects associated with existing technologies for profiling transcripts or DNA loci in cells, particularly for single cells. Moreover, the present invention provides new technologies for achieving effective such profiling, including of single cells. Provided technologies are broadly useful, including for example for profiling of isolated cells, cells in tissues, cells in organs, and/or cells in organisms.

[0006] For example, the present invention provides the insight that existing technologies such as single cell RNA-seq or qPCR require single cells to be isolated and put into multi-well format, which is a multiple step process that can be cost prohibitive, labor intensive and prone to artifacts. Furthermore, the present invention recognizes that existing *in situ* sequencing technologies that use enzymatic reactions to convert the mRNA into a DNA template first can be highly inefficient (for example in the mRNA to DNA conversion process), so that, often, only a small fraction of the RNAs are converted and detected. The present invention provides the particular insight that one major downside of such low efficiency, which is estimated at 1% for RT and 10% for PLA, is that it can introduce significant noise ad bias in the gene expression measurements. The present invention further recognizes that existing spectral mRNA barcoding technologies that utilize single molecule fluorescence in situ hybridization (smFISH) require distinct fluorophores for scale up, and may be limited in the number of barcodes that can be

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generated. smFISH also requires splitting probes into barcoding subsets during hybridization. Because smFISH often uses two or more colors for a target, it produces high density of objects in the image, which can increase the complexity of data analysis.

[0007] Among other things, the present inventions provides new technologies for profiling, for example, transcripts and/or DNA loci, that overcome one or more or all of the problems associated with methods prior to the present invention. In some embodiments, the present invention provides methods for detecting multiple targets, *e.g.*, transcripts or DNA loci, in a cell through a sequential barcoding scheme that permits multiplexing of different targets.

[0008] In one aspect, disclosed herein is a method of barcoding molecular targets. The method comprises: identifying N molecular targets in a biological sample, wherein the N molecular targets are immobilized; associating a unique barcode to each molecular target via n sequential barcoding rounds (where $n \geq 2$), wherein each barcoding round comprises m serial hybridizations of probes collectively bound to the N molecular targets (where $m \geq 2$), and optionally removing probes between two barcoding rounds. In some embodiments, each serial hybridization in turn further comprises: contacting one or more groups of probes to a subset of the N molecular targets, the total number of groups of probes corresponding to the number of molecular targets in the subset, where probes in each group comprise one or more binding sequences specifically targeting a molecular target in the subset, where each probe is capable of generating at least one detectable visual signal representing binding of the probe to a molecular target in the subset, and where probes in the one or more groups generate one or more different detectable visual signals corresponding to the number of molecular targets in the subset; detecting the detectable visual signals that reflect the binding between the one or more groups of probes and the subset of the N molecular targets; and removing the visual signals, when applicable, prior to the next serial hybridization; wherein the unique barcode to each molecular target consists of n components, each component is assigned from S unique symbols, where S is an integer that equal to or greater than $\sqrt[n]{N}$. N and n are both integer.

[0009] In some embodiments, the detecting the detectable visual signals comprises: capturing, for each serial hybridization round, an image of the detectable visual signals that

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reflect the binding between the one or more groups of probes and the subset of the *N* molecular targets.

[0010] In some embodiments, the method further comprises: generating, for each barcoding round, a composite image by superimposing *m* images corresponding to the *m* serial hybridizations, wherein the *m* images are aligned based on one or more alignment references whose positions remain constant relative to the biological sample.

[0011] In some embodiments, the method further comprises: applying Gaussian analysis to super-localize the detectable visual signals in an image.

[0012] In some embodiments, the method further comprises: decoding the detectable visual signals in each composite image based on the unique barcodes for the N molecular targets and the S unique symbols.

[0013] In some embodiments, the method further comprises: detecting reference visual signals associated with the one or more alignment reference.

[0014] In some embodiments, the one or more alignment references comprise one or more selected from the group consisting of an oligonucleotide sequence immobilized on the coverslips and detected by a complementary oligo, a common sequence embedded in all probes, a microscopic object, a metal bead, a gold bead, a polystyrene bead, a PCR handle sequence on a primary binding probe, and combinations thereof.

[0015] In some embodiments, the *n* sequential barcoding rounds includes *x* round for error correction, where *x* is an integer equal or greater than 1; and wherein assigning unique barcodes for each of *N* molecular targets requires *S* unique symbols, where *S* is an integer equal or greater than $\sqrt[n-x]{N}$.

[0016] In some embodiments, the biological sample comprises a tissue sample, a cell sample, a cell extract sample, a nucleic acid sample, an RNA transcript sample, a protein sample, an mRNA sample, DNA molecules, protein molecules, RNA and DNA isoform molecules, single nucleotide polymorphism molecules, or combinations thereof.

[0017] In some embodiments, the method further comprises: determining a secondary molecular target that are associated with the *N* molecular targets by contacting the biological sample with molecules specifically binding to the secondary molecular target.

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[0018] In some embodiments, the secondary molecular target comprises one selected from the group consisting of a RNA binding protein molecule, ribosome, a DNA binding protein molecule, a transcription factor, a chromatin binding protein, a protein binding molecule, a scaffold protein, and combinations thereof.

[0019] In some embodiments, probes in the one or more groups of probes further comprise: one or more binding sequences each specifically targeting one or more sites within a molecular target in the biological sample; and n unique readout sequences associated with the one or more binding sequences, wherein, in each barcoding round, only one unique readout sequence is associated with a detectable visual signal for a particular molecular target.

[0020] In some embodiments, the one or more binding sequences target multiple different sites within the same molecular target. In some embodiments, the one or more binding sequences target multiple different sites within different molecular targets.

[0021] In some embodiments, each probe comprises one or more of the *n* unique readout sequences.

[0022] In some embodiments, at least one of the *n* unique readout sequences is located in an overhang sequence directly connected to the binding sequence of a probe.

[0023] In some embodiments, at least one of the n unique readout sequences is indirectly connected to the binding sequence of a probe via one or more intermediate molecules.

[0024] In some embodiments, the one or more intermediate molecules comprise an RNA bridge probe, a DNA bridge probe, a protein bridge probe, a probe for hybridization chain reaction (HCR), a hairpin nucleic acid probe, an HCR initiator, an HCR polymer, or combinations thereof.

[0025] In some embodiments, the one or more binding sequences specifically target one or more non-nucleic acid sites in the molecular target, and wherein the n unique readout sequences comprising nucleic acid sequences that are directly or indirectly connected to the binding sequences of the probes.

[0026] In some embodiments, the detectable visual signal is connected to the binding sequence of a probe or an intermediate molecule via a cleavable linker.

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[0027] In some embodiments, the one or more binding sequences comprises a peptide sequence binding to a specific antigen within a particular molecular target, an aptmer, or click chemistry group.

[0028] In some embodiments, the S unique symbols comprise colors, numbers, letters, shapes, or combinations thereof.

[0029] In some embodiments, for each serial hybridization, the one or more groups of probes to a non-overlapping subset of the *N* molecular targets.

[0030] In one aspect, disclosed herein is a method of hybridization analysis of binding between labeled probes and molecular targets in a biological sample. The method comprises: generating multiple composite images of labeled probes bound to a plurality of molecular targets in the biological sample, wherein each composite image is generated from a plurality of images of labeled probes collectively bound to the plurality of molecular targets, wherein the plurality of molecular targets are immobilized within the biological sample, and wherein each image of the plurality of images reveals: labeled probes bound to a subset of molecular targets within the plurality of molecular targets, wherein the labeled probes comprise one or more groups of probes, the total number of groups of probes corresponding to the number of molecular targets in the subset, wherein probes in each group comprise one or more binding sequences specifically targeting a molecular target in the subset, and wherein each labeled probe is capable of generating a visual signal representing binding of the probe to a molecular target; and one or more reference targets whose positions remain constant in the biological sample for aligning the plurality of images.

[0031] In some embodiments, the biological sample comprises a tissue sample, a cell sample, a cell extract sample, a nucleic acid sample, an RNA transcript sample, a protein sample, an mRNA sample, DNA molecules, protein molecules, RNA and DNA isoform molecules, single nucleotide polymorphism molecules, or combinations thereof.

[0032] In some embodiments, in each image, the labelled probes bind to a non-overlapping subset of molecular targets within the plurality of molecular targets.

[0033] In some embodiments, the method further comprises: contacting the one or more groups of probes with the subset of molecular targets of the plurality of molecular targets; detecting visual signals that reflect the binding between the one or more groups of probes and

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molecular targets in the subset; and removing the visual signals, when applicable, prior to a next round of hybridization of labeled probes binding to a new subset of molecular targets within the plurality of molecular targets.

[0034] In some embodiments, the method further comprises: detecting reference visual signals associated with the one or more alignment references.

[0035] In some embodiments, the method further comprises: aligning the plurality of images based on the positions of the one or more alignment references.

[0036] In one aspect, disclosed herein is a sequential hybridization method that comprises the steps of: identifying a plurality of target genes; and associating, via sequential hybridization of binding probes to the plurality of target genes, a first plurality of unique codes with the plurality of target genes, where each target gene in the plurality of target genes is represented by a unique code in the first plurality of unique codes, where the sequential hybridization comprises n rounds of hybridization (where $n \geq 2$). Here, each round of hybridization in n rounds of hybridization in turn comprises the steps of contacting the plurality of target genes with a plurality of binding probes, where each probe in the plurality of binding probes comprises: a binding sequence that specifically binds a target sequence in a gene in the plurality of target genes, where target genes from the plurality of target genes are spatially transfixed from each other, and where each probe is capable of emitting a detectable visual signal upon binding of the probe to a target sequence; detecting visual signals that reflect the binding between the plurality of binding probes and the plurality of target genes; and removing the visual signals, when applicable, prior to the next round of hybridization. In some embodiments, probes used in the n rounds of hybridization are capable of emitting at least F types of detectable visual signals (where $F \geq 2$ and F^n is greater than the number of target genes in the plurality of target genes). In some embodiments, a unique code in the first plurality of unique codes for a target gene consists of n components. In some embodiments, each component is determined by visual signals that reflect the binding between binding probes and the target gene during one of the n rounds of hybridization. In some embodiments, the n rounds of hybridization include m error correction round ($m \geq 1$). In some embodiments, a second plurality of unique codes for the plurality of target genes is generated after the m error correction round is removed from the n

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rounds of hybridization. In some embodiments, each unique code in the second plurality of unique codes consists of $(n - m)$ components and uniquely represents a target gene in the plurality of target genes.

[0037] In some embodiments, the plurality of target genes are located on immobilized nucleic acids selected from the group consisting of mRNAs, chromosomal DNAs and combinations thereof. In some embodiments, n is 4 or greater, 5 or greater, or 10 or greater. In some embodiments, the m error correction round comprises one round of the n rounds of hybridization. In some embodiments, the one round of the n rounds of hybridization is a repeat of one of the remaining one or more $(n - 1)$ rounds of the n rounds of hybridization. In some embodiments, where $m \leq 0.5n$.

[0038] In some embodiments, the at least F types of detectable visual signals comprises one selected from the group consisting of a fluorescence signal, a color signal, a red signal, a green signal, a yellow signal, a combined color signal representing two or more colors, and combinations thereof.

[0039] In some embodiments, a probe in the plurality of binding probes further comprises a signal moiety that emits a detectable visual signal upon binding of the probe to a target sequence.

[0040] In some embodiments, the signal moiety is connected to the binding sequence of the probe via a cleavable linker.

[0041] In some embodiments, each component of a n-component unique code in the first plurality of unique codes is assigned a numerical value that corresponds to one of the at least F types of detectable visual signals; and where at least one component of the n-component unique code is determined based on the numerical values of all or some of the other n-1 components. In some embodiments, the n-component unique code is determined as:

$\{j_1, j_2, \ldots (a_1*j_1 + a_2*j_2 \ldots + a_n*j_n + C) \bmod F, \ldots, j_n\}$, where j_1 is a numerical value that corresponds the detectable visual signals used in the first round of hybridization, j_2 is a numerical value that corresponds the detectable visual signals used in the second round of hybridization, and j_n is a numerical value that corresponds the detectable visual signals used in the nth round of hybridization; and where $j_1, j_2, \ldots j_n, a_1, a_2, \ldots a_n$ and n are none zero integers and C is an integer.

[0042] In some embodiments, m, n, F, i, j and k are all integers.

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[0043] In one aspect disclosed herein is a hybridization method that comprises the steps of: identifying a plurality of target genes; performing sequential hybridization of binding probes to the plurality of target genes, where the sequential hybridization comprises n rounds of hybridization (where $n \geq 2$). Here, each round of hybridization in n rounds of hybridization in turn comprises: contacting the plurality of target genes with a plurality of binding probes, where each probe in the plurality of binding probes comprises: a binding sequence that specifically binds a target sequence in a gene in the plurality of target genes, where target genes from the plurality of target genes are spatially transfixed from each other, and where each probe is capable of emitting a detectable visual signal upon binding of the probe to a target sequence; detecting visual signals that reflect the binding between the plurality of binding probes and the plurality of target genes, where each target gene in the plurality of target genes is represented by visual signals that are unique for the target gene, and where probes used in the n rounds of hybridization are capable of emitting at least F types of detectable visual signals (where $F \geq 2$, and F^n is greater than the number of target genes in the plurality of target genes); and removing the visual signals, when applicable, prior to the next round of hybridization; and performing serial hybridizations against one or more serial target genes, where the expression level of each serial target gene is above a predetermined threshold value, and where each serial hybridization in turn comprises: contacting the one or more serial target genes with a plurality of binding probes, where each probe in the plurality of binding probes comprises: a binding sequence that specifically binds a target sequence in a serial target gene in the one or more serial target genes, where one or more serial target genes are spatially transfixed from each other, where each probe is capable of emitting a detectable visual signal upon binding of the probe to the target sequence, and where probes binding to target sequences in the same serial target gene emit the same detectable visual signals; and detecting visual signals that reflect the binding between the plurality of binding probes and the one or more serial target gene.

[0044] In some embodiments, the n rounds of hybridization generate a first plurality of unique codes, where each target gene in the plurality of target genes is represented by a unique code in the first plurality of unique codes.

[0045] In some embodiments, where a unique code in the first plurality of unique codes for a target gene consists of n components, and where each component is determined by visual

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signals that reflect the binding between binding probes and the target gene during one of the n rounds of hybridization.

[0046] In some embodiments, the n rounds of hybridization include m error correction round ($m \geq 1$), where a second plurality of unique codes for the plurality of target genes is generated after the m error correction round is removed from the n rounds of hybridization, and where each unique code in the second plurality of unique codes consists of $(n - m)$ components and uniquely represents a target gene in the plurality of target genes.

In some embodiments, the method of hybridization further comprises the step of: identifying the one or more serial target genes based on expression levels of candidate target genes.

In some embodiments, the plurality of target genes are located on immobilized nucleic acids selected from the group consisting of mRNAs, chromosomal DNAs and combinations thereof.

[0047] In some embodiments, the one or more serial target genes are located on immobilized nucleic acids selected from the group consisting of mRNAs, chromosomal DNAs and combinations thereof.

[0048] In some embodiments, each unique code in the first plurality of unique codes consists of n component, where each component of a n-component unique code in the first plurality of unique codes is assigned a numerical value that corresponds to one of the at least F types of detectable visual signals; and where at least one component of the n-component unique code is determined based on the numerical values of all or some of the other n-1 components.

In some embodiments, the n-component unique code is determined as:

$\{j_1, j_2, \ldots (a_1*j_1 + a_2*j_2 \ldots + a_n*j_n + C) \text{mod } F, \ldots, j_n\}$, where j_1 is a numerical value that corresponds the detectable visual signals used in the first round of hybridization, j_2 is a numerical value that corresponds the detectable visual signals used in the second round of hybridization, and j_n is a numerical value that corresponds the detectable visual signals used in the nth round of hybridization; and where $j_1, j_2, \ldots j_n, a_1, a_2, \ldots a_n$ are none zero integers and C is an integer.

[0049] In one aspect, disclosed herein is a non-transitory computer-readable medium containing instructions that, when executed by a computer processor, cause the computer processor to: associate, via sequential hybridization of binding probes to a plurality of target genes, a first plurality of unique codes with the plurality of target genes, where each target gene in the plurality of target genes is represented by a unique code in the first plurality of unique codes,

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where the sequential hybridization comprises n rounds of hybridization (where $n \geq 2$). Here each round of hybridization in n rounds of hybridization in turn comprises: contacting the plurality of target genes with a plurality of binding probes, where each probe in the plurality of binding probes comprises: a binding sequence that specifically binds a target sequence in a gene in the plurality of target genes, where target genes from the plurality of target genes are spatially transfixed from each other, and where each probe is capable of emitting a detectable visual signal upon binding of the probe to a target sequence; detecting visual signals that reflect the binding between the plurality of binding probes and the plurality of target genes; and removing the visual signals, when applicable, prior to the next round of hybridization.

[0050] In some embodiments, probes used in the n rounds of hybridization are capable of emitting at least F types of detectable visual signals (where $F \geq 2$ and F^n is greater than the number of target genes in the plurality of target genes). In some embodiments, a unique code in the first plurality of unique codes for a target gene consists of n components. In some embodiments, each component is determined by visual signals that reflect the binding between binding probes and the target gene during one of the n rounds of hybridization. In some embodiments, the n rounds of hybridization include m error correction round ($m \geq 1$). In some embodiments, a second plurality of unique codes for the plurality of target genes is generated after the m error correction round is removed from the n rounds of hybridization. In some embodiments, each unique code in the second plurality of unique codes consists of $(n - m)$ components and uniquely represents a target gene in the plurality of target genes.

[0051] In one aspect, disclosed herein is a non-transitory computer-readable medium containing instructions that, when executed by a computer processor, cause the computer processor to: perform sequential hybridization of binding probes to a plurality of target genes, where the sequential hybridization comprises n rounds of hybridization (where $n \geq 2$).

[0052] Here, each round of hybridization in n rounds of hybridization comprises: contacting the plurality of target genes with a plurality of binding probes, where each probe in the plurality of binding probes comprises: a binding sequence that specifically binds a target sequence in a gene in the plurality of target genes, where target genes from the plurality of target genes are spatially transfixed from each other, and where each probe is capable of emitting a detectable visual signal upon binding of the probe to a target sequence; detecting visual signals

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that reflect the binding between the plurality of binding probes and the plurality of target genes, where each target gene in the plurality of target genes is represented by visual signals that are unique for the target gene, and where probes used in the n rounds of hybridization are capable of emitting at least F types of detectable visual signals (where $F \geq 2$, and F^n is greater than the number of target genes in the plurality of target genes); and removing the visual signals, when applicable, prior to the next round of hybridization; and perform serial hybridizations against one or more serial target genes, where the expression level of each serial target gene is above a predetermined threshold value, where each serial hybridization comprises: contacting the one or more serial target genes with a plurality of binding probes, where each probe in the plurality of binding probes comprises: a binding sequence that specifically binds a target sequence in a serial target gene in the one or more serial target genes, where one or more serial target genes are spatially transfixed from each other, where each probe is capable of emitting a detectable visual signal upon binding of the probe to the target sequence, and where probes binding to target sequences in the same serial target gene emit the same detectable visual signals; and detecting visual signals that reflect the binding between the plurality of binding probes and the one or more serial target gene.

[0053] In any of the embodiments disclosed herein, m, n, F, i, j and k are all integers. Embodiments disclosed herein can be applied individually or in combination in any aspect disclosed herein.

[0054] In one aspect, disclosed herein is a composition comprising a plurality of primary probes, a first plurality of bridge probes, and first plurality of readout probes.

[0055] In some embodiments, each primary probe in the plurality of primary probes comprises: a primary binding sequence that binds to a complementary target sequence in a target nucleic acid molecule, and a first overhang sequence connected to one end of the primary binding sequence.

[0056] In some embodiments, each bridge probe in the first plurality of bridge probes comprises a binding sequence that specifically binds to all or a part of the first overhang sequence of a primary probe of the plurality of primary probes, and one or more readout binding targets connected in series and linked to the binding sequence.

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[0057] In some embodiments, each readout probe in the first plurality of readout probes comprises: a readout binding sequence that specifically binds to a first readout binding target of the one or more readout binding targets of a bridge probe of the first plurality of bridge probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0058] In these embodiments, the signal moiety is capable of emitting a first detectable visual signal upon binding of each readout probe from the first plurality of readout probes to the first readout binding target of one of the one or more readout binding targets.

[0059] In some embodiments, the composition further comprises: a second plurality of readout probes, wherein each readout probe comprises: a readout binding sequence that specifically binds to a second readout binding target of the one or more readout binding targets in a bridge probe of the first plurality of bridge probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0060] In these embodiments, the signal moiety is capable of emitting a second detectable visual signal upon binding of each readout probe from the second plurality of readout probes to the second readout binding target of the one or more readout binding targets.

[0061] In some embodiments, the composition further comprises: a second overhang sequence, linked to the other end of the primary binding sequence.

[0062] In some embodiments, the composition further comprises: a second plurality of bridge probes, wherein each bridge probe comprises: a binding sequence that specifically binds to all or a part of the second overhang sequence of a primary probe of the plurality of primary probes, and one or more additional readout binding targets connected in series and linked to the binding sequence.

[0063] In some embodiments, the composition further comprises: a third plurality of readout probes, wherein each readout probe comprises: a readout binding sequence that specifically binds to a first additional readout binding target of the one or more additional readout binding targets in a bridge probe of the second plurality of bridge probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0064] In these embodiments, the signal moiety is capable of emitting a third detectable visual signal upon binding of each readout probe from the third plurality of readout probes to the first additional readout binding target of the one or more additional readout binding targets.

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[0065] In some embodiments, the composition further comprises: a fourth plurality of readout probes. Each readout probe in the fourth plurality of readout probes comprises: a readout binding sequence that specifically binds to a second additional readout binding target of the one or more additional readout binding targets in a bridge probe of the second plurality of bridge probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0066] In these embodiments, the signal moiety is capable of emitting a fourth detectable visual signal upon binding of each readout probe from the fourth plurality of readout probes to the second additional readout binding target of the one or more additional readout binding targets.

[0067] In some embodiments, the cleavable linker is selected from the group consisting of an enzyme cleavable linker, a nucleophile/base sensitive linker, reduction sensitive linker, a photo-cleavable linker, an electrophile/acid sensitive linker, a metal-assisted cleavable linker, and an oxidation sensitive linker.

[0068] In some embodiments, the cleavable linker is a disulfide bond or a nucleic acid restriction site. In some embodiments, the one or more readout binding targets comprises three or more readout binding targets.

[0069] In some embodiments where second overhang is present, the additional one or more readout binding targets comprises three or more readout binding targets.

[0070] In one aspect, disclosed herein is a sequential hybridization method utilizing a plurality of primary probes, a first plurality of bridge probes, and first plurality of readout probes. In some embodiments, the method comprises the steps of: a) contacting a target nucleic acid molecule with a plurality of primary probes, where each primary probe comprises: a primary binding sequence that binds to a complementary target sequence within the target nucleic acid molecule, and a first overhang sequence connected to one end of the primary binding sequence; b) contacting, after step a) the target nucleic acid molecule with a first plurality of bridge probes, where each bridge probe comprises: a binding sequence that specifically binds to all or a part of the first overhang sequence of a primary probe of the plurality of primary probes, and one or more readout binding targets connected in series and linked to the binding sequence; and c) contacting, after step b) the target nucleic acid molecule with a first plurality of readout probes, wherein each readout probe comprises: a readout binding sequence that specifically binds to a

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first readout binding target of the one or more readout binding targets of a primary probe of the plurality of primary probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0071] In these embodiments, the signal moiety is capable of emitting a first detectable visual signal upon binding of each readout probe from the first plurality of readout probes to the first readout binding target of the one or more readout binding targets of a bridge probe of the first plurality of bridge probes.

[0072] In some embodiments, the method further comprises the steps of: c1) imaging the target nucleic acid molecule after step c) so that interactions between the first plurality of readout probes and the first readout binding target of the one or more readout binding targets of a primary bridge probe are detected by the presence of first detectable visual signal; and c2) applying, after step c1) a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the first plurality of readout probes.

[0073] In some embodiments, the method further comprises: d) contacting, after step c), the target nucleic acid molecule with a second plurality of readout probes. Each readout probe comprises: a readout binding sequence that specifically binds to a second readout binding target of the one or more readout binding targets of a bridge probe, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0074] In these embodiments, the signal moiety is capable of emitting a second detectable visual signal upon binding of each readout probe from the second plurality of readout probes to the second readout binding target of the one or more readout binding targets of a bridge probe of the first plurality of bridge probes.

[0075] In some embodiments, the method further comprises: d1) imaging the target nucleic acid molecule after step d) so that interactions between the second plurality of readout probes and the second readout binding target of the one or more readout binding targets of a bridge probe are detected by the presence of second detectable visual signal; and d2) applying a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the second plurality of readout probes.

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[0076] In some embodiments, each primary probe in the plurality of primary probes further comprises: a second overhang sequence connected to the other end of the primary binding sequence.

[0077] In some embodiments, the method further comprises: e) contacting, after step d), the target nucleic acid molecule with a second plurality of bridge probes. Each bridge probe comprises: a binding sequence that specifically binds to all or a part of the second overhang sequence of a primary probe of the plurality of primary probes, and one or more additional readout binding targets connected in series and linked to the binding sequence.

[0078] In some embodiments, the method further comprises: f) contacting, after step e), the target nucleic acid molecule with a third plurality of readout probes. Each readout probe comprises: a readout binding sequence that specifically binds to a first additional readout binding target of the one or more additional readout binding targets of a bridge probe in the second plurality of bridge probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0079] In these embodiments, the signal moiety is capable of emitting a third detectable visual signal upon binding of each readout probe from the third plurality of readout probes to the first additional readout binding target of the one or more additional readout binding targets.

[0080] In some embodiments, the method further comprises: f1) imaging the target nucleic acid molecule after step f) so that interactions between the third plurality of readout probes and the first additional readout binding target of the one or more additional readout binding targets of a bridge probe in the second plurality of bridge probes are detected by the presence of the third detectable visual signal; and f2) applying a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the third plurality of readout probes.

[0081] In some embodiments, the method further comprises: g) contacting, after step f), the target nucleic acid molecule with a fourth plurality of readout probes. Each readout probe comprises: a readout binding sequence that specifically binds to a second additional readout binding target of the one or more additional readout binding targets of a bridge probe in the second plurality of bridge probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

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[0082] In these embodiments, the signal moiety is capable of emitting a fourth detectable visual signal upon binding of each readout probe from the fourth plurality of readout probes to the second additional readout binding target of the one or more additional readout binding targets.

[0083] In some embodiments, the method further comprises: h1) imaging the target nucleic acid molecule after step g) so that interactions between the fourth plurality of readout probes and the second additional readout binding target of the one or more additional readout binding targets of a bridge probe in the second plurality of bridge probes are detected by the presence of the fourth detectable visual signal; and h2) applying a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the fourth plurality of readout probes.

[0084] In some embodiments, the target nucleic acid molecule is an mRNA or a DNA. In some embodiments, the target nucleic acid molecule is within an intact mammalian cell. In some embodiments, the intact mammalian cell is a human cell.

[0085] In these embodiments, the cleavable linker is selected from the group consisting of an enzyme cleavable linker, a nucleophile/base sensitive linker, reduction sensitive linker, a photo-cleavable linker, an electrophile/acid sensitive linker, a metal-assisted cleavable linker, and an oxidation sensitive linker. In these embodiments, the cleavable linker is a disulfide bond or a nucleic acid restriction site. In these embodiments, the one or more readout binding targets comprises three or more readout binding targets.

[0086] In these embodiments where a second overhang is present, the additional one or more readout binding targets comprises three or more readout binding targets.

[0087] In one aspect, disclosed herein is a composition that comprises a plurality of primary probes and a first plurality of readout probes. In these embodiments, each primary probe comprises: a primary binding sequence that binds to a complementary target sequence in a target nucleic acid molecule, and a first overhang sequence connected to one end of the primary binding sequence, wherein the first overhang sequence comprises one or more readout binding targets connected in series. Also in these embodiments, each readout probe comprises: a readout binding sequence that specifically binds to a first readout binding target of the one or more readout binding targets in a first overhang sequence, and a signal moiety linked to the readout

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binding sequence via a cleavable linker. In these embodiments, the signal moiety is capable of emitting a first detectable visual signal upon binding of each readout probe from the first plurality of readout probes to the first readout binding target of one of the one or more readout binding targets.

[0088] In some embodiments, the composition further comprises: a second plurality of readout probes, where each readout probe comprises: a readout binding sequence that specifically binds to a second readout binding target of the one or more readout binding targets in a first overhang sequence, and a signal moiety linked to the readout binding sequence via a cleavable linker. In these embodiments, the signal moiety is capable of emitting a second detectable visual signal upon binding of each readout probe from the second plurality of readout probes to the second readout binding target of the one or more readout binding targets.

[0089] In some embodiments, a primary probe further comprises: a second overhang sequence, linked to the other end of the primary binding sequence, where the second overhang sequence comprises one or more additional readout binding targets connected in series.

[0090] In some embodiments, the composition further comprises a third plurality of readout probes, where each readout probe comprises: a readout binding sequence that specifically binds to a first additional readout binding target of the one or more additional readout binding targets in a second overhang sequence, and a signal moiety linked to the readout binding sequence via a cleavable linker. In these embodiments, the signal moiety is capable of emitting a third detectable visual signal upon binding of each readout probe from the third plurality of readout probes to the first additional readout binding target of the one or more additional readout binding targets.

[0091] In some embodiments, the composition further comprises a fourth plurality of readout probes, where each readout probe comprises: a readout binding sequence that specifically binds to a second additional readout binding target of the one or more additional readout binding targets in a second overhang sequence, and a signal moiety linked to the readout binding sequence via a cleavable linker. In these embodiments, the signal moiety is capable of emitting a fourth detectable visual signal upon binding of each readout probe from the fourth plurality of readout probes to the second additional readout binding target of the one or more additional readout binding targets.

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[0092] In any embodiments disclosed herein, the cleavable linker is selected from the group consisting of an enzyme cleavable linker, a nucleophile/base sensitive linker, reduction sensitive linker, a photo-cleavable linker, an electrophile/acid sensitive linker, a metal-assisted cleavable linker, and an oxidation sensitive linker.

[0093] In any embodiments disclosed herein, the cleavable linker is a disulfide bond or a nucleic acid restriction site.

[0094] In any embodiments disclosed herein, the one or more readout binding targets comprises three or more readout binding targets.

[0095] In embodiments where a second overhang sequence is present, the additional one or more readout binding targets comprises three or more readout binding targets.

[0096] In some embodiments, the target nucleic acid molecule is an mRNA or a DNA. In some embodiments, the target nucleic acid molecule is within an intact mammalian cell. In some embodiments, the intact mammalian cell is a human cell.

[0097] In one aspect, disclosed herein is a sequential hybridization method utilizing with a plurality of primary probes and a first plurality of readout probes. The method comprises the steps of: a) contacting a target nucleic acid molecule with a plurality of primary probes. Each primary probe comprises: a primary binding sequence that binds to a complementary target sequence within the target nucleic acid molecule, and a first overhang sequence connected to one end of the primary binding sequence, wherein the first overhang sequence comprises one or more readout binding targets connected in series; and b) contacting, after step a) the target nucleic acid molecule with a first plurality of readout probes. Each readout probe comprises: a readout binding sequence that specifically binds to a first readout binding target of the one or more readout binding targets of a primary probe of the plurality of primary probes, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[0098] In these embodiments, the signal moiety is capable of emitting a first detectable visual signal upon binding of each readout probe from the first plurality of readout probes to the first readout binding target of one of the one or more readout binding targets.

[0099] In some embodiments, the method further comprises the steps of: b1) imaging the target nucleic acid molecule after step b) so that interactions between the first plurality of readout probes and the first readout binding target of the one or more readout binding targets of a

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primary bridge probe are detected by the presence of the first detectable visual signal; and b2) applying a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the first plurality of readout probes.

[00100] In some embodiments, the method further comprises the steps of: c) contacting, after step b), the target nucleic acid molecule with a second plurality of readout probes. Each readout probe comprises: a readout binding sequence that specifically binds to a second readout binding target of the one or more readout binding targets of a primary probe, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[00101] In these embodiments, the signal moiety is capable of emitting a second detectable visual signal upon binding of each readout probe from the second plurality of readout probes to the second readout binding target of the one or more readout binding targets.

[00102] In some embodiments, the method further comprises the steps of: c1) imaging the target nucleic acid molecule after step c) so that interactions between the second plurality of readout probes and the second readout binding target of the one or more readout binding targets of a primary probe are detected by the presence of the second detectable visual signal; and c2) applying a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the second plurality of readout probes.

[00103] In some embodiments, each primary probe in the plurality of primary probes further comprises: a second overhang sequence connected to the other end of the primary binding sequence, wherein the second overhang sequence comprises one or more additional readout binding targets connected in series.

[00104] In some embodiments, the method further comprises the steps of: d) contacting, after step c), the target nucleic acid molecule with a third plurality of readout probes. Each readout probe comprises: a readout binding sequence that specifically binds to a first additional readout binding target of the one or more additional readout binding targets of a primary probe, and a signal moiety linked to the readout binding sequence via a cleavable linker.

[00105] In these embodiments, the signal moiety is capable of emitting a third detectable visual signal upon binding of each readout probe from the third plurality of readout probes to the first additional readout binding target of the one or more additional readout binding targets.

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[00106] In some embodiments, the method further comprises the steps of: d1) imaging the target nucleic acid molecule after step d) so that interactions between the second plurality of readout probes and the second readout binding target of the one or more readout binding targets of a primary probe are detected by the presence of the second detectable visual signal; and d2) applying a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the second plurality of readout probes.

[00107] In some embodiments, the method further comprises the steps of: e) contacting, after step d), the target nucleic acid molecule with a fourth plurality of readout probes. Each readout probe comprises: a readout binding sequence that specifically binds to a second additional readout binding target of the one or more additional readout binding targets of a primary probe, and a signal moiety linked to the readout binding sequence via a cleavable linker,

[00108] In these embodiments, the signal moiety is capable of emitting a fourth detectable visual signal upon binding of each readout probe from the fourth plurality of readout probes to the second additional readout binding target of the one or more additional readout binding targets.

[00109] In some embodiments, the method further comprises the steps of: e1) imaging the mRNA after step d) so that interactions between the fourth plurality of readout probes and the second additional readout binding target of the one or more additional readout binding targets of a primary probe are detected by the presence of the fourth detectable visual signal; and e2) applying a cleaving agent to cleave the linker, thereby eliminating the signal moiety from each readout probe in the fourth plurality of readout probes.

[00110] In some embodiments, the target nucleic acid molecule is an mRNA or a DNA. In some embodiments, the target nucleic acid molecule is within an intact mammalian cell. In some embodiments, the intact mammalian cell is a human cell.

[00111] In some embodiments, the cleavable linker is selected from the group consisting of an enzyme cleavable linker, a nucleophile/base sensitive linker, reduction sensitive linker, a photo-cleavable linker, an electrophile/acid sensitive linker, a metal-assisted cleavable linker, and an oxidation sensitive linker. In some embodiments, the cleavable linker is a disulfide bond or a nucleic acid restriction site.

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[00112] In some embodiments, the one or more readout binding targets comprises three or more readout binding targets.

[00113] In some embodiments where the second overhang sequence is present, the additional one or more readout binding targets comprises three or more readout binding targets.

[00114] In one aspect, disclosed herein is a composition comprising a first plurality of nucleic acid detection probes and an extendible signal motif formed by a first plurality populations of extender probes $\{EP_1, EP_2, \ldots, EP_n\}$. In some embodiments, each nucleic acid detection probe in the first plurality of nucleic acid detection probes comprises: a binding region comprising a binding sequence that binds to a first target sequence; and an initiator sequence linked to the binding region with a cleavable linker. In some embodiments, each population of extender probes is represented by EP_1, EP_2, …, EP_n, respectively, where each extender probe in EP_1 comprises: a binding sequence that binds to all or a part of the initiator sequence; one or more target sequences for extender probes in EP_2 and subsequent populations of extender probes, and a signal moiety capable of emitting a first detectable signal. In some embodiments, each probe in EP_2 and subsequent populations of extender probes comprises: a binding sequence that binds to all or a part of the previous extender sequence; one or more target sequences for probes in subsequent populations of extender probes; and a signal moiety capable of emitting the first detectable signal.

[00115] In some embodiments, the first target sequence is within a primary probe that directly binds to a target nucleic acid molecule. In some embodiments, the first target sequence is within a secondary probe that binds to a primary probe that directly binds to a target nucleic acid molecule. In some embodiments, the first target sequence is within a tertiary probe that binds to a secondary probe that binds to a primary probe that directly binds to a target nucleic acid molecule.

[00116] In some embodiments, the target nucleic acid molecule is an mRNA or a DNA. In some embodiments, the target nucleic acid molecule is within an intact mammalian cell. In some embodiments, the intact mammalian cell is a human cell.

[00117] In some embodiments, the cleavable linker is selected from the group consisting of an enzyme cleavable linker, a nucleophile/base sensitive linker, reduction sensitive linker, a photo-cleavable linker, an electrophile/acid sensitive linker, a metal-assisted cleavable linker,

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and an oxidation sensitive linker. In some embodiments, the cleavable linker is a disulfide bond or a nucleic acid restriction site.

[00118] In some embodiments, each extender probe of the plurality of extender probes comprises a binding sequence that is complementary to all or a part of the initiator sequence in the nucleic acid detection probe, wherein each extender probe forms a hairpin structure, and wherein the presence of the initiator sequence causes the hairpin structure to unfold and initiates a hybridization chain reaction.

[00119] In some embodiments, the composition further comprises a second plurality of nucleic acid detection probes and an extendible signal motif formed by a second plurality populations of extender probes $\{EP_{1'}, EP_{2'}, \ldots, EP_{n'}\}$. In some embodiments, each nucleic acid detection probe in the second plurality of nucleic acid detection probes comprises: a binding region comprising a binding sequence that binds to a second target sequence; and an initiator sequence linked to the binding region with a cleavable linker. In some embodiments, each population of extender probes is represented by $EP_{1'}$, $EP_{2'}$, …, $EP_{n'}$, respectively, wherein each extender probe in $EP_{1'}$ comprises: a binding sequence that binds to all or a part of the initiator sequence; one or more target sequences for extender probes in $EP_{2'}$ and subsequent populations of extender probes; and a signal moiety capable of emitting a second detectable signal. In some embodiments, each probe in $EP_{2'}$ and subsequent populations of extender probes comprises: a binding sequence that binds to all or a part of the previous extender sequence; one or more target sequences for probes in subsequent populations of extender probes; and a signal moiety capable of emitting the second detectable signal.

[00120] In one aspect, disclosed herein is a sequential hybridization method. The method comprises the steps of: a) contacting a target nucleic acid molecule with a first plurality of nucleic acid detection probes and b) contacting, after step a) the target nucleic acid molecule with a first plurality populations of extender probes $\{EP_1, EP_2, \ldots, EP_n\}$. In some embodiments, each nucleic acid detection probe in the first plurality of nucleic acid detection probes comprises: a binding region comprising a binding sequence that binds to a first target sequence; and an initiator sequence linked to the binding region with a cleavable linker. In some embodiments, each population of extender probes is represented by EP_1, EP_2, …, EP_n, respectively, where each extender probe in EP_1 comprises: a binding sequence that binds to all or a part of the initiator

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sequence; one or more target sequences for extender probes in EP_2 and subsequent populations of extender probes; and a signal moiety capable of emitting a first detectable signal. In some embodiments, each probe in EP_2 and subsequent populations of extender probes comprises: a binding sequence that binds to all or a part of the previous extender sequence; one or more target sequences for probes in subsequent populations of extender probes; and a signal moiety capable of emitting the first detectable signal.

[00121] In some embodiments, the method further comprises: b1) imaging the target nucleic acid molecule after step b) so that interactions between the first plurality of nucleic acid detection probes and first target sequences are detected by the presence of the first detectable visual signal; and b2) applying a cleaving agent to cleave the linker, thereby eliminating the extendible signal motif.

[00122] In some embodiments, the method further comprises: c) contacting an target nucleic acid molecule with a second plurality of nucleic acid detection probes. In some embodiment, each nucleic acid detection probe in the second plurality of nucleic acid detection probes comprises: a binding region comprising a binding sequence that binds to a second target sequence; and an initiator sequence linked to the binding region with a cleavable linker.

[00123] In some embodiments, the method further comprises: d) contacting, after step c) the target nucleic acid molecule with a second plurality populations of extender probes $\{EP_{1'}, EP_{2'}, \ldots, EP_{n'}\}$, where each population of extender probes is represented by $EP_{1'}$, $EP_{2'}$, …, and $EP_{n'}$, respectively. In some embodiments, each extender probe in $EP_{1'}$ comprises: a binding sequence that binds to all or a part of the initiator sequence; one or more target sequences for extender probes in $EP_{2'}$ and subsequent populations of extender probes; and a signal moiety capable of emitting a second detectable signal. In some embodiments, each probe in $EP_{2'}$ and subsequent populations of extender probes comprises: a binding sequence that binds to all or a part of the previous extender sequence; one or more target sequences for probes in subsequent populations of extender probes; and a signal moiety capable of emitting the second detectable signal.

[00124] In some embodiments, the method further comprises: d1) imaging the target nucleic acid molecule after step d) so that interactions between the second plurality of nucleic acid detection probes and second target sequences are detected by the presence of the second

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detectable visual signal; and d2) applying a cleaving agent to cleave the linker, thereby eliminating the extendible signal motif.

[00125] In some embodiments, the second target sequence is within a primary probe that directly binds to a target nucleic acid molecule. In some embodiments, the second target sequence is within a secondary probe that binds to a primary probe that directly binds to a target nucleic acid molecule. In some embodiments, the second target sequence is within a tertiary probe that binds to a secondary probe that binds to a primary probe that directly binds to a target nucleic acid molecule.

[00126] In some embodiments, the target nucleic acid molecule is an mRNA or a DNA. In some embodiments, the target nucleic acid molecule is within an intact mammalian cell. In some embodiments, the intact mammalian cell is a human cell.

[00127] In some embodiments, the cleavable linker is selected from the group consisting of an enzyme cleavable linker, a nucleophile/base sensitive linker, reduction sensitive linker, a photo-cleavable linker, an electrophile/acid sensitive linker, a metal-assisted cleavable linker, and an oxidation sensitive linker. In some embodiments, the cleavable linker is a disulfide bond or a nucleic acid restriction site.

[00128] In some embodiments, each extender probe of the plurality of extender probes comprises a binding sequence that is complementary to all or a part of the initiator sequence in the nucleic acid detection probe, where each extender probe forms a hairpin structure, and where the presence of the initiator sequence causes the hairpin structure to unfold and initiates a hybridization chain reaction.

[00129] The compositions and methods disclosed herein can be used in sequential hybridizations to identify any suitable cellular targets within an intact cell or in an *in vitro* setting. In some embodiments, the cellular targets can be mRNAs or DNAs. In some embodiments, the cellular targets can be proteins. For example, the initial target-binding primary probe can be an antibody conjugated with nucleic acid sequence for subsequent bindings.

[00130] One of skill in the art would understand that embodiments disclosed herein can be applied or combined in any aspect when applicable.

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DEFINITIONS

[00131] *Animal:* As used herein, the term "animal" refers to any member of the animal kingdom. In some embodiments, "animal" refers to humans, at any stage of development. In some embodiments, "animal" refers to non-human animals, at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and/or worms. In some embodiments, an animal may be a transgenic animal, a genetically-engineered animal, and/or a clone.

[00132] *Approximately:* As used herein, the terms "approximately" or "about" in reference to a number are generally taken to include numbers that fall within a range of 5%, 10%, 15%, or 20% in either direction (greater than or less than) of the number unless otherwise stated or otherwise evident from the context (except where such number would be less than 0% or exceed 100% of a possible value). In some embodiments, use of the term "about" in reference to dosages means ± 5 mg/kg/day.

[00133] *Homology*: "Homology" or "identity" or "similarity" refers to sequence similarity between two nucleic acid molecules. Homology and identity can each be determined by comparing a position in each sequence which can be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar nucleic acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar nucleic acids at positions shared by the compared sequences. A sequence which is "unrelated" or "non-homologous" shares less than 40% identity, less than 35% identity, less than 30% identity, or less than 25% identity with a sequence described herein. In comparing two sequences, the absence of residues (amino acids or nucleic acids) or presence of extra residues also decreases the identity and homology/similarity.

[00134] In some embodiments, the term "homology" describes a mathematically based comparison of sequence similarities which is used to identify genes with similar functions or

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motifs. The nucleic acid sequences described herein can be used as a "query sequence" to perform a search against public databases, for example, to identify other family members, related sequences or homologs. In some embodiments, such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. In some embodiments, BLAST nucleotide searches can be performed with the NBLAST program, score=100, word length =12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. In some embodiments, to obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and BLAST) can be used (See www.ncbi.nlm.nih.gov).

[00135] *Identity*: As used herein, "identity" means the percentage of identical nucleotide residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions. Identity can be readily calculated by known methods, including but not limited to those described in (Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Molec. Biol. 215: 403-410 (1990) and Altschul et al. Nuc. Acids Res. 25: 3389-3402 (1997)). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., J. Mol. Biol. 215:

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403-410 (1990). The well-known Smith Waterman algorithm can also be used to determine identity.

[00136] *In vitro*: As used herein, the term "*in vitro*" refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, *etc.*, rather than within an organism (*e.g.,* animal, plant, and/or microbe).

[00137] *In vivo:* As used herein, the term "*in vivo*" refers to events that occur within an organism (*e.g.,* animal, plant, and/or microbe).

[00138] *Oligonucleotide:* the term "oligonucleotide" refers to a polymer or oligomer of nucleotide monomers, containing any combination of nucleobases, modified nucleobases, sugars, modified sugars, phosphate bridges, or modified bridges.

[00139] Oligonucleotides of the present invention can be of various lengths. In particular embodiments, oligonucleotides can range from about 2 to about 200 nucleotides in length. In various related embodiments, oligonucleotides, single-stranded, double-stranded, and triple-stranded, can range in length from about 4 to about 10 nucleotides, from about 10 to about 50 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, from about 20 to about 30 nucleotides in length. In some embodiments, the oligonucleotide is from about 9 to about 39 nucleotides in length. In some embodiments, the oligonucleotide is at least 4 nucleotides in length. In some embodiments, the oligonucleotide is at least 5 nucleotides in length. In some embodiments, the oligonucleotide is at least 6 nucleotides in length. In some embodiments, the oligonucleotide is at least 7 nucleotides in length. In some embodiments, the oligonucleotide is at least 8 nucleotides in length. In some embodiments, the oligonucleotide is at least 9 nucleotides in length. In some embodiments, the oligonucleotide is at least 10 nucleotides in length. In some embodiments, the oligonucleotide is at least 11 nucleotides in length. In some embodiments, the oligonucleotide is at least 12 nucleotides in length. In some embodiments, the oligonucleotide is at least 15 nucleotides in length. In some embodiments, the oligonucleotide is at least 20 nucleotides in length. In some embodiments, the oligonucleotide is at least 25 nucleotides in length. In some embodiments, the oligonucleotide is at least 30 nucleotides in length. In some embodiments, the oligonucleotide is a duplex of complementary strands of at least 18 nucleotides in length. In some embodiments, the oligonucleotide is a duplex of complementary strands of at least 21 nucleotides in length.

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[00140] *Predetermined:* By predetermined is meant deliberately selected, for example as opposed to randomly occurring or achieved. A composition that may contain certain individual oligonucleotides because they happen to have been generated through a process that cannot be controlled to intentionally generate the particular oligonucleotides is not a "predetermined" composition. In some embodiments, a predetermined composition is one that can be intentionally reproduced (e.g., through repetition of a controlled process).

[00141] *Probe*: As used herein, the term "probe" or "probes" refers to any molecules, synthetic or naturally occurring, that can attach themselves directly or indirectly to a molecular target (e.g., an mRNA sample, DNA molecules, protein molecules, RNA and DNA isoform molecules, single nucleotide polymorphism molecules, and etc.). For example, a probe can include an nucleic acid molecule, an oligonucleotide, a protein (e.g., an antibody or an antigen binding sequence), or combinations thereof. For example, a protein probe may be connected with one or more nucleic acid molecules to for a probe that is a chimera. As disclosed herein, in some embodiments, a probe itself can produce a detectable signal. In some embodiments, a probe is connected, directly or indirectly via an intermediate molecule, with a signal moiety (e.g., a dye or fluorophore) that can produce a detectable signal.

[00142] *Sample*: As used herein, the term "sample" refers to a biological sample obtained or derived from a source of interest, as described herein. In some embodiments, a source of interest comprises an organism, such as an animal or human. In some embodiments, a biological sample comprises biological tissue or fluid. In some embodiments, a biological sample is or comprises bone marrow; blood; blood cells; ascites; tissue or fine needle biopsy samples; cell-containing body fluids; free floating nucleic acids; sputum; saliva; urine; cerebrospinal fluid, peritoneal fluid; pleural fluid; feces; lymph; gynecological fluids; skin swabs; vaginal swabs; oral swabs; nasal swabs; washings or lavages such as a ductal lavages or broncheoalveolar lavages; aspirates; scrapings; bone marrow specimens; tissue biopsy specimens; surgical specimens; feces, other body fluids, secretions, and/or excretions; and/or cells therefrom, *etc*. In some embodiments, a biological sample is or comprises cells obtained from an individual. In some embodiments, a sample is a "primary sample" obtained directly from a source of interest by any appropriate means. For example, in some embodiments, a primary biological sample is obtained by methods selected from the group consisting of biopsy (*e.g.*, fine needle aspiration or

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tissue biopsy), surgery, collection of body fluid (*e.g.,* blood, lymph, feces *etc.*), *etc.* In some embodiments, as will be clear from context, the term "sample" refers to a preparation that is obtained by processing (e.g., by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, filtering using a semi-permeable membrane. Such a "processed sample" may comprise, for example nucleic acids or proteins extracted from a sample or obtained by subjecting a primary sample to techniques such as amplification or reverse transcription of mRNA, isolation and/or purification of certain components, etc.

[00143] *Subject:* As used herein, the term "subject" or "test subject" refers to any organism to which a provided compound or composition is administered in accordance with the present invention *e.g.*, for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates, and humans; insects; worms; *etc.*) and plants. In some embodiments, a subject may be suffering from, and/or susceptible to a disease, disorder, and/or condition.

[00144] *Substantially:* As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and/or chemical phenomena.

[00145] *Suffering from*: An individual who is "suffering from" a disease, disorder, and/or condition has been diagnosed with and/or displays one or more symptoms of a disease, disorder, and/or condition.

[00146] *Susceptible to*: An individual who is "susceptible to" a disease, disorder, and/or condition is one who has a higher risk of developing the disease, disorder, and/or condition than does a member of the general public. In some embodiments, an individual who is susceptible to a disease, disorder and/or condition may not have been diagnosed with the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition may exhibit symptoms of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition may not exhibit symptoms of the disease, disorder, and/or condition. In some embodiments, an

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individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[00147] *Treat:* As used herein, the term "treat," "treatment," or "treating" refers to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of, and/or reduce incidence of one or more symptoms or features of a disease, disorder, and/or condition. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition. In some embodiments, treatment may be administered to a subject who exhibits only early signs of the disease, disorder, and/or condition, for example for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

[00148] *Wild-type:* As used herein, the term "wild-type" has its art-understood meaning that refers to an entity having a structure and/or activity as found in nature in a "normal" (as contrasted with mutant, diseased, altered, etc.) state or context. Those of ordinary skill in the art will appreciate that wild type genes and polypeptides often exist in multiple different forms (e.g., alleles).

BRIEF DESCRIPTION OF THE DRAWINGS

[00149] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[00150] Those of skill in the art will understand that the drawings, described below, are for illustrative purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[00151] Figure 1 depicts exemplary embodiments of known methods for coding molecular targets.

[00152] Figure 2 depicts exemplary sequential barcoding of provided methods. (a) Schematic of sequential barcoding. In each round of hybridization, multiple probes (*e.g.*, 24)were hybridized on each transcript, imaged and then stripped by DNase I treatment. The same probe sequences could be used in different rounds of hybridization, but probes were

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coupled to different fluorophores. (b) Composite four-color FISH Data from 3 rounds of hybridizations on multiple yeast cells. Twelve genes were encoded by 2 rounds of hybridization, with the third hybridization using the same probes as hybridization 1. The boxed regions were magnified in the bottom right corner of each image. The matching spots were shown and barcodes were extracted. Spots without co-localization, without the intention to be limited by theory, could be due to nonspecific binding of probes in the cell as well as mis-hybridization. The number of each barcode were quantified to provide the abundances of the corresponding transcripts in single cells. (c) Exemplary barcodes. mRNA 1: Yellow-Blue-Yellow; mRNA 2: Green-Purple-Green; mRNA 3: Purple-Blue-Purple; and mRNA 4: Blue-Purple-Blue.

[00153] Figure 3A illustrates aspects of an exemplary hybridization experiment.

[00154] Figure 3B depicts an exemplary process for performing a pseudo-color based barcoding scheme.

[00155] Figure 4 depicts an overview illustrating a method for performing a pseudo-color based barcoding scheme: SPOTs (Sequential Probing Of Targets). (a) RNA molecules are captured on Locked Nucleic Acid(LNA) poly(dT) functionalized coverslips followed by hybridization of 28-32 gene specific primary probes. (b) Schematic to generate 12 pseudocolors used in decoding each RNA species. In each round of serial hybridizations, 3 'colors' are generated by imaging 3 unique readout probes conjugated with dye Alexa 647, Alexa 594, and Cy3b. After 4 rounds of serial hybridizations, the images are collapsed into 1 image to generate an image with 12 pseudo-colors. (c) Schematic of decoding 5 barcoded rounds based on 12 pseudo-colors coding scheme. (d) Digitized image of 12 pseudo-colors switching based on actual experimental image. After decoding 5 barcoded hyb, up to 20736 unique RNA species can be decoded with high accuracy and low error rates.

[00156] Figure 5 illustrates mRNA transcripts immobilized on a surface through poly-A-tail or hydrogel embedment.

[00157] Figure 6 illustrates an exemplary embodiment of gene specific primary probes design and sequential barcoding hybridization on mRNA immobilized on a surface. (a) Design of gene specific primary probe. (b) primary probe hybridizations on mRNA on the surface, alternatively, the primary probes hybridizations can also be done in solution. (c) sequential barcoding hybridization on the surface. One or multiple 18-30mers secondary readout probes

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conjugated with fluorophore will hybridize to the primary probes during each round of hybridization.

[00158] Figure 7 illustrates different arrangements of secondary probe binding sites on primary probes. Assume 24 primary probes are used for each gene, 4 rounds of barcoding hybridization with 4 different unique secondary probe binding sequences, there are various combinations of arranging the secondary probe binding sequences on the primary probe. (a) all 4 unique binding sequences a, b, c, d are placed on all 24 primary probes. (b) each unique binding sequence are placed separately into each primary probe. In this case, 6 primary probes have unique binding sequence a, 6 primary probes will get unique binding sequence b, 6 primary probes will have unique binding sequence c, and 6 primary probes will have unique binding sequence d. (c). a combination of unique binding sequences is placed on different primary probes for a gene. In this case, the unique binding sequences combination can be (a,b),(a,c),(a,d),(b,c),(b,d),(c,d) on each 4 primary probes.

[00159] Figure 8 depicts possible ways to extinguish fluorescent signals via (a) flowing in high concentration of formamide to 'melt' off the secondary readout probe or (b) chemical cleavage.

[00160] Figure 9 depicts a schematic illustration of serial hybridization to scale up the number of available 'fluorophore'. For example, by using 3 fluorophores, and a total of 12 unique secondary readout sequences, 3 unique secondary readout probes were serially flown in. The probes code for hyb1 at a time, image, and then extinguish the signal. Then, another 3 unique secondary readout probes were be flowed in and undergo the same process. With a total of 4 rounds of serial hybridizations (n= 4), 12 unique secondary readouts with 3 colors are used to generate the barcode color for hyb1. The same process was repeated for barcoding hyb 2, … to hyb N. Essentially, a combination of serial hybridization and sequential barcoding hybridization allows us increase the number of fluorophore (F) from 3 to 12, and if number of barcoding hybridization (N) = 4, the barcode capacity will be $(3 \times 4)^4 = 20736$ which can cover almost the entire transcriptome.

[00161] Figure 10 depicts a table showing possible barcodes obtained using the pseudo-color barcode scheme.

[00162] Figure 11 depicts an exemplary embodiment of data analysis.

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[00163] Figure 12 depicts an exemplary a raw image from a barcoding experiment.

[00164] Figure 13 depicts exemplary results from data analysis.

[00165] Figure 14 depicts exemplary results from data analysis.

[00166] Figure 15 depicts exemplary results from translational profiling analysis.

[00167] Figure 16 depicts exemplary results from translational profiling analysis.

[00168] Figure 17 illustrates results of gene specific primary probes design. (a) Each primary probe comprises a 25-nt gene specific sequences complementary to the mRNA, 4 readout sequences, and 2 primers binding sites. Each gene is targeted by a minimum of 28 to 32 primary probes. (b) Both priming regions (grey in the probe schematic) used in synthesizing gene specific primary probes are also used as a registration marker through the hybridization of Alexa 488 conjugated readout probes. Majority of the fluorescent spots stay even after 20 rounds of hybridizations. (Scale bars: 2μm.)

[00169] Figure 18 illustrates fluorescent switching through cleavage of disulfide conjugate dye on readout probes is highly efficient (a) 20 rounds of hybridization are accomplished by extinguishing fluorescent signals through reduction of disulfide conjugated dye to readout probes using TCEP, followed by re-hybridization of next unique secondary readout probes. The amide bond between the Alexa 488 dye (shown in yellow) and primer readout probes used as a registration marker is not affected by TCEP. (b) The fluorescent signals in each channel after treatment of 50mM of TCEP for 5 minutes at room temperature is reduced to minimal to none. (Scale bars: 5μm.)

[00170] Figure 19 illustrates RNA Sequential Probing Of Targets (SPOTs) measurement of 10,212 genes. Correlation of RNA SPOTs with RNAseq in fibroblasts with Pearson r coefficient of 0.86. (b Two SPOTs replicate experiments, signifies its high reproducibility (c) Correlation of RNA SPOTs measurement with RNAseq in mESCs, with Pearson r coefficient of 0.90. (d) Correlation of averaged smFISH counts for 24 genes in mESCs. RNA SPOTs has single molecule sensitivity and highly accurate in transcriptome profiling.

[00171] Figure 20 illustrates RNA SPOTs analysis at lower depth. (a) Correlation between RNA-seq FPKM and RNA SPOTs SPM from another replicate is high when a total of 376,781 spots are counted. SPM, spots per million; FPKM, fragments per kilobase per million reads. (b)

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High reproducibility of RNA SPOTs between the two replicates in profiling ES-E14 cell gene expression (n1=376,781 spots, n2=1,688,747 spots).

[00172] Figure 21 shows raw images of 20 rounds of fluorescent switching in channel 647. Bright dots are the real targets while dim dots are due to nonspecific binding. The switching between each round of hybridization is complete, with minimal retention of fluorescent signals from previous round. (Scale bars: 2μm.)

[00173] Figure 22 depicts assessment of primary probes non-specific binding. (a) Raw images of 532 channel with the presence of mRNA on coverslips through LNA poly(d)T capturing. (b) No bright fluorescent signals is observed in the absence of mRNA on coverslips as a control. The left image has the same contrast as (a) while the right image contrast has been increased 4.5 fold to illustrate better the non-specific fluorescent signals. (c) Quantitative measurement of fluorescent intensity in channel 647 with and without the presence of mRNA. A threshold can be set to distinguish between the two populations to identify the real signals. (d) & (e) same as (c) but for channel 594 and channel 532.

[00174] Figure 23 depicts smFISH measurement in single cells correlates with RNA SPOTs measurement in NIH/3T3 cells. (a) Raw images of the 7 genes measured by smFISH in NIH/3T3 cells. (Scale bars: 5μm.) (b) The averaged RNA smFISH counts agrees with RNA SPOTs SPM (spots per million) with a Pearson correlation coefficient of 0.88, indicating RNA SPOTs quantitation is accurate. Error bars represents the standard error of the mean (SEM) across different single cells.

[00175] Figure 24 depicts that mRNA can be immobilized by polyacrylamide hydrogel on a bind-silane treated coverslips. (a) mRNA is trapped in the hydrogel mesh once acrylamide and bis-acrylamide monomers crosslink completely on the coverslip. (b) smFISH detection of ACTB once the total RNA is captured on a coverslip through LNA poly(d)T capturing (left) or polyacrylamide hydrogel (right). Negative control (channel 488) shows that the fluorescent signals are not coming from nonspecific sources. (Scale bars: 5μm.)

[00176] Figure 25 depicts exemplary reaction scheme for synthesizing DNA probes conjugated to dye through cleavable disulfide linker.

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[00177] Figure 26. An exemplary embodiment illustrating sequential barcoding using gene specific primary probes, secondary bridge probes and tertiary readout probes. (a) Sequential barcoding FISH (seqFISH) with DNA readout probes conjugated with dyes through disulfide linkage. The scheme begins with hybridization of gene specific primary probes, followed by secondary bridges with readout binding sites, and a unique tertiary readout probes with disulfide-linked dye. Once imaged, reducing agent such as TCEP/DTT is used to eliminate the fluorescent signals. Subsequent hybridization gives fluorescent signals which is not interfered by previous fluorescent spots. The secondary bridges can be stripped off by using high concentration of formamide, and replaced by a new set of secondary bridges. (b) An exemplary embodiment illustrating primary probes with two overhang sequences. One of the alternate designs of gene specific primary probes and secondary bridges. For example, with 2 overhangs on primary probes, each overhang can bind 1 secondary bridge which consists of 3 unique tertiary readout probes binding sites. By using 4 different colors of fluorophore, one can scale up the barcodes to $4^6 = 4096$ with this design.

[00178] Figure 27A depicts an exemplary hybridization chain reaction (HCR).

[00179] Figure 27B depicts an exemplary readout probe.

[00180] Figure 27C depicts an exemplary hybridization chain reaction based on readout probes with cleavable linkers.

[00181] Figure 28 depicts an exemplary embodiment illustrating rehybridization in mouse embryonic stem cells (mESCs). (a) First hybridization with tertiary readout probes conjugated with A647. Real fluorescent spots are shown in red dashed box. (b) No fluorescent spots are observed in channel 594 during the first hybridization. (c) After TCEP treatment and washing steps, channel 647 is reimaged. The observed dim dots are non-specific sticking of dyes which does not interfere with subsequent real fluorescent spots identification. (d) Second unique readout probes are hybridized to the secondary bridge binding sites to give real fluorescent spots that appear as the same positions in (a).

[00182] We also capture in vitro transcribed polyA-tailed dCAS9-EGFP mRNA on a dT20 Locked Nucleic Acid (LNA) surface-modified coverslips to show the rehybridization scheme works on the coverslips.

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[00183] Figure 29 depicts an exemplary embodiments illustrating rehybridization on mRNA captured on a dT20 LNA surface-modified coverslips. (a) (Left) First round of hybridization with tertiary probes conjugated with A647. (Right) No fluorescent spots are observed in channel 594 during first hybridization. (b) (Left) Channel 647 has minimal to no leftover fluorescent signals. (Right) Fluorescent spots from second round of hybridization which appear as the same positions as first hybridization.

[00184] Figure 30 illustrates an exemplary process for error correction.

[00185] Figure 31 illustrate an exemplary computer system for implementing the error correction methods disclosed herein.

[00186] Figure 32 depicts an overview of the Sequential barcode FISH (seqFISH) in brain slices. A). A coronal section from a mouse brain was mounted on a slide and imaged in all boxed areas. Each image was taken at 60x magnification. B). Example of barcoding hybridizations from one cell in field from A. The same points are re-probed through a sequence of 4 hybridizations (numbered). The sequence of colors at a given location provides a barcode readout for that mRNA ("barcode composite"). These barcodes are identified through referencing a lookup table abbreviated in D and quantified to obtain single cell expression. In principle, the maximum number of transcripts that can be identified with this approach scales to FN, where F is the number of fluorophores and N is the number of hybridizations. Error correction adds another round of hybridization. C). Serial smHCR is an alternative detection method where 5 genes are quantified in each hybridization and repeated N times. Serial hybridization scales as F*N. D). Schematic for multiplexing 125 genes in single cells. 100 genes are multiplexed in 4 hybridizations by seqFISH barcoding. This barcode scheme is tolerant to loss of any round of hybridization in the experiment. 25 genes are serially hybridized 5 genes at a time by 5 rounds of hybridization. Each number represents a color channel in single molecule HCR. As a control, 5 genes are measured both by double rounds of smHCR as well as barcoding in the same cell. E. SmHCR amplifies signal from individual mRNAs. After imaging, DNAse strips the smHCR probes from the mRNA, enabling rehybridization on the same mRNA (step a). The "color" of an mRNA can be modulated by hybridizing probes that trigger HCR polymers labeled with different dyes (step b). mRNA are amplified following

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hybridization by adding the complementary hairpin pair (step c). The DNAse smHCR cycle is repeated on the same mRNAs to construct a predefined barcode over time.

[00187] Figure 33 illustrates an example accurate in situ quantification of mRNA levels generated by seqFISH. A). Image of seqFISH barcoding 100 genes in the outer layer of the mouse cortex. RNA dots in the image are z projected over 15μm. Individual mRNA points are shown across 4 hybridizations in the inset images. White squares correspond to identified barcodes, yellow squares correspond to missing transcripts in a particular hybridization, red squares correspond to spurious false positives and are not counted in any barcode measurements. Numbers in the squares correspond to barcode indices. B). seqFISH correlates with smHCR counts. After barcoding, 5 target mRNAs were measured twice by smHCR in the same cells, providing absolute counts of the transcripts. The two techniques correlate with an R=0.85 and a slope (m) of 0.84 (n=3851 measurements). The 2D histogram intensity shows the distribution of points around the regression line. A high density of points is seen along the regression line. The density falls off steeply around the regression line. C). Error correction results in a median gain of 373 (25%) counts per cell (n=3497). Red and blue curves correspond to the total barcode counts per cell before and after error correction. D). Dropped and off-target barcodes represent a small source of error in seqFISH. 100 on-target barcodes and 525 off-target barcodes are measured per cell. Dropped barcodes are due to at least two overlapping dots appearing within the same region. E. Off-target barcodes are rarely observed and contribute minimally to the expression profile in single cells. Each of the 100 on-target barcodes (blue) and 525 off-target barcodes (red) are quantified per cell. The mean is shown with shaded regions corresponding to 1 SD (N=41 imaged regions).

[00188] Figure 34 depicts an example illustrating that distinct clusters of cells exhibit different regional localization in the brain. A). Gene expression of 14,908 cells presented as a Z-score normalized heatmap. B). Regional compositions of 13 cell clusters are visualized as stacked bar plots with the area corresponding the number of cells in each region. Hippocampal regions are: CA3, CA1, Dentate Gyrus (DG). Cortical regions: parietal and temporal. Box plot of the Z scores of 21 representative genes are plotted for each cell class. The major tick marks correspond to Z score 0 while every minor tick is a z score interval of 1. Cell type assignments are shown on the dendrogram. Abbreviations: Hippocampus pyramidal (Hipp), cortex (Cort),

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Dentate Gyrus (DG), Interneurons (Int), Astrocyes(Astro), Microglia (μGlia). C). Subclusters of cluster 6 cells and their regional localization and gene expression profile displayed under the dendrogram. Subcluster 6.1 is enriched in the CA3, while 6.7 is enriched in the DG. D). Subclusters of cluster 7 cells are shown. Almost all cells are localized in the GCL but have different combinatorial expression profiles. Note Calb1 expression, which marks out granule cell maturation, differs amongst subclusters. E). Any random subset of 25 genes can recapitulate approximately 50% of the information in the correlation amongst cells (red), but a larger number of genes are required to accurately assign cells to cluster using a random forest algorithm (blue) (n=10 bootstrap replicates; shading is 95% CI), indicating that fine structures in the data require quantitative measurements of combinatorial expression of many genes. F). Similar to E, while the first ten PCs explain the coarse structure, a larger number of principal components (PCs) are required to describe the full data. Expected variation (green) and accuracy in predicting cell identity using a random forest model (blue).

[00189] Figure 35 depicts an example embodiment, illustrating spatial layering of cell classes in the Dentate Gyrus (DG). A-B). Suprapyramidal and infrapyramidal blades of DG. Cells of the subgranular zone (SGZ) and granule cell layer (GCL) are arranged in lamina layers in mirror symmetric patterns on the upper and lower blades. C). The SGZ stays on the inner layer of the DG fork. D). Cells are patterned in the crest. Numbered color key corresponds to cluster numbers in Figure 34b. E). Letters in the cartoon of DG correspond to images. F). 3D image of the fork region shown in C).

[00190] Figure 36 depicts an example embodiment, illustrating that subregions of the hippocampus are composed of distinct compositions of cell classes based on the first 125 gene experiment. Upper right panel. Cartoon of hippocampus with imaged regions labeled. Color key corresponds to the classes in Figure 39b. A-D). These images are regions from the CA1d. Astrocytes (Astro) are marked in image A) and a microglia cell (μGlia) is marked in image B). Moving along the hippocampus from CA1 dorsal to ventral, cell classes transition from a homogenous dorsal population (C to D) to a mixed population in the CA1 intermediate (E-F) to regions of even larger cellular diversity in the CA1 ventral region (G-I). The dotted line in D) marks the transition point of the CA1d to the CA1i. E) shows two laterally segregated cell classes (marked by a dotted line) in the CA1i along with cholinergic interneurons (Int) on the

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interior surface of the CA1i. The ventral (J-K) and intermediate CA3 (L-M) have similar cell classes compositions to the CA1v and CA1i. The two last regions (O-P) of the dorsal CA3 shows distinct cell classes compositions that are relatively homogeneous within a field but are different than other fields of CA3. The cell class composition of field P is similar to that of the CA1d, but these cluster 6 cells are grouped into a distinct subcluster.

[00191] Figure 37 depicts an example embodiment, showing mapping of cell types to a second brain slice with 125 genes. Upper right panel. Cartoon of hippocampus with imaged regions labeled. Color key corresponds to the classes in Figure 34b. A-D. Similar to the cell class compositions shown for the hippocampus in Figure 36, CA1d in this second coronal section from a second mouse is composed of mostly cluster 6 cells. (E) CA1i region and (F-G) the CA1 ventral regions are again composed of similar cell classes to that shown in Figure 36 with increasing diversity of cell class compositions from the CA1d to the CA1i to finally the CA1v. (H-J) CA3 regions. (K-M) DG regions showing the same cell classes and layer pattern of the GCL and SGZ shown in Figure 35.

[00192] Figure 38 depicts an example embodiments, showing mapping of cell types to a third brain slice with 249 genes. Upper right panel. Cartoon of hippocampus with imaged regions labeled. Color key corresponds to the classes in Figure 46C. A-C). Similar to the slice shown in Figures 36 and 37, CA1d is relatively homogenous in cell cluster composition. D-G). Images from the CA1i region show that the cell class composition is different from that of the CA1d. H-K). Again, similar to Figures 36 and 37, images from the CA1 ventral regions shows a much more complicated cellular composition and a high degree of cellular heterogeneity. L-R). Images from the CA3 region show that the cellular compositions also creates 3-4 subregions within the CA3. The cellular heterogeneity of the CA3 subregions mirrors that of the CA1, where the ventral region of the CA3 is very heterogenous while the dorsal region of the CA3 is relatively homogenous. S-T). The DG regions show the distinct SGZ versus GCL layering pattern seen in the previous two brains.

[00193] Figure 39 depicts an example embodiment, showing correlations of the transcription profile across the pyramidal layer A). mRNA counts in the cell bodies in the Stratum Pyramidale (SP) are grouped within each field of view. A single cell in the Stratum Radiatum (SR) is shown to illustrate individual mRNA localization. Stratum Oriens (SO) is

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labeled for orientation. B). mRNAs in different subregions of pyramidal layer show both long-distance spatial correlations as well as local correlations between neighboring fields. Both CA1 and Dentate Gyrus (DG) show high regional correlations. Correlation is calculated based on the 125 gene experiment. C). Illustration of regional and long distance correlation patterns observed in B. Correlated regions are colored and long distance correlations are shown as dotted lines with their median correlation coefficient written over the dotted line.

[00194] Figure 40 depicts an example embodiment, showing barcode assignments for all genes in the combined hybridization experiment (Figure 32). Barcode assignments in the 125-gene seqFISH and serial experiment (Figure 32). 125 genes are profiled, 100 of which are barcoded and 25 are identified by serial smHCR hybridizations. Five control genes (Hdx, Vps13c, Zfp715, Fbll1, Slc4a8) were quantified by both techniques. The smHCR round of hybridization of control genes were performed twice to co-localize signal to obtain an absolute count.

[00195] Figure 41 depicts an example embodiment, showing smHCR performance metrics as compared to smFISH, (related to Figure 32). A). Raw data of Pgk1 transcripts imaged in a brain slice. The transcript was targeted with 2 hcr probes sets and 1 smFISH probe set, each consisted of 24 oligonucleotide probes. The probe sets were hybridized together and were imaged in 3 different channels. Green circles are transcripts detected in all channels, yellow circles signify transcripts detected in 2 out of 3 channels, and red circles represent signal found in only 1 channel (false positives due to nonspecific binding). T hese images show that smHCR and smFISH have similar sensitivity, specificity, and spot size. B). Gain of smHCR vs smFISH. The mean gain of smHCR is 22.1 ± 11.55 vs smFISH (n=1338). C). True positive detection rate of smHCR and smFISH per channel. The percent of true positives (transcripts detected with at least 2 out of 3 probe sets) detected with each probe set (n=1338). D). False positive rate of smHCR and smFISH. Percent of total dots in a channel not detected in any other channel for 3 color Pgk1 (n=1338). E). All the regions imaged in the coronal section are boxed. Each box represents a field of 216 um x 216 um. The brain section used for Figures 32 and 33 is shown on the left. The middle section is used for Figure 34 and the right section is used for Figure 38.

[00196] Figure 42 depicts an example embodiment, showing quantitation of seqFISH (related to Figure 33). A). All control genes show high correlations between seqFISH and

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smHCR. B). Number of dropped hybridizations from the barcode. Blue bars represent measured probability and the red bars represent inferred values from binomial distribution fitting of measured probability. The ratio of the full barcodes (4 hybridizations) vs 3 hybridization barcodes indicate that transcripts that are mis-hybridized in 2 rounds are rare. Transcripts missed in 2 or more hybridizations (red bars) could not be recovered from the error-correction algorithm and would be dropped from our quantifications (N=2,115,477 total barcodes). C). Intensity of barcode hybridizations overtime. All dots belonging to barcodes are quantified in each hybridization and their mean intensity is plotted over time normalized to the first hybridization. 99% CI ratio of mean is plotted as a bar over points, but is not visible due to its small size (n=60143 to 111284 points per channel). D). Barcoding confidence ratio. Barcode classes in D) are compared to a null model of barcode observations where random chance observation should give a ratio of 1. Off target barcodes are observed 0.005 times less than expected, suggesting that seqFISH has high accuracy in correctly counting barcoded transcripts (n=3493 cells). Dark bars on top of bar plots correspond to 99.999% confidence interval determined by bootstrap resampling. E). Comparison of average copy numbers per gene as measured by Zeisel et al. and seqFISH. Single cell RNA-seq underestimates copy numbers compared to seqFISH.

[00197] Figure 43 depicts an example embodiment, showing gene expression patterns and clustering of the 125-gene dataset (related to Figure 34). A). Overview of 125 gene expression. Plots show the distribution of each transcript in all 14,908 imaged cells. Note the last 25 genes have higher expression and were imaged with serial hybridization. B). Violin plots of Z-score distribution for 125 genes. C). Sub-cluster hierarchy of each of the 13 clusters identified in Figure 34B. D). PCA eigenvalue analysis of the cell-to-cell correlation matrix. First 125 PC and their eigenvalues are shown. As observed in Figure 31, the first 10 PCs explain 59.5% of the variation in the data, while the remaining 115 PCs are needed to explain remaining data. Reflecting this, the eigenvalues of the first 10 components are high, while the remaining eigenvalues are uniform. E). Correlation between gene expression and spatial localization. Each dot represents a pair of cell classes and their correlations in gene expression space (x) and spatial localization patterns (y) (N=153 pairwise correlations between classes, R=0.67). Classes that are similar in expression have similar localization patterns. F). PCA decomposition separates cells

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into coherent clusters corresponding to cell classes. Cells are colored according to the clusters displayed in the dendrogram.

[00198] Figure 44 depicts an example embodiment, showing robustness of cell classes to downsampling of cells (related to Figure 34). To measure how well cluster assignments perform with a limited number of cells, a random forest model was trained on the cell-to-cell correlation matrix of the 6872 cells in the center field of view. The robustness of the clusters was calculated by applying this model to classify the remaining cells and determining the percent accuracy of correct assignment to the clusters presented in Figure 34b. While some classes can be assigned accurately even with a small number of cells as the initial training set, several classes require large number of cells to accurately assign (n=10 bootstrap replicates, S.E.)

[00199] Figure 45 depicts an example embodiment, showing cell-to-cell correlation analysis as a function of dropping genes (related to Figure 34). A). Clustered gene to gene correlation map for all 125 genes. There are many blocks of highly correlated genes. A few genes do not fall into any blocks. B). The full cell-to-cell correlation map using all genes in the data set. C). Representative cell-to-cell correlation with the indicated number of genes used to construct the matrix indicated above each plot. Dropping genes from the data results in degradation of the fine structure of the correlation map.

[00200] Figure 46 depicts an example embodiment, showing gene expression patterns and clustering of the 249-gene dataset (related to Figure 38). A). Overview of 249-gene expression. Plots show the distribution of each transcript in all 2050 imaged cells in the hippocampus. Note the last 35 genes have higher expression and were imaged with serial hybridization. B). Violin plots of *Z*-score distribution for 249 genes. C). Dendogram with regional localization of the 18 cell clusters for the 249-gene experiment. D). Correlation of seqFISH counts to smHCR counts for the 249-gene experiment. The 2D density histogram shows a high density of points around the regression line that fall off towards the edges of the distribution. E). Cell-to-cell correlation for all 2050 cells in the 249-gene dataset. F). Heat map of the percentage of each cell class in each region of the hippocampus for both the 125-gene experiments. These heat maps show that in both 125-gene experiments the same cell classes are used in roughly the same proportions in each subregion. G). Heat map of the percentage of each cell class in each region of the

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hippocampus for the 249-gene experiment. The same patterns are seen as the 125 gene experiment (i.e., different regions use different cell classes in varying amounts).

[00201] Figure 47 depicts an example embodiment, showing marker genes expression in the hippocampus (related to Figure 38). A). The top panel outlines the region of the hippocampus being shown in a yellow box. The images show the raw gene expression patterns seen using smHCR in our data at the dorsal most tip of the CA3 for a representative set of cell identity markers used in the 249 gene experiment. The transcript expression profile is shown in red, Nissl staining is shown in green, and DAPI staining is shown in blue. Each image shown is the full field of view and a maximum intensity projection over 15 um. B). Set of images showing the distinction between the GCL and SGZ. The GCL shows a high level of Nissl staining and expression of neuronal genes such as slc17a7 and camkII. The SGZ shows an absence of Nissl staining and terminal neuron marker genes. The transcript expression profile is shown in red, Nissl staining is shown in green, and DAPI staining is shown in blue. Each image shown is the full field of view (216 um x 216 um) and a maximum intensity projection over 15 um.

[00202] Figure 48 depicts an example embodiment, showing comparison of SeqFISH expression data to Allen Brain Atlas expression data (related to Figure 39). A). ISH data from the Allen Brain Atlas for genes seen to be enriched in the SGZ in the 125 and 249 gene seqFISH experiments. In the 125 gene experiment, mertk and mfge8 were found to be enriched in the SGZ. In the 249 gene experiment, nfia and sox11 were seen to be enriched in the SGZ. ABA ISH data shows similar patterns to those observed with seqFISH for the SGZ. B-C). Comparison of averaged z-score values per cell from seqFISH to ABA data across hippocampus. B). Amigo2 Z-score profile found across the different fields of the hippocampus using seqFISH is shown on top and the ABA ISH image for Amigo2 is shown on the bottom. C). Gpc4 Z-score profile found across the different fields of the hippocampus using seqFISH is shown on top and ABA ISH image for Gpc4 is shown on the bottom.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[00203] Among other things, the present invention provides new methods, compositions and/or kits for profiling nucleic acids (*e.g.*, transcripts and/or DNA loci) in cells.

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[00204] In some embodiments, the present invention provides methods for profiling nucleic acids (e.g., transcripts and/or DNA loci) in cells. In some embodiments, provide methods profile multiple targets in single cells. Provided methods can, among other things, profile a large number of targets (transcripts, DNA loci or combinations thereof), with a limited number of detectable labels through sequential barcoding.

Pseudo-Color Based Barcoding

[00205] In one aspect, disclosed herein are pseudo-color based barcoding method. For example, pre-designed barcodes are associated specific molecular targets through sequential hybridization experiments. A pseudo-color based barcoding scheme is developed to overcome limitations in the previous generation of the technology such as lack of visual signals that can be associated with the probes or small internal within cell when carrying out in situ experiments.. The current method can be applied to both in vitro and in situ analysis. According to the method, each barcoding round comprises multiple serial hybridizations where a small number of colored signals (that are associated with probes) are used in each hybridization experiment within a serial hybridization round. Images from each serial hybridization experiment within the same serial hybridization round are combined to form a composite image for each barcoding round. In each barcoding round, the same set of molecular targets are analyzed. After all barcoding rounds are completed, associated of the barcode with these molecular targets is completed.

[00206] To distinguish from existing FISH methods, the currently pseudo-color based barcoding methods is referred to as Sequential Probing Of Targets ("SPOTs").

[00207] SPOTs offers numerous advantageous over existing barcoding methods. For example, it does not require a user to have a large number of detectable label molecules, which can save time and money.

[00208] Pseudo-color scheme can overcome this density problem in both in situ and in vitro applications. In the in vitro cases, implemented with SPOTS, capturing transcripts onto an oligonucleotide dT surface and adjusting the dilution factors can easily remove the optical crowding problems and allow the transcriptome to be imaged by seqFISH.

[00209] In addition, pseudo-color is more efficient in terms of imaging time than all existing imaging methods including expansion microscopy. For example, with 3 fluorophores, it

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takes 3^(10-1)=19,683 to code for the transcriptome with one round of error correction. Thus, a total of 30 frames of imaging is required. In the pseudo color scheme, 20 pseudo-colors can be used for 4 rounds of hybridization to code for 20^(4-1)=8000 genes in each of the three fluorophore channels for a total of 24,000 genes. This requires 3x20x4=240 frames to image, a 8 fold increase in the imaging time. However, because the density of mRNA is effectively diluted into 3x20=60 pseudo-channels instead of 3 fluorophore without pseudo-color coding, the density problem is alleviated with a factor of 20. The target spots can be localized to nanometer precision by Gaussian fitting to decrease the density in each pseudo-color channel before barcode alignment. Thus, the benefit of pseudo-color coding is to decrease the density of the target spots in the cell, while saving imaging time compared to expansion microscopy, where expanding the sample by 20 folds requires an additional 20 fold increase in imaging time. Since imaging time is rate limiting in any sequential imaging method, pseudo-color solves a major problem in implementing transcriptome profiling in situ.

Overview

[00210] In fluorescence in situ hybridization (FISH) methods, fluorescence labels that are capable of producing different visual signals are used to detect multiple molecular targets (such as mRNA transcripts) at the same time in one hybridization experiment.

[00211] Figure 1 illustrates three types of hybridization methods, where combinations of visual signals (barcoding schemes) are used to specifically encode individual molecular targets. Figure 1a) shows a spatial barcode scheme where different colored probes are bound to the same target. The probes of the same color are grouped and spatially separated from another color.

[00212] Figure 1b) depicts a spectral barcode scheme where different colored probes are no longer separated in groups according to their respective colors. Probes bound along a target are resolved by spectral methods to create a barcode.

[00213] The approaches in Figures 1a) and 1b) both rely on combinations of signals (colors) and are heavily limited by the types of signals that are available and the resolution at which microscopic instruments can resolve such signals.

[00214] As noted in Figure 1, when 5 different color dyes are associated with probes to identify different molecular targets, the spatial barcoding scheme in Figure 1a) of Figure 1 will

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lead a barcoding capacity of 720; i.e., up to 720 molecular targets can be uniquely coded. Using the approach of Figure 1b), only a maximum of 31 molecular targets can be uniquely coded.

[00215] The approach in Figure 1c), known seqFISH, greatly expanded coding capacity. Here, instead of cramming all coding signals into a single hybridization experiment, barcodes are formed via temporally separately sequential hybridization experiments. The color of probes binding to a target can remain the same or change to a different color in different rounds. The barcode for a target is determined by the order by which each color appears during sequential rounds of hybridization. Details of this barcoding scheme are depicted in Figure 2 and can be found in International Patent Publication No. WO 2014/182528, which hereby incorporate by reference in its entirely.

[00216] This method involves lysing cells and immobilized the mRNA on a surface for single molecule quantification. However, this technology is not limited to profiling only the mRNA species, but also applicable to other molecules such as DNA and proteins.

[00217] Since mRNAs are immobilized on a surface, they can be barcoded by introducing several rounds of hybridizations, imaging the fluorescent spots, and extinguishing the signal for the next round of hybridization. The number of barcodes available scales as F^N where F is the number of fluorophores and N is the number of barcoding hybridization rounds. For example, with 3 fluorophores and 9 rounds of hybridizations, the available barcodes are 3^9=19683. This method achieves single molecule sensitivity which allows the determination of gene expression through counting each identified mRNA molecules on the surface.

[00218] As shown in Figure 2a), in each round of hybridization, multiple probes (e.g., 20-30) were hybridized to each transcript, imaged and then stripped by DNase I treatment before another set of probes are used in the next round of hybridization. Probes with different binding sequences can be used in different rounds of hybridization. Also, the same probe sequences could be used in different rounds of hybridization, but probes were coupled to different fluorophores. A barcode for this mRNA is formed by combining the fluorescent colors associated with the probes in each round of hybridization according to the order or sequence by which the colors are detected. In the example illustrated in Figure 2a, the barcode for the mRNA in N rounds of hybridization will be {purple-blue-…-green}, with each color representing a particular hybridization round.

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[00219] Figure 2b) illustrates exemplary composite four-color FISH images from 3 rounds of hybridizations on multiple yeast cells. As illustration, the same small region is magnified to reveal colored spots representing binding to probes to mRNA transcripts. Colored spots corresponding to the same target from different rounds of hybridization are matched to render a color barcode for the target. In each round of hybridization, the same spots were detected, but the dye associated with the transcript can change. The identity of an mRNA is encoded in the temporal sequence of dyes hybridized. For example, three rounds of hybridizations created the barcodes in Figure 1c): mRNA 1: Yellow-Blue-Yellow; mRNA 2: Green-Purple-Green; mRNA 3: Purple-Blue-Purple; and mRNA 4: Blue-Purple-Blue.

[00220] As noted above, the method of Figures 1c and 2 has significantly increased coding capacity. Based on 5 different visual signals and 7 rounds of hybridization experiments, a total of 78,125 targets (e.g., genes or mRNA transcripts) can be unique coded.

[00221] However, the types of color signals that can be used in these sequential hybridization experiments are limited. In order to encode the human genome, which includes about 20,000 protein encoding genes, at least 7 rounds of hybridization experiments are required. This many number of hybridization rounds result in long and complex barcodes that are hard to distinguish from each other, thus resulting in errors and inaccuracies. If one would like to include error correction in the barcodes, at least 8 rounds of hybridization experiments are needed, leading to even more complex barcodes and likelihood of more errors and inaccuracies.

[00222] Efforts made for creating more types of visual signals have not led to significant improvement. More improvements are need to increase coding capacities, enhance detection accuracies and sensitivities, and reduce errors.

[00223] In one aspect, a novel barcoding scheme is provided. Here, instead of developing different colors that are suitable for probe design, a counter-intuitive approach is adopted to create pseudo-colors to expand capacity of barcoding. Although the term "pseudo-color" is used, one is not limited to using colors in this new coding scheme, symbols, letters, numbers, 2D barcodes, 3D barcodes, and combinations thereof can be used to uniquely identify molecular targets. As disclosed herein the number of pseudo-colors far exceeds the number of actual colors that are associated with the detection probes used in the hybridization experiments.

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[00224] Figure 3A illustrates exemplary aspects that may contribute to error correction during a sequential hybridization process. Such aspects include but not limited to sample processing 302, probe design, barcode design, hybridization, image collection, signal removal and re-hybridization, and data analysis. In practice, any or all of these aspect can contribute to the quality of analysis.

[00225] Barcoding by sequential hybridization includes multiple rounds of hybridization. Each round of hybridization in turn is a multiple step process including most or all of the aspects outline above. Errors and inaccuracies can be introduced at any step during any round of hybridization. Such errors can lead to misidentification of target genes in a sample.

[00226] Prior to hybridization, samples that will be subject to analysis are processed. The main purpose of such processing is to immobilize target molecules; for example, mRNAs, chromosomal DNAs, and proteins. It is essential that the target molecules remain spatially fixed through different rounds of hybridization.

[00227] Probe design contributes to specificity of binding between the probes and target sequences. It is possible to apply hybridization chain reaction to allow multiple probes to bind at the same target sequence to amplify detectable signals. Additionally, as illustrated in Figures 26 and 27, it is possible to insert a cleavable linker between the binding sequence (that binds a target sequence) and signal moiety (that emits visible signals) of a probe. Here, error can be reduced because no removal of probes is needed for the next round of hybridization. Instead, only visible signals are switched.

[00228] Barcodes implemented during the analysis are unique. Nonspecific binding or other mistakes can render the results from one or more rounds of hybridization unreliable. A simple solution is to remove data that are unreliable. However, if data from one or more rounds of hybridization are eliminated from analysis, some of the barcodes would become indistinguishable from each other.

[00229] During and after hybridization of probes to target sequences, there are also aspects that are important for improving the quality of the sequential analysis. For example, the hybridization conditions should be designed to avoid non-specific bindings. This can also be achieved through sample processing and probe design. Similarly, image collection can also be affected by a number of factors including sample processing, probe design and barcode design.

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As described above, probes with too many types of color signal in many rounds of hybridization can lead to barcodes that are hard to resolve or even errors.

[00230] Between hybridization rounds, old probes can be removed before new probes are added. Here, the removal process can also be associated with errors. For example, if the removal condition is too harsh, immobilized biological samples can be disturbed. As a result, positions of visual signals in images from different rounds of hybridization experiments would change.

[00231] Some of the errors may be corrected or reduced by data analysis. For example, in most scenario, bindings between probes and target sequences are observed as colored bright spots over relatively darker background where no binding is observed. The spots are brightest in the center and fade away at the edges. Gaussian distribution analysis can be used to focus on the most significant portion of an image thus leading to better resolved image data. In addition, noise reduction can be used to reduce background signals.

[00232] Figure 3B depicts an exemplary process for performing a pseudo-color based barcoding scheme. Exemplary embodiment 300 provides an example of a pseudo-color based barcoding process. Here, instead of using a large number of actual detectable color signals, which are not available or impractical to obtain, a small number of color probes are repeatedly used in multiple serial hybridization experiments where non-overlapping sets of molecular targets are analyzed. Images from the serial hybridization experiment are combined to form a composite image representing a single barcoding round. In the composite image, at least some of the would-be redundant colors are replaced with predefined symbols in a pseudo-color based scheme. The process is repeated multiple times to produce the final multi-component barcodes.

[00233] At step 302 liquid molecular targets in a biological sample that needs to be barcoded will be identified. In particular, the number of the molecular targets and their respective identity, such as the name of the target and sequence information of the molecular target, will be identified to provide information that will be used for specific binding probe design.

[00234] Prior to actual hybridization experiments, the molecular targets in the biological sample will be immobilized; for example, on a glass cover slip. As disclosed herein, exemplary

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biological sample includes but is not limited to a tissue sample, a cell sample, a cell extract sample, a nucleic acid sample, a RNA transcript sample, a protein sample, or an mRNA sample.

[00235] At step 304, depending on the total number of the biological molecular targets, the number of barcoding rounds will be decided. Because the number of barcoding rounds corresponds to the size of the barcode that will result, the user should decide on how many rounds will be needed depending on the sample size of the molecular targets. In some embodiments, a user may take into consideration the types of color probes that are available. In some embodiment, three or more barcoding rounds will be used. In some embodiments, four or more barcoding rounds will be used. In some embodiments, five or more barcoding rounds will be used. In some embodiments, six or more barcoding rounds will be used. As disclosed air in one advantage of The method disclosed herein is providing relatively simpler barcodes that are more error resistant. In general, a five component barcode is more preferred then an eight component barcode.

[00236] At step 306, the number of symbols in a pseudo-color scheme will be determined based on the total number of the molecular targets (e.g., $N \gg 2$) and the intended for number of barcoding rounds (e.g., $n \geq 2$). As disclosed here in the minimum number of pseudo-color symbols (e.g., S) can be determined according to the following equation:

$$S \geq \sqrt[n]{N}, \text{ where S, n and N are all integers.} \quad (1)$$

[00237] In some embodiments, one or more error correction coding rounds (e.g., x rounds where $x \geq 1$) will be implemented. As such, one may choose a higher number of pseudo-color symbols to avoid too many barcoding rounds. The minimum number of pseudo-color symbols, when one or more error reaction rounds, can be determined according to the following equation:

$$S \geq \sqrt[n-x]{N}, \text{ where S, n, x, and N are all integers.} \quad (2)$$

[00238] Although the term pseudo-color is used, the actual codes used in barcodes are not limited to colors. Any symbol or combination of symbols can be used as codes in a barcode so long as such symbol or combination of symbols are unique. Exemplary symbol or combination of symbols include but are not limited to colors, numbers, letters, shapes, or combinations thereof. It would be understood that a color based scheme would be preferred because

[00239] At step 308, once the total number of symbols to be used in a pseudo-color barcoding scheme and the number of barcoding rounds are determined, non-redundant and

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unique barcodes will be created. For example, in a n-component barcode using S pseudo-color symbols, a barcode can be expressed as: $\{B_1, B_2, \ldots, B_n\}$, where each of B_1, B_2 through B_n is selected from the S pseudo-color symbols. Standard code design algorithms can be used such that the results barcodes are unique.

[00240] During hybridization analysis, barcodes can be shortened due to loss of data from one or more rounds of hybridization, For example, after the loss of one round of hybridization, n-component barcodes can become n-1- component barcodes.

[00241] When error correction algorithm is not implemented during probe design, the resulting n-1- component barcodes may now include redundant codes, making it difficult or impossible to decode targets based on the coding scheme useless. In a more specific example using colors as symbols in a pseudo-color coding scheme, two distinct six-component barcodes: {Red-Blue-Green-Red-Yellow-Blue} and {Red-Blue-Green-Green-Yellow-Blue} can become {Red-Blue-Green-~~Red~~-Yellow-Blue} and {Red-Blue-Green-~~Green~~-Yellow-Blue} if data from the fourth hybridization round becomes unavailable. As a result, the results from the entire set of experiments will be discard, wasting time and resource.

[00242] The current disclosure also creates barcodes that are drop or error-resistant. In some embodiments, one or more rounds of error correction can be implemented. Additional code design algorithms can be applied such that the resulting barcodes are error resistant; for example, the loss of any one or more rounds of hybridization data would still lead to shortened barcodes. However, the shortened barcodes are still unique and can be used to specifically identify a molecular targets.

[00243] In some embodiments, implementing an error correction mechanism involves removing barcodes that are vulnerable to loss of data. As such, the number of targets that can detected using the error-corrected scheme will be lower than that of the original scheme without error correction. To detect the same number of genes, more hybridization rounds would generally be needed.

[00244] The current method is advantageous in that it does not require additional hybridization rounds. Instead, coding capacity of the same number of hybridization rounds can be increased by designating more pseudo-color symbols.

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[00245] At step 308, barcodes for an entire set of hybridization experiments will be created each corresponding to a particular molecular target. For example, a set of hybridization experiments including includes 4 barcoding rounds, each of which in turn including includes 4 serial hybridization experiments. In each hybridization experiment, 3 different detectable signals (such as three different fluorophores) will be associated with probes that bind to a set of molecular targets. Here, each detectable signal in the same serial hybridization experiment can be associated with multiple molecular targets; for example, hundreds or even more molecular targets. For example, 100 or more molecular targets can be assigned barcodes that start with the same pseudo-color symbol; i.e., all the molecular targets can be labeled with the same detectable signal. The unique identity of each molecular target will be reflected by symbols used in the subsequent three positions in the barcodes. To complete the barcoding in the example above will require that each target 3 molecular target will have a total of 48 hybridization experiments in four barcoding runs (see, Figure 10). These 48 hybridization experiments can detect a total of 20,736 molecular target (12^4) based on 12 pseudo-colors. If one error correction round is implemented, only up to 1728 (12^3) molecular targets can be detected. If 1728 different mRNA transcripts are to be detected with error correction, 1728 unique barcodes with embedded error-correct mechanism for one round of data loss will be created. Actual hybridization experiments will be carried out based on these predesigned barcodes.

[00246] At step 310, after unique barcodes are generated, each identified molecular target will be assigned one of the unique barcodes. For example, for a five component barcode based on 12 pseudo-color symbols, the barcodes will be generated using the 12 symbols. The first code can be any one of the 12 symbols, but each subsequent symbol and each subsequent barcode will be generated taking into consideration the symbols and barcodes that have already been used in order to avoid redundant barcodes. In some embodiment, additional error correction algorithms are implemented in barcode design such that the resulting barcodes are resistant to mistakes such as the loss of data from an entire barcoding round.

[00247] In some embodiments, probes can be synthesized according to the predetermined probe designs. Each probe can include a binding sequence that specifically target a site in an intended molecular target. For each molecular targets, multiple probes with different binding sequences targeting different sites of the same molecular target can be synthesized. When

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probes with different binding sequences targeting the same molecular target are used in one hybridization rounds, the probes will be associated with the same detectable signal.

[00248] In some embodiments, a probe can include one or more readout sequences that can be connected, directly or indirectly, to either side of the binding sequence. In some embodiments, one or more readout sequences are directly connected to the binding sequence as one or more overhangs. In some embodiments, one or more readout sequences are indirectly connected to the binding sequence of the probe via one or more intermediate molecules; for example, as one or more overhangs of a bridge probe. Exemplary intermediate molecules include but are not limited to an RNA bridge probe, a DNA bridge probe, a protein bridge probe, a probe for hybridization chain reaction (HCR), a hairpin nucleic acid probe, an HCR initiator, an HCR polymer, other known amplification methods, or combinations thereof.

[00249] In some embodiments, the number of different readout sequences is the same as the number of barcoding rounds. In some embodiments, the number of readout sequences is greater than a number of barcoding rounds. In some and body months, each type of readout sequence, when activated, is capable of generating one color signal. In some embodiments, different readout sequence, when activated, are capable of generating the same color signal. Any detectable signals suitable for hybridization experiments can be used.

[00250] For five barcoding rounds, five groups of probes can be designed and synthesized for each molecular target. Probes in each group would increase food binding sequences that target specific sequences within a molecular target. In some embodiments, probes in the same group will bind to the same target sequence. In other and more preferred embodiments, probes in the same group will bind to multiple target sequences. As disclosed herein, each barcoding around 1 to 100 probes can be used targeting a specific molecular target. In some embodiments, 100 or fewer, 80 or fewer, 60 or fewer, 50 or fewer, 40 or fewer, 30 or fewer, 20 or fewer, or 10 or fewer probes can be used. In some embodiments, between 5 and 40 probes are used.

[00251] In some embodiments, these probes will bind to two or more target sequences, three or more target sequences, four or more target sequences, five or more target sequences, or six or more target sequences in the same molecular target. And some embodiments, the probes will be concentrated in one area of the molecular target. In some embodiments the probes will be spread out along the entire length of the molecular target.

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[00252] At step 310, all probes required to carry out the entire set of hybridization experiments will be synthesized, each associated via direct or indirect connection to a primary binding sequence. For example, a set of hybridization experiments including five barcoding round each including 12 serial hybridizations will have a total of 60 hybridization experiments. After step 310, all probes required for each hybridization will be designed and synthesized.

[00253] At step 312, serial hybridization experiments can be performed using probes synthesized in the previous step. Barcodes associated with these probes are stored in a database or library. Each probe will be associated with a code in the designated barcode for the molecular target of the probe. The number of hybridization experiments within a serial hybridization round will be determined based on the availability of different types of detectable signals (such as colors) and the total number of symbols in the pseudo-color barcoding scheme. As an example, hybridization experiments based on a 60 pseudo-color scheme will be carried out s using 4 different types of color signals. Then 5 rounds of barcoding with the 60 pseudo-color will be carried out for $60^5 = 7.776 \times 10^8$ number of barcodes. In some embodiments, in each barcoding round, 15 serial hybridization rounds will be performed. In each of the 15 hybridization round, all four color signals are used to each identify a different molecular target. Here, after the particular round of serial hybridization experiments, 60 unique molecular targets will be labeled with color signals. In some embodiments, 20 serials hybridization arounds will be performed using three of the four color signals, each color representing a different molecular target. Again, after the particular round of serial hybridization experiments, each 60 unique molecular targets are decoded and associated with a previously assigned pseudo-color symbol. In some embodiments, any combinations of three colors can be used in the serial hybridization experiments. In some embodiments, in each hybridization round, two to four color signals can be used, each representing each representing a different molecular target. In some embodiments, one color signals can be used in one or more hybridization experiments in a serial hybridization round. Here, one color must be present in separate hybridization experiments in order to represent different molecular targets. For example, if one is limited only one type of color signal, hybridization experiment can be carried out 60 times in each barcoding round. Each hybridization experiment identifies only one molecular target. It would be understood that performing a high number of hybridization experiments may not be desired because the

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biological sample being analyzed can be degraded, for example, by enzymes that digest nucleic acids.

[00254] In some embodiments, during each hybridization experiment in each serial hybridization round, reference visual signals are introduced. These reference visual signals are associated with one or more alignment references that are associated with a biological sample and do not change their positions between hybridization rounds. These alignment references can serve as standard for subsequent image analysis. They can be part of the biological sample or external material added to and immobilized with the biological sample. In some embodiments, alignment references are immobilized at the same time as the biological sample. In some embodiments, alignment references are immobilized at a different time from when the biological sample is immobilized. Exemplary alignment references include but are not limited to beads, oligonucleotide sequences immobilized on the coverslips and detected by a complementary oligo, microscopic objects (e.g., a metal bead, a gold bead, a polystyrene bead loaded with a dye), PCR handle sequences on the primary probe, or combinations thereof. As disclosed herein, the reference signals are fiducial markers for alignment between the different serial and barcoding hybridizations. They are present throughout all of the hybridizations. In some embodiments, at least one marker is present per image. They can be probes targeting all of the primary probes (as shown in Figure 18a, yellow probes), such that all the dots are observed in all of the hybridizations. In some embodiments, the reference signals can be produced from beads attached to the cover glass, or other molecules or particles. In some applications, where "super-resolution" capabilities are used, signals form the beads can be Gaussian fitted to nanometer resolution, and images between hybridizations can be aligned to that precision. In some embodiments, this becomes useful for the in situ application where Gaussian fitting allow more molecular targets to be detected in each pseudo-color and then collapsed into the composite image for barcoding.

[00255] At step 314, an image of visible probes bound to the biological sample will be taken after each hybridization experiment in a serial hybridization round. All images from hybridization experiment in a serial hybridization round will be aligned using reference visual signals to create a composite image.

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[00256] At step 316, it will be determined whether there are one or more barcoding rounds remaining based on the barcodes pre-designed at step 308 If yes, the method returns to steps 312 and 314 and more serial hybridization experiments are carried out to constitute another barcoding round and produce another composite image.

[00257] If no, the hybridization portion of the analysis is complete and the method proceeds to step 318. Images and composite images will be subject to further data analysis. For example, in a sample with limited space, e.g., in a single cell, Gaussian localization analysis can be carried to generate, for example, more focused color spots each representing binding to a molecular target. Other data processing methods can also be used to enhance data quality, for example, a de-noise mechanism can be applied to reduce back signals.

[00258] The method concludes at step 320.

[00259] Figure 4 depicts a schematic overview of Sequential Probing Of Targets (SPOTs), targeting RNA samples using a pseudo-color barcode scheme. The method performs transcriptome level profiling of mRNAs with single molecule sensitivity and high accuracy using a method based on sequential FISH (seqFISH) [Lubeck 2014]. The initial demonstration of seqFISH showed promising coding capacity to barcode a large number of molecular species in cells and tissues [Shah 2016]; however, a major limitation has been identified: when seqFISH is performed in cells, optical diffraction limit prevents many mRNAs to be resolved simultaneously due to the limited space in a cell.

[00260] As shown in Figure 4, there are a total of 5 barcoding runs and each barcoding run has four serial hybridizations. Three different fluorophores (e.g., red, green and blue) are used in each serial hybridization experiment. For simplicity, each serial hybridization experiment in Figure 4b only shows one molecular target for each fluorophore. However, in practice, each fluorophore can represent tens, hundreds or even more molecular targets. In addition, unlike the illustration in Figure 4b which shows that the same number of targets are tagged in each serial hybridization, different numbers of molecular targets can be tagged in different serial hybridizations. For example, for a 1,500 mRNA experiment of 4 barcoding runs, serial hybridization No. 1 of barcoding round 1 can tag 400 mRNA transcripts with three different fluorophores (e.g., red, green and blue). Serial hybridization No. 2 of the same barcoding round can tag 280 mRNA transcripts with the same three fluorophores. 320 mRNA transcripts can be

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tagged the same three fluorophores in serial hybridization No. 3. And 500 mRNA transcripts can be tagged the same three fluorophores in serial hybridization No. 4. As the numbers suggest, within each serial hybridization, multiple mRNA transcripts can be tagged with the same fluorophore. In some embodiments, a fluorophore is attached to a probe via multiple readout sequences, which in turn are connected to a primary binding sequence targeting a specific site in a molecular target. In some embodiments, the readout sequences can be connected directly to the primary sequence. In some embodiments, the readout sequences can be connected indirectly to the primary sequence, for example, via one or more intermediate molecules. In some embodiments, the one or more intermediate molecules comprise an RNA bridge probe, a DNA bridge probe, a protein bridge probe, a probe for hybridization chain reaction (HCR), a hairpin nucleic acid probe, an HCR initiator, an HCR polymer, or combinations thereof.

[00261] In addition, there can be different number of serial hybridizations in two different barcoding rounds. For example, in the example presented above, 4 serial hybridizations are included in barcoding round 1. Barcoding round 2 can have 4 or 6 or 3 serial hybridizations, so long as the results from the two sets of serial hybridizations can reveal the same 1,500 mRNA transcripts. For example, images from all serial hybridizations in the same barcoding round can be compiled into a composite image for the barcoding round. In this barcode scheme, 12 pseudo-colors are created, which can be assigned numbers as 1 through 12. In some embodiments, 12 different colors are assigned as symbols of the 12 pseudo-colors. After all 5 barcoding rounds are completed, the 1,500 mRNA transcripts will be fully coded.

[00262] In some embodiments, the total number of targets revealed in two different barcoding rounds can differ. For example, there are some problems and only 1,400 mRNA transcripts are tagged in in barcoding round 1 while 1,350 mRNA are tagged in barcoding run 2. Subsequent image and data analysis can reveal that only 1,200 of the mRNA transcripts overlap between the two barcoding rounds. And ultimately only 1,200 mRNA transcripts are fully coded in all 5 barcoding rounds.

[00263] The pseudo-color scheme can overcome this density problem in both in situ and in vitro applications. In the in vitro cases, implemented with SPOTS, capturing transcripts onto an oligonucleotide dT surface and adjusting the dilution factors can easily remove the optical crowding problems and allow the transcriptome to be imaged by seqFISH.

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[00264] In addition, pseudo-color is more efficient in terms of imaging time than all existing imaging methods including expansion microscopy. For example, with 3 fluorophores, it takes $3^{(10-1)}$=19,683 to code for the transcriptome with one round of error correction. Thus, a total of 30 frames of imaging is required. In a pseudo-color scheme, 20 pseudo-colors can be used for 4 rounds of hybridization to code for $20^{(4-1)}$=8000 genes in each of the three fluorophore channels for a total of 24,000 genes. This requires 3x20x4=240 frames to image, a 8 fold increase in the imaging time. However, because the density of mRNA is effectively diluted into 3x20=60 pseudo-channels instead of 3 fluorophore without pseudo-color coding, the density problem is alleviated with a factor of 20. The target spots can be localized to nanometer precision by Gaussian fitting to decrease the density in each pseudo-color channel before barcode alignment. Thus, the benefit of pseudo-color coding is to decrease the density of the target spots in the cell, while saving imaging time compared to expansion microscopy, where expanding the sample by 20 folds requires an additional 20 fold increase in imaging time. Since imaging time is rate limiting in any sequential imaging method, pseudo-color solves a major problem in implementing transcriptome profiling in situ.

[00265] In some embodiments, for in situ experiments based on a pseudo-color scheme, fewer number of different detectable signals can be used so that they can be resolved from each other more clearly due to the limited amount of real estate within a cell. In some embodiments, with super resolution microscopy, even densely populated detectable signals can be resolved from each other.

[00266] As an example, three different color signals are used in an in situ experiment. In some embodiments, for each color, hybridization experiments can be repeated 20 times to generate a 20 pseudo-color image. Here, each 20 pseudo-color image is compiled from only 1 real color channel although multiple colors can be used. The single-color approach offers several advantages during imaging analysis. Significantly, there are chromatic aberrations in a microscope, and aligning images across different actual color channel can be difficult and lead to errors or inaccuracies. In the current embodiments, all 20 pseudo-color can be from the same color channel (e.g., the Cy5 channel) then all images are by definition aberration free.

[00267] Using an mRNA hybridization experiment as an example, mRNA transcripts bound to activated color probes are visualized as dots in the image. In some embodiments,

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Gaussian fitting is applied to localize the RNA dots in cells and generate a higher resolution image or less dense image of the cell. In some embodiments, Gaussian localization can take place at one or more time points; for example, for each image corresponding to a serial hybridization experiment, for the images from all serial hybridization experiments in a barcoding round; for each composite image corresponding to a barcoding round. In some embodiments, Gaussian localization can be repeated multiple times.

[00268] In some embodiments, color beads can be used to align the pseudo-color images to create composite images.

[00269] When multiple color probes are used (e.g., 4 or 5 colors), the resulting image can be too dense and it can be difficult or impossible to apply the Gaussian fitting process, which requires discrete dots. By virtually diluting the samples to 60 pseudo-color, then the density becomes much less of an issue. After the first barcoding round, DNAse can be used to strip the probes, which carries the RNA targeting sequence and one round of readout sequence. This ensures that when barcoding is done in cells (in situ), there is no nonspecific binding. A new set of probes with the 2nd barcode sequence is then hybridized. Another 20 rounds of serial hybridization with 3 colors is done. In some embodiments, images from the hybridization experiments are subject to Gaussian fit, and be compiled into 3 sets of 20 color pseudo-color images.

[00270] This process can be repeated 4 times to generate a 20x20x20x20 barcode in each color. So 8000 error corrected barcodes are available in each color, to encode a total of 24000 barcodes.

[00271] In this example, the barcode scheme can be actually 3 separate pieces of 8000 barcodes in each of the colors. There can be 60 readout sequences total. Because of the DNAse stripping step, the readout sequences used in the 2nd barcoding hybridization can be the same as 1st barcoding hybridization.

[00272] For in vitro analysis, all the readout sequences can be added onto the primary binding probes. In those cases, the readout sequences for the different barcoding hybridizations have to be different. The approach is possible in vitro because there is very little nonspecific binding, since the biological sample has often been extracted out of the cells. For in situ, more stringent conditions are needed to avoid nonspecific binding in cells.

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[00273] An alternative approach is to generate 100 readout sequences all in one color and just code for 10,000 targets with 3 rounds of hybridization (100x100x100) with one round of error correction. As disclosed herein, any manageable number of readout sequences can be used; for example, 50 or fewer, 60 or fewer, 80 or fewer, 100 or fewer, 150 or fewer, 200 or fewer, 250 or fewer, 300 or fewer, 400 or fewer, or 500 or fewer. In some embodiments, more than 500 readout sequences can be used.

[00274] As disclosed herein, the pseudo-color scheme is better than expansion microscopy in solving the density issue. Expansion microscopy is a new technology for imaging biological samples with fine detail by physically making the samples bigger through a chemical process that preserves nanoscale isotropy. Expansion microscopy enables super-resolution imaging on a conventional light microscope. However, expansion is a complicated procedure and the sample can contract and expand during the rehybridization process, which makes aligning the barcodes hard. Also, if the tissue sample is expanded 10 times, the tissue will be imaged 10 times longer. So even though pseudo-color appears to require more hybridizations, the total imaging time is comparable or even more favorable compared to if one had to expand the sample. For example, the 60 pseudo-color scheme is basically 20x expansion compared to just a 3 color scheme.

[00275] In a seqFISH experiment, 10 rounds of hybridization are needed to encode 20,000 genes with error correction with 3 colors ($3^{(10-1)}$=19,683). As disclosed herein, the pseudo-color scheme use 20x4=80 rounds of hybridizations for each channel. Thus, the number of hybridizations gone up by 8 fold, but the density is diluted down by a factor of 20. Thus the current approach is more efficient than expansion microscopy. For example, one can also use 1 channel and 100 pseudo-color and three rounds of hybridization, which correspond to 33.3 fold (100 pseudo-colors/3 colors) improvement in dilution and only 10 fold increase in imaging time. As disclosed herein, 3 color signals and 20 pseudo-color in each channel have produced excellent results. In practice, one can balance the "dilution" factor with the number of targets being analyze to determine the number of the actual color signals and the number of pseudo-colors.

[00276] In some embodiments, the current approach can also be combined with correlation FISH (corrFISH, Coskun and Cai, Nature Methods 2016) to quantify the abundances of highly expressed mRNAs. corrFISH can be applied to decoding samples where the number of

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target dots are so high that they are individually resolvable. This approach can decode the abundance of the genes, but trade off in spatial resolution. As an example of implementing the corrFISH scheme in addition to the existing pseudocolor scheme, a 4th channel can be dedicated to barcode highly abundant transcripts by corrFISH in addition to the 3 colors channels used for generating the pseudo-colors. The 4th channels can generate 20 pseudo-color through 20 rounds of serial hybridization. Then corrFISH can be used to code for another 8000 genes, but highly expressed.

[00277] In some implementations, primary probes can be stripped and signals extinguished after the pseudocolor schemes and probes targeting another set of targets can be hybridized. The utility of this can be that multiple types of targets can be probed sequentially in the same cells. For example, 20,000 intronic RNAs can be first targeted through the pseudo-color scheme. Then 20,000 mRNAs can follow, with 20,000 proteins after that. Or alternatively, probes can be used to target specific highly expressed genes to measure their localizations in cells, especially cells with polarization, such as processes in neurons. Or probes can be used to target specific isoforms or combination of isoforms of specific targets.

[00278] As disclosed herein, the approached disclosed herein can also work with amplification methods, such as HCR, to implement this work robustly in tissues.

[00279] It will be noted that although the approach above is described in connection with an in situ experiment, one of more aspects of it can be applied to in vitro experiments as well.

[00280] In one aspect, disclosed herein are readout probes with cleavable linkers. Figure 25 depicts exemplary chemical reactions for synthesizing a readout probe with a disulfide linker.

[00281] In one aspect, sequential barcoding FISH (seqFISH) is performed by using nucleic acid readout probes that are conjugated with a signal moiety via a cleavable linker. Any suitable cleavable linkers can be used, including but not limited to an enzyme cleavable linker, a nucleophile/base sensitive linker, reduction sensitive linker, a photo-cleavable linker, an electrophile/acid sensitive linker, a metal-assisted cleavable linker, or an oxidation sensitive linker. Exemplary linkers can be found in Leriche et al., 2012, "Cleavable linkers in chemical biology," Bioorganic & Medicinal Chemistry 20:571–582, which is hereby incorporated herein in its entirety.

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[00282] In some embodiments, the cleavable linker is a disulfide linkage. In some embodiments, the cleavable linker is a nucleic acid restriction site. In some embodiments, the cleavable linker is a protease cleavage site.

[00283] An exemplary system utilizing nucleic acid readout probes is shown in Figure 26. As depicted, a gene specific primary probe binds to a target site; e.g., in an mRNA molecule under an in situ or in vitro setting. Besides a binding sequence, the primary probe further includes an overhang sequence at one end of the binding sequence. In some embodiments, a second overhang sequence is included at the other end of the binding sequence.

[00284] In some embodiments, an overhang sequence includes one or more target sequences to which one or more nucleic acid readout probes bind. In some embodiments, each target sequence uniquely interacts with a set of readout probes with specific readout binding sequences. As disclosed herein, an overhang sequence may include two target sequences, three target sequences, five or fewer target sequences, seven or fewer target sequences, or ten or fewer target sequences. In some embodiments, an overhang sequence may include ten or more target sequences. Similar arrangements can be implemented where there are two overhang sequences.

[00285] In some embodiments, an overhang sequence binds to a bridge probe that provides target sequences for one or more readout probes to bind, as depicted in Figure 26. A bridge probe can be interchangeably called an intermediate bridge probe or a secondary bridge probe. A bridge probe includes a binding sequence that binds to all or a portion of an overhang sequence in a primary probe. In some embodiments, a bridge probe further includes one or more readout binding targets that are connection in series and linked to the binding sequence.

[00286] In some embodiments, as depicted in Figure 26, two bridge probes can bind to the same primary probe via two overhang sequences.

[00287] As disclosed herein, a bridge probe may include two readout binding targets, three readout binding targets, five or fewer readout binding targets, seven or fewer readout binding targets, or ten or fewer readout binding targets. In some embodiments, an overhang sequence may include ten or more readout binding targets. Similar arrangements can be implemented where there are two bridge probes bound to overhang sequences.

[00288] Exemplary rehybridization schemes utilizing the readout probes are illustrated in Figure 26. For example, the first round of rehybridization (hyb1) begins with the hybridization

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of gene specific primary probes to the target mRNA. Each gene specific primary probes contains one or more "overhang" sequences that allows the secondary bridge probes to hybridize against. The secondary bridges contain two or more tertiary readouts binding sites which is the key to efficient and quick rehybridization. In the first hybridization, unique tertiary readout probes conjugated with blue dye are hybridized to their unique binding sites on the secondary bridge probe. Once imaged, the sample is treated with reducing agent such as TCEP or DTT to cleave off the disulfide-linked dyes. Then, the sample is washed with wash buffers. During the second round of hybridization, a second set of unique tertiary readout probes with red dye is hybridized to its unique binding site on the secondary bridge. After two rounds of hybridizations, a particular mRNA is then barcoded with a color barcode of red and blue. Additional rounds of hybridization can be applied to create more sophisticated barcoding sequences. Technically, the scaling factor of seqFISH with this rehybridization method depends on the number of available secondary bridges with its number of unique tertiary probes binding sites. For example, by incorporating 2 secondary bridges with total 8 unique tertiary readout binding sites (N=8) , and with 4 fluorophores (F=4), one can generate up over 64,000 unique barcodes ($F^N = 4^8 = 65,536$). Moreover, in embodiments where bridge probes are used, it is possible to strip off the secondary bridges with high concentration of formamide, and flow in another unique set of secondary bridges to continue the scaling process, which further increases the upper limit of the scaling factor.

[00289] In one aspect, disclosed herein are methods and systems for amplifying visual signals during each round of hybridization during sequential hybridization reactions, based on hybridization chain reaction (HCR). An exemplary embodiment of HCR is illustrated in Figure 27A. During hybridization round 1, probes with overhang initiator sequences are added to a nucleic acid target molecule such as an mRNA or a DNA. Also added are hairpin nucleic acid probes bearing sequences complementary to those of the initiator sequences. The presence of initiator sequences cause unfolding of the hairpin nucleic acid probes and result in chain reactions that lead to self-assembled extended HCR polymers. Because each hairpin nucleic acid probe bears a signal, self-assembled extended HCR polymers result in amplification of signals and better detection of target sites.

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[00290] Figure 27B illustrates an exemplary readout probe embedded with a cleavable linker. Here, the cleavable linker is a disulfide bond. At one end of the cleavable linker, a readout probe as disclosed herein includes a binding sequence that allows it to bind to a specific nucleic acid target. In some embodiments, the nucleic acid target is an mRNA or a DNA. In some embodiments, the nucleic acid target is within an intact cell or as part of cell extract. In some embodiments, the nucleic acid target is within a primary binding probe that directly binds to a target site in an mRNA. In some embodiments, the nucleic acid target is within a secondary binding probe that binds to a primary binding probe that directly binds to a target site in an mRNA. In some embodiments, the nucleic acid target is within a tertiary or quaternary binding probe. One of skill in the art can apply the principle to any level of binding and interaction.

[00291] At the other end of the cleavable linker, a readout probe as disclosed herein further includes an HCR initiator sequence. When exposed to hairpin nucleic acids bearing partial or complete complementary sequences, the initiator sequence can trigger a chair reaction that allows a signal motif formed by multiple extender probes. Each extender probe includes a signal moiety. Aggregation of multiple extender probes enhances signal detection.

[00292] An exemplary scheme for forming a signal motif with multiple extender probes during a sequential hybridization process is illustrated in Figure 27C. During the first round of hybridization, nucleic acid detection probes with embedded cleavable linkers binds to a first target site within a nucleic acid target sequence. In some embodiments, extender probes are added after the initial binding of nucleic acid detection probes to the first target sequences. In some embodiments, extender probes form an aggregate before the aggregated polymer is added to the reaction mix and binds to the imitator sequence in the nucleic acid detection probes.

[00293] In some embodiments, extender probes are standard hairpin probes each including a sequence that is partly or completely complementary to the initiator sequence in the readout probes. In these embodiments, extender probes are very similar or identical to each other. The size of the resulting extendible signal motif may be controlled by the concentration or absolute quantity of the extender probes added.

[00294] In some embodiments, extender probes including different types of nucleic acid sequences can be used to achieve controlled signal amplification. For example, the signal can be amplified five times if five populations of extender probes are used: {EP_1, EP_2, EP_3, EP_4, and

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EP_5}. The first population of extender probes includes a binding sequence that binds to all or a part of the initiator sequence. The second population of extender probes includes a binding sequence that binds to a region in the first population of extender sequence. The third population of extender probes includes a binding sequence that binds to a region in the second population of extender sequence. The fourth population of extender probes includes a binding sequence that binds to a region in the third population of extender sequence. The fifth population of extender probes includes a binding sequence that binds to a region in the fourth population of extender sequence. In such embodiments of linear amplification, the size of the resulting extendible signal motif can be controlled by the number of populations of extender probes that are provided.

[00295] In some embodiments, an extender probe may include multiple binding sites for binding subsequent extender probes. For example, besides binding to the initiator sequence, EP_1 may include two or more binding sites for EP_2, thus allowing further amplification of the signal. This form of amplification may occur at any level. For example, in the example above, multiple binding sites for subsequent or downstream extender probes can be implemented in any one or combinations of EP_1, EP_2, EP_3, or EP_4. For example, extender probes from EP_2, EP_3, or EP_4 can all bind to target sites in EP_1, which in turn binds to the initiator sequence.

[00296] In some embodiments, the amplification occurs at multiple levels. Generally, when m populations of extender probes are present, multiple binding sites for subsequent or downstream extender probes cam be implemented in any one or combinations of EP_1, EP_2, …, or EP_{m-1}. Additionally, when multiple binding sites are present, they can be connected in series or arranged in a non-linear fashion (e.g., in a branched or circular arrangement). Depending on the number and configuration of the binding sites, the resulting extendible signal motif can be a stick, a ball, a net or in any other applicable form.

[00297] One of skill in the art would understand that any suitable number of populations of extender probes can be added to achieve an optimal signal to noise ratio for the best imaging effects. For example, the extender probes can include five or fewer, seven or few, 10 or fewer, 15 or fewer, 20 or fewer, 25 or fewer, 30 or fewer, 40 or fewer, 50 or fewer populations.

[00298] In some embodiments, the extender probes are mixed together prior to being mixed with the readout probes having the initiator sequence. In some embodiments, the extender

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probes are sequentially added to the readout probes having the initiator sequence where the readout probes are already bound to its nucleic acid targets.

[00299] As shown in Figure 27C, after imaging analysis, a cleaving agent can be applied to sever the linker between the binding sequence and the imitator sequence in a readout probe. The amplified polymers can then be cleaved off and washed away.

[00300] During a second round of rehybridization, new nucleic acid detection probes are applied. The new nucleic acid detection probes include a different binding sequence that binds to a second and different target site in the nucleic acid target sequence. The new nucleic acid detection probes also include a cleavable linker and an initiator sequence. The initiator sequence can be the same as or different from the initiator sequence from the previous set of nucleic acid detection probes.

[00301] The new extender probes are used, as described hereinabove, to form amplified polymers to enhance signal detection. After imaging analysis, the new set of amplified polymers can be cleaved off and washed away. By using extender probes bearing a different type of visual signals, barcodes can be established for nucleic acid targets. Depending on the availability of target sites within a nucleic acid target, multiple rounds of hybridizations can be performed to create more complex barcodes. For example, there can be three rounds of hybridizations, four rounds of hybridizations, five rounds of hybridizations, seven or fewer rounds of hybridizations, 10 or fewer rounds of hybridizations, 12 or fewer rounds of hybridizations, 15 or fewer rounds of hybridizations, 20 or fewer rounds of hybridizations, 30 or fewer rounds of hybridizations, 40 or fewer rounds of hybridizations, or 50 or fewer rounds of hybridizations.

[00302] The compositions and methods disclosed herein can be used in sequential hybridizations to identify any suitable cellular targets within an intact cell or in an *in vitro* setting. In some embodiments, the cellular targets can be mRNAs or DNAs. In some embodiments, the cellular targets can be proteins. For example, the initial target-binding primary probe can be an antibody conjugated with nucleic acid sequence for subsequent bindings.

[00303] As exemplified herein, provided technologies work for a wide variety of samples. For example, HCR-seqFISH worked in brain slices and that SPIMs can robustly detect single mRNAs in CLARITY brain slices. In some embodiments, provided technologies are useful for

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profiling targets in mouse models of neurodegenerative diseases, or human brains. No other technology prior to the present invention can deliver the same quality and quantity of data.

Error Correction Mechanism

[00304] Figure 3A illustrates general aspects of a sequential hybridization analysis that may contribute to quality of the analysis. Sequential hybridization includes multiple rounds of hybridization, where each round of hybridization is a multiple step process. Errors can be introduced at any step during any round of hybridization. Such errors can lead to misidentification of target genes in a sample.

Barcodes and Error Correction

[00305] In one aspect, disclosed herein are methods for designing barcodes with built-in error correction mechanisms such that the multi-component barcodes can withstand the loss of the data from one or more rounds of hybridization (i.e., drop-safe). As disclosed herein the terms "barcode" and "code" are used interchangeably.

[00306] As disclosed herein, by using probes that are associated with F detectable visual signals ($F \geq 2$), a sequential hybridization of N rounds ($N \geq 2$) can generate a total of F^N combinations of visual signals. In some embodiments, these combinations of visual signals can be used as barcodes to uniquely identify cellular targets such as mRNA, DNA, or even protein.

[00307] Figure 30 illustrates an exemplary process 3000 for generating drop safe barcodes.

[00308] At step 3010, the total number of genes that will be analyzed during the hybridization experiments is determined. This number sets the threshold values for the number of detectable visual signals (F) and the total number of rounds in the sequential hybridization (N).

[00309] Once the total number of genes is determined, steps 3020 and 3030 are performed simultaneously. The number of genes being analyzed must be smaller than the total number of possible combinations of visual signals (F^N). Practical aspects of the hybridization analysis need to be considered when selecting values for F and N. One would tend to reduce the number of rounds of hybridization to as few as possible. Theoretically, this can be achieved by using a high number of detectable visual signals (F). In practice, however, too many different types of visual

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signals may interfere with each other. For example, overlapping of visual signals can lead to barcode misidentification.

[00310] At step 3040, a library of drop-safe unique barcodes are generated by implementing one or more error correction mechanisms.

[00311] In some embodiments, a repeat round can be performed for any round during a sequential hybridization of N rounds, rendering a new sequential hybridization of $(N+1)$ rounds. The extra repeat round can be an error correction round. The repeat round can be a duplicate of any round of the n rounds sequential hybridization. The repeat round can take place as any round during the sequential hybridization $(N+1)$ rounds.

[00312] After the repeat, there are two rounds of hybridization that should be identical to each other. Consequently, the complete loss of one of the repeat rounds does not affect the outcome of the sequential hybridization. As such, either of the repeat rounds is a drop-safe round.

[00313] Figure 2 illustrates an experiment where 3 rounds of hybridization using probes with 4 types of detectable visual signals (red: R, yellow: Y, green: G, and cyan: C) are used to create barcodes for 4 different mRNA molecules. Hybridization round 3 is a repeat of hybridization round 1, as summarized in Table 1 below.

Table 1. Illustration of the effect of repeat hybridization rounds.

mRNA molecules	Color barcodes (3 rounds)	Color barcodes (dropping round 1)	Color barcodes (dropping round 3)
mRNA1	Y-C-Y	C-Y	Y-C
mRNA2	G-R-G	R-G	G-R
mRNA3	R-C-R	C-R	R-C
mRNA4	C-R-C	R-C	C-R

[00314] As shown in the table above, data from one of the repeat rounds can be dropped completely in case of major experiment error, barcodes derived from the remaining rounds of hybridization still uniquely represent the mRNA molecules.

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[00315] In some embodiments, even in a questionable hybridization round, most of the information is still reliable. Only some of the bindings between probes and target sequences include inaccurate information. In some embodiments, partial data from a questionable round of hybridization are used. For example, in the illustration above, binding signals can be missing or ambiguous for a particular location during hybridization round 1, which can produce an incomplete three letter barcode *-C-Y for the particular location, where * remains undetermined. In the scheme illustrated, the identity of * is not needed to decipher that the code is for mRNA1. Similarly, binding signals can be missing or ambiguous for a particular location during hybridization round 2, which can produce an incomplete three letter barcode R-*-R for the particular location, where * remains undetermined. Once again, the identity of * is not needed to decipher that the code is for mRNA3.

[00316] Additionally, data from repeat rounds can validate each other. For example, in Figure 2C, a circle highlights a cyan data point in the image corresponding to hybridization round 2. In the same location, the image corresponding to hybridization round 3 reveals a yellow data point. Based on only information from hybridization rounds 2 and 3, this location would be identified as part of mRNA1. However, no signals are identified at the location during hybridization round 1, which suggests that the highlighted data points may be due to non-specific binding.

[00317] In some embodiments, a sophisticated barcode generating algorithm is used such that the resulting barcodes can withstand the loss of any round or even multiple rounds of hybridization data. In some embodiments, a barcode generator is used to generate the drop-safe barcodes. For example, Figure 32 illustrates an example, where probes with 5 different visual signals (blue, green, red, purple and yellow) are used in 4 rounds of hybridization. One of the hybridization round is an error correction round where barcodes are generated based on barcodes from the previous 3 rounds. The following is an example that illustrates how barcodes are generated.

[00318] Designing an error correction code to correct for m number of errors in a message of n length is analogous to packing as many spheres of radius m in a n dimensional cube. There are examples of "perfect codes" such as Golay and Hamming codes that can be as efficient as possible in this packing design. These perfect codes are important in digital communication

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because the word lengths are long, up to billions of letters for gigabytes of data, and many forms of errors can occur, including deletion and insertions. However, in the seqFISH experiments, as the code lengths are short, a perfect code correction system is not necessary, especially as the "correct" codes are already defined. One of the major source of error is deletions due to loss of a hybridization. Thus, it is possible to design simple correction schemes that are not completely efficient (i.e. obtain the tightest packing density for the n-spheres) but can achieve good error correction with just a few extra rounds of hybridization.

[00319] To design a barcode scheme that can tolerate loss of a single round of hybridization is akin to a problem where any n-dimensional hypercube is collapsed by 1 dimension to a n-1 dimensional hypercube without having any two points on the n-dimensional hypercube mapping to the same point. In order for this to be true, no two barcodes can be connected by a 1D line running parallel to any of the axes. There are many solutions to generate this 1 round loss tolerant code.

[00320] In this example, 4 rounds of hybridization is used. Here, 5 different visual signals (blue, green, red, purple and yellow) are assigned numerical values. In some embodiments, the numerical values are integers. For example, blue = 1; green = 2; red = 3; purple = 4; and yellow = 5. It would be understood that these are mere sample values. Any non-redundant numerical values can be assigned to represent the different types of visual signals. In some embodiments, a barcode generator is used to generate the barcodes used in the experiment. In the exemplary embodiment, a drop-safe barcode for a particular target gene can be defined as a four-component linear array: $\{i, (i + j + k) mod\ 5, j, k\}$. Here, mod (modulo operation or modulus) finds the remainder after division of one number by another. For example, 8 mod 5 is 3. 5 mod 5 is 0, which is equivalent to 5.

[00321] In this example, i represents the numerical values corresponding to the visual signals observed for the particular target gene during the first round of hybridization. The scheme $(i + j + k) mod\ 5$ represents the numerical values corresponding to the visual signals observed for the particular target gene during the second round of hybridization. j represents the numerical values corresponding to the visual signals observed for the particular target gene during the third round of hybridization. k represents the numerical values corresponding to the visual signals observed for the particular target gene during the found round of hybridization. In

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this example, i, j, and k each can be 1, 2, 3, 4 or 5,or any one of the numerical values that have been assigned to the five types of visual signals used in the experiment.

[00322] In this example, $(i + j + k) mod\ 5$ is determined as the error correction round. However, once complete barcodes are generated, any of round 1 through round 4 can be dropped to yield unique 3-component barcodes. As such, the barcodes determined by this method can be used to correct errors in any round.

[00323] The following table illustrates how the 1 drop tolerant barcodes can be generated using the equation $(i + j + k) mod\ 5$.

Table 2. Illustration of the effect of repeat hybridization rounds.

Genes	**1st round of hyb***	**2nd round of hyb**	**3rd round of hyb**	**4th round of hyb $(i + j + k) mod\ 5$**
mRNA1	1	2	4	2
mRNA2	3	3	1	2
mRNA 3	5	1	2	3
mRNA 4	2	3	5	5
…	…	…	…	…
mRNA125	5	2	1	3

*The term "hyb." stands for hybridization. Numerical values are assigned to color signals as follows: blue = 1; green = 2; red = 3; purple = 4; and yellow = 5.

[00324] As illustrated above, although the 4th round of hybridization is generated using an error correction algorithm, any one round of four rounds of hybridization in Table 2 can be dropped and still yield a unique set of barcodes for 125 genes.

[00325] More generally, a barcode that can resist the elimination of one round of hybridization can be defined as:

$$\{j_1, j_2, \ldots (a_1 * j_1 + a_2 * j_2 + \cdots + a_n * j_n + C)\ mod\ F, \ldots, j_n\} \qquad (1)$$

where j_1 is a numerical value that corresponds the detectable visual signals used in the first round of hybridization, j_2 is a numerical value that corresponds the detectable visual signals used in the second round of hybridization, and j_n is a numerical value that corresponds the detectable visual signals used in the *n*th round of hybridization. In some embodiments, $j_1, j_2, \ldots j_n$ are non-redundant integers. In some embodiments, $a_1, a_2, \ldots a_n$ can be any integers that are not none zero. In some embodiments, C is a constant integer. In some embodiments, C is zero. The remainder of F divided by F is 0 (F mod F = 0), so F and 0 are equivalent. There is no limitation on the number of hybridization. One of such examples is shown in Figure 37.

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[00326] Array (1) is a general representation of a barcode that is safe against the drop or loss of one round of hybridization. Although $(a_1 * j_1 + a_2 * j_2 + \cdots + a_n * j_n + C)\ mod\ F$ is the designated error correction round, in some embodiments, the barcode is safe against the loss or drop of any round of hybridization.

[00327] As disclosed herein, array (1) consists of n-component, each corresponding to the visual signals from a particular round of hybridization. In some embodiments, probes binding to a particular gene are all associated with the same detectable visual signal, for example, red, green or blue. In some embodiments, probes binding to a particular gene are all associated with multiple types of detectable visual signal, for example, green + yellow or blue + red. Through combinations of visual signals, the total number of different types of detectable visual signals can be further expanded.

[00328] In some embodiments, barcodes can be designed such that drop or loss of data from two rounds of hybridization can be tolerated. Using 2 additional rounds of hybridization does not correct for all possible 2 drops, but it does correct for a large fraction of the 2 drops. For example, for detecting 100 genes with F=5 dyes, 3 rounds of hybridization are needed for basic barcoding of these genes. When adding two rounds of hybridization, the error correction code:

$$\{i, j, k, (i + j + k)\ mod\ F, (i - j)\ mod\ F\} \qquad (2)$$

[00329] Such codes can correct for 2 drops all except dropping hybridization round 3 and round 4 together. Here, each component in the 5-member array represents one round of hybridization.

[00330] Similarly, an error correction code such as

$$\{i, j, k, (i + j + k)\ mod\ F, (i - k)\ mod\ F\} \qquad (3)$$

can correct for dropping hybridization round 2 and hybridization round 4 together. Again, each component in the 5-member array represents one round of hybridization.

[00331] For example, to code for most of the transcriptome, only 6 rounds of hybridization are needed when F=5 (6^5=15,625). When adding two rounds of hybridization, the following error correction code is generated:

$$\{i, j, k, l, m, n, (i + k + l + m + n)\ mod\ F, (i - j - k - l + n)\ mod\ F\} \qquad (4)$$

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[00332] There are a total of 28 combinations of how 2 rounds of hybridization can be lost or dropped. This type of code can correct for 24 out of the total 28 combinations. Here, each component in the 8-member array represents one round of hybridization. Similarly, the 1st error correction round can be any liner combination of 5 out of 6 rounds of hybridization (e.g., without j) and 2nd error correction can be a subset of the linear combination of 5 out of 6 rounds of hybridization (e.g., without m). In these embodiments, in the 2nd error correction round, indices include different coefficients as long as the it is not exactly the same 5 indices used in the 1st error correction round.

[00333] To correct for all combinations of drop or loss of 2 rounds of hybridization (2 drops) fully, 3 additional hybridizations are needed. Again for 6 rounds of hybridization with 5 types of detectable signals (F=5), three extra rounds of hybridizations are added to create the full 9-member error correction code:

$$\{i, j, k, l, m, n, (i + j + k + l + m + n)\ mod\ F, (i - j - k - l)\ mod\ F, (m - n - j + k)\ mod\ F\} \quad (5)$$

[00334] In some embodiments, there are many equivalent codes that can correct for 2 drops with 3 additional rounds of hybridization. They can be all empirically determined. The number of hybridization for any reasonable number can be simulated to determine the complete correcting barcode.

[00335] In some embodiments, three additional hybridization can correct for majority of the errors due to drop or loss of three rounds of hybridization. For example, for 6 rounds of hybridization with 5 types of detectable signals (F=5), three extra rounds of hybridizations are added to create the full 9-member error correction code:

$$\{i, j, k, l, m, n, (k + i - l + m - n)\ mod\ F, (i - l + j - k + m)\ mod\ F, (l - n - j - k + i)\ mod\ F\} \quad (6)$$

[00336] Similar to the previous example, 3 additional rounds of hybridizations can correct for a majority of the loss or drop of 3 rounds of hybridization. There are a total of 84 combinations how 3 rounds of hybridization can be lost or dropped. A 9-componenet code as illustrated in (6) can correct for 72 out of the 84 combinations.

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[00337] In some embodiments, 4 additional rounds of hybridizations can correct for the drop or loss of all and any three rounds of hybridization. An example 10-component code is as follows:

$$\{i, j, k, l, m, n, (k+i+l+m+n)\ mod\ F, (i-l+j-k+m)\ mod\ F,\ (l-n-j-k+i)\ mod\ F, (n-k-i-j+m)\ mod\ F\} \quad (7)$$

[00338] It will be understood that there are many other solutions that can be determined empirically. For higher number of drops, similar correction schemes can be determined empirically.

[00339] For 16,000 species, this scheme allows 10 hybs with the ability to correct 3 drops. In comparison, in MERFISH, 16 hybs are needed to target 140 species, with only 2 round correction ability. Because the more round of hybridization one implements, the more mistakes can be made, keeping the number of hybs low is crucial. Thus, this error correction scheme is very powerful compared to the Hamming Distance scheme used in MERFISH. This is because hamming distance correction is used in telecommunications with binary numbers, which uses much longer strings of 0,1.

[00340] As described above, the design disclosed above can correct for loss of 1 hybridization for an arbitrarily long barcode sequence with minimal extra effort. In this example, only one round of error correction is needed in a total of 4 rounds of hybridization that analyzes 100 genes, which below the capacity of 54 (625).

[00341] For example, 7 rounds of hybridization with 5 colors can cover 5^7= 78,125 transcripts, more than the transcriptome, with 8 hybridizations the entire transcriptome can be coded with error correction using the barcoding system disclosed herein.

[00342] Another consideration in designing error-tolerant barcodes is that the mechanism of re-hybridization should guide the robustness of error correction. In the merFISH implementation of seqFISH (Chen 2015), null signal, or "0", along with "1" which is cy5 fluorescence, is used to form a binary barcode. However, it is difficult to determine whether no signal is due to mis-hybridization or actual null signal. In the seqFISH implementation using positive signals as readouts during each round of hybridization reduces the need for error correction because false positive signal is unlikely to re-occur in the same position during another hybridization due to DNAse stripping between hybridizations. Thus, implementation of

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seqFISH with 5 colors and 1 extra round of hybridization to error correct is both efficient and accurate, and allows imaging of a large tissue sections since imaging time is ultimately limiting in multiplexing experiments.

[00343] At step 3050, sequential hybridization is carried out to associate or assign barcodes from step 3040 to target genes in a sample. As disclosed herein, the sample can be immobilized mRNAs, DNAs, chromosomal DNAs, and combinations thereof. For example, in the 100-gene sequential hybridization example (see Figure 32 and Figure 40), 4 rounds of hybridization are carried out using probes associated with 5 different types of visual signals. Barcodes are assigned through selection of probes during the 4 rounds of hybridization experiment on immobilized nucleic acid samples.

[00344] At step 3060, after hybridization, visual signals are collected and used in further analysis. For example, images are collected from different hybridization are used to readout the barcodes for specific locations on the immobilized nucleic acid samples. Such barcodes can then be used to decipher the identity of the nucleic acid targets (see, for example, Figures 2, 32, 33, 40 and 41).

[00345] In one aspect, sequential hybridization and serial hybridization are combined for gene identification. In serial hybridization, only one round of hybridization is used to identify target genes. The method is particularly helpful when analyzing genes whose expression level is too high. In some embodiments, genes that are highly expressed, if included in hybridization analysis with genes that are not so highly expressed, would overpower the signals for the genes that are not so highly expression. In some embodiment, the method can also applied to genes whose expression level is too low.

[00346] In some embodiments, expression levels of genes are pre-determined. For example, gene expression levels (e.g., measured by mRNA transcription level) can be already available for certain species. It is possible to identify highly expressed genes by mining publically available data, thus obviating the need to conduct additional experiments to measure expression level.

[00347] In some embodiments, initial experiments are performed to determine relative expression level of candidate genes. In some embodiments, genes are grouped according to their expression levels. For example, genes with moderate or low expression levels can be grouped

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together and subject to sequential hybridization analysis. Genes that are highly expressed can be subject to serial hybridization analysis. In some embodiments, expression levels of different genes are compared to the same control gene to derive a relative expression level. For example, the expression level of actin can be used as a control. It will be understood that gene expression level may vary by organisms and can change with respect to different internal and environmental controls. In some embodiments, data from existing expression analysis can be used in identifying highly expressed gene. In some embodiments, preliminary expression analysis is carried out before sequential and/or serial hybridization analysis.

[00348] In some embodiments, a threshold value is set for high expression. Any genes having expression level above the threshold will be excluded from sequential hybridization.

[00349] Depending on types of detectable visual signals that are available, a serial hybridization experiment can detect as many target genes as the number of types of detectable visual signals. For example, in the experiment illustrated in Figures 32 and 40, 5 genes are analyzed at the same time during one serial hybridization experiment.

[00350] In some embodiments, when multiple target genes are present in one serial hybridization round, the number of probes that recognize each target gene is selected such that overlapping of signals is minimize or avoided. In some embodiments, the concentration of probes are selected to avoid or minimize overlapping of detectable signals.

Computer System

[00351] Figure 31 depicts a diagram of an example system architecture for implementing the features and processes of the method disclosed herein, in particular the barcode design functionalities with embedded error correct mechanism.

[00352] In one aspect, some embodiments can employ a computer system (such as the computer system 3100) to perform methods in accordance with various embodiments of the invention. An exemplary embodiment of computer system 3100, includes a bus 3102, one or more processors 3112, one or more storage devices 3114, at least an input device 3116, at least an output device 3118, a communication subsystem 3120, working memory 3130 which includes an operating system 3132, device drivers, executable libraries, and/or other code, such as one or more application(s) 3134 (one or more for implementing the methods disclosed herein).

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[00353] According to a set of embodiments, some or all of the procedures of such methods are performed by the computer system 3100 in response to processor 3112 executing one or more sequences of one or more instructions (which might be incorporated into operating system 3132 and/or other code, such as an application program 3134) contained in working memory 3130. Such instructions can be read into the working memory 3130 from another computer-readable medium, such as one or more of storage device(s) 3114. Merely by way of example, execution of the sequences of instructions contained in working memory 3130 might cause processor(s) 3112 to perform one or more procedures of the methods described herein. Additionally or alternatively, portions of the methods described herein can be executed through specialized hardware. Merely by way of example, a portion of one or more procedures described with respect to the method(s) discussed above, such as method 3000, and methods illustrated in Figures 3B and 30, might be implemented by processor 3112.

[00354] In some embodiments, computer system 3100 can further include (and/or be in communication with) one or more non-transitory storage devices 3114, which can comprise, without limitation, local and/or network accessible storage, and/or can include, without limitation, a disk drive, a drive array, an optical storage device, a solid-state storage device, such as a random access memory ("RAM"), and/or a read-only memory ("ROM"), which can be programmable, flash-updateable, and/or the like. Such storage devices can be configured to implement any appropriate data stores, including without limitation, various file systems, database structures, and/or the like. In some embodiments, the storage device 3114 can be example of local database of a user device, or the server database of a server.

[00355] In some embodiments, computer system 3100 can further include one or more input devices 3116, which can comprise, without limitation, any input device that allows a computer device to receive commands from a user such as a request for barcode design.

[00356] In some embodiments, computer system 3100 can further include one or more input output devices 3118, which can comprise, without limitation, any output device that can receive information from a computer device and communicate such information to a user, to another computer device, to the environment of the computer device, or to a functional component communicably connected with the computer device. Examples of input devices include but are not limited to a display, a keyboard, a mouse and etc. For example, the results of

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the barcoding analysis can be presented on any one or more of the output devices, for example, as two dimensional heat map, cluster map, a list, a table, and etc.

[00357] It would be understood that any applicable input/output devices or components, such as those disclosed in connection with one or more user device or server, can be applied to input device 3116 and output device 3118.

[00358] In some embodiments, computer system 3100 might also include a communications subsystem 3120, which can include without limitation a modem, a network card (wireless or wired), an infrared communication device, a wireless communication device, and/or a chipset (such as a Bluetooth.TM. device, an 802.11 device, a WiFi device, a WiMax device, cellular communication facilities, etc.), and/or the like. Communications subsystem 620 can include one or more input and/or output communication interfaces to permit data to be exchanged with a network, other computer systems, and/or any other electrical devices/peripherals. In many embodiments, computer system 3100 will further comprise a working memory 3130, which can include a RAM or ROM device, as described above.

[00359] In some embodiments, computer system 3100 also can comprise software elements, shown as being currently located within the working memory 3130, including an operating system 3132, device drivers, executable libraries, and/or other code, such as one or more application(s) 3134, which can comprise computer programs provided by various embodiments, and/or can be designed to implement methods, and/or configure systems, provided by other embodiments, as described herein. Merely by way of example, a portion of one or more procedures described with respect to the method(s) discussed above, such as the methods described in relation to Figure 30, can be implemented as code and/or instructions executable by a computer (and/or a processing unit within a computer); in an aspect, then, such code and/or instructions can be used to configure. and/or adapt a general purpose computer (or other device) to perform one or more operations in accordance with the described methods.

[00360] A set of these instructions and/or code might be stored on a non-transitory computer-readable storage medium, such as storage device(s) 3114 described above. In some cases, the storage medium might be incorporated within a computer system, such as computer system 3100. In other embodiments, the storage medium might be separate from a computer system (e.g., a removable medium, such as an optical disc), and/or provided in an installation

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package, such that the storage medium can be used to program, configure, and/or adapt a general purpose computer with the instructions/code stored thereon. These instructions might take the form of executable code, which is executable by computer system 3100 and/or might take the form of source and/or installable code, which, upon compilation and/or installation on the computer system 3100 (e.g., using any of a variety of generally available compilers, installation programs, compression/decompression utilities, etc.), then takes the form of executable code.

[00361] It will be apparent to those skilled in the art that substantial variations can be made in accordance with specific requirements. For example, customized hardware might also be used, and/or particular elements might be implemented in hardware, software (including portable software, such as applets, etc.), or both. Further, connection to other computing devices such as network input/output devices can be employed.

[00362] The terms "machine-readable medium" and "computer-readable medium," as used herein, refer to any medium that participates in providing data that causes a machine to operate in a specific fashion. In an embodiment implemented using computer system 3100, various computer-readable media might be involved in providing instructions/code to processor(s) 3112 for execution and/or might be used to store and/or carry such instructions/code. In many implementations, a computer-readable medium is a physical and/or tangible storage medium. Such a medium can take the form of a non-volatile media or volatile media. Non-volatile media include, for example, optical and/or magnetic disks, such as storage device(s) 3114. Volatile media include, without limitation, dynamic memory, such as working memory 3130.

[00363] Common forms of physical and/or tangible computer-readable media include, for example, a floppy disk, a flexible disk, hard disk, magnetic tape, or any other magnetic medium, a CD-ROM, any other optical medium, any other physical medium with patterns of holes, a RAM, a PROM, EPROM, a FLASH-EPROM, any other memory chip or cartridge, or any other medium from which a computer can read instructions and/or code.

[00364] Various forms of computer-readable media can be involved in carrying one or more sequences of one or more instructions to processor(s) 3112 for execution. Merely by way of example, the instructions can initially be carried on a magnetic disk and/or optical disc of a remote computer. A remote computer might load the instructions into its dynamic memory and

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send the instructions as signals over a transmission medium to be received and/or executed by computer system 3100.

[00365] Communications subsystem 3120 (and/or components thereof) generally will receive signals, and bus 3102 then might carry the signals (and/or the data, instructions, etc. carried by the signals) to working memory 3130, from which processor(s) 3112 retrieves and executes the instructions. The instructions received by working memory 1330 can optionally be stored on non-transitory storage device 3114 either before or after execution by processor(s) 3112.

[00366] The methods and systems are provided by way of illustration only. They should in no way limit the scope of the present invention.

[00367] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

[00368] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

EXEMPLIFICATION

[00369] The foregoing has been a description of certain non–limiting embodiments of the invention. Accordingly, it is to be understood that the embodiments of the invention herein described are merely illustrative of the application of the principles of the invention. Reference herein to details of the illustrated embodiments is not intended to limit the scope of the claims.

EXAMPLE 1

Pseudo-color Based Barcoding

Sample preparation

[00370] Figure 5 illustrates mRNA transcripts immobilized on a surface through poly-A-tail or hydrogel embedment. Once cells are lysed, the cell lysate or purified total RNA can be immobilized on a surface through a coverslips functionalized by DNA or LNA Poly-T by

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capturing the poly-A tail of mRNA. Alternatively, the mRNA can be mixed with hydrogel such as polyacrylamide gel and allowed gelation on a coverslips surface which the pore size formed would trap the mRNA molecules on the surface.

Design of primary probes

[00371] Figure 6 illustrates an exemplary embodiment of gene specific primary probes design and sequential barcoding hybridization on mRNA immobilized on a surface. In particular, Figure 6a shows that primary probes are not directly labeled with fluorophore. The primary probe contains (i) a gene specific targeting region of the mRNA which is 20nt to 35nt, (ii) one or multiple secondary readout probe binding sites, (iii) PCR primer pairs, and optionally (iv) a restriction enzyme cutting sites and (v) spacers nucleotides. Each target mRNA requires at least 20 or more primary probes. These primary probes are synthesized from a complex oligo pool which later is amplified by the unique PCR primers to obtain a complete set of probes. The primary probes can be attached to target sequence either on the surface or in solution (Figure 6b). Different primary binding sequences are associated with different readout sequences, which can be selectively turned on during hybridization rounds.

[00372] In this case, mRNA 1 is barcoded with red color and mRNA 2 is barcoded with blue color in the first round of hybridization. After imaging, a signal extinguishing step is performed, followed by the next round of barcoding hybridization. Each mRNA will receive 4 colors in total which gives their unique identity to differentiate between each other. The barcode capacity scales up as F^N, with F is the number of fluorophore and N is the number of barcoding hybridization (Figure 6c).

Arrangement of secondary probe binding sites

[00373] Figure 7 illustrates different arrangements of secondary probe binding sites on primary probes. Assume 24 primary probes are used for each gene, 4 rounds of barcoding hybridization with 4 different unique secondary probe binding sequences, there are various combinations of arranging the secondary probe binding sequences on the primary probe. Multiple or all unique secondary probe binding sequences can be found in one probe; for

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example, as overhang sequences connected to the primary binding sequence in various combinations (Figure 7a-7c).

Signal Extinguishing Steps

[00374] Figure 8 depicts possible ways to extinguish fluorescent signals. For example either high concentration of formamide can be introduced to 'melt' or strip off the secondary readout probe (Figure 8a). Alternatively, chemical cleavage can be applied (Figure 8b); for example, one implementation can be disulfide conjugated dye on secondary readout probes can be reduced by TCEP or DTT and other reducing agents to cleave off the linker, and thus getting rid the fluorophore from the primary probe..

Scaling up the number of 'fluorophore' by serial hybridization

[00375] Figure 10 depicts a table showing possible barcodes obtained using the pseudo-color barcode scheme. In this example, 3 different color signals, 4 serial hybridization rounds and 4 barcoding rounds were used. In this implementation, 16 rounds of imaging will be implemented with a total of 48 unique secondary readout adapters conjugated to one of the 3 fluorophores.

[00376] One can adjust F, n, and N to achieve the best strategy to generate a desired barcode capacity. For example, in order to reduce the number of barcoding rounds to 3, 30 unique secondary readout sequences are needed at each round of barcoding, as 30^3=27,000. This scheme can allow lower density of targets detected per channel in each hybridization round, allowing a larger number of genes to be detected in situ.

Time efficient error correction scheme

[00377] A time efficient error correction scheme can be implemented in the current coding scheme to tolerate 1 drop of 'color' in decoding the whole color codes due to loss of hybridization. It is possible to design a highly efficient correction schemes that are not perfect by obtaining the tightest packing density for the n-spheres. A barcode generator (i, (i+j+k) mod 5,j,k) can be used to generate barcodes that can tolerate 1 round of mis-hybridization. For example, 6 rounds of hybridization with 5 color can cover 15625 genes, one can design the

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barcode scheme to have an extra round of hybridization to still be able to identify the 15625 genes even though any one round of hybridizations is lost in the barcode. More rounds can be corrected with additional error correction hybridization rounds. For example, barcodes can be designed such that shortened barcodes are still unique after data for two or more rounds of hybridizations are lost.

Hybridization experiment

[00378] To show the entire idea works, we targeted 64 genes in mouse NIH3T3 cells (Figure 10). These 64genes were chosen randomly with a range of FPKM values from bulk RNA-sequencing data. A set of 24 primary probes for 1 gene were designed as illustrated in Figure2 which contain 35nt of gene specific targeting region, 3 unique secondary readout binding sequences, a pair of PCR primers, and a 1nucleotide-'T' spacer. The primary probes were synthesized in a complex oligo pool and were amplified by PCR. The barcodes were generated as $4^3 = 64$, without implementing an error correction scheme. 3 fluorophores were used: Cy3b, Alexa594, and Alexa 647 and the number of fluorophores were scaled up as described in Figure 4 with slight variation. Briefly, in each round of barcoding hybridization, 4 unique secondary readout probes were coupled to the 3 fluorophores + one of the fluorophores hybridized in a later hybridization. In this experiment, the fourth unique secondary readout probes in each barcoding hybridization were coupled to Alexa 647, Alexa594, and Cy3B so that only one extra round of imaging is needed to scale up the number of 'fluorophores'. The length of readout probes used in this proof-of-concept experiment is 18mers. These secondary readout probes were conjugated to dye through an amine-NHS reaction. The fluorescent signals in each hybridization was extinguished by high concentration of formamide (60% formamide).

[00379] This proof-of-principle shows that the secondary probe readout scheme using a combination of serial and barcoding steps can be scaled up to perform whole transcriptome profiling. Probes can be designed to target over 20,000 genes in the transcriptome and can be readout with 4 barcoded rounds of hybridization with 12 based "colors", performed in similar fashion as this 64-gene experiment.

[00380] Figure 11 depicts an exemplary embodiment of data analysis. Pearson's correlation plot between the copy number determined by this technology vs FPKM values

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determined from bulk RNA-seq. This shows that the measurements by the sequential hybridization barcoding experiments are highly accurate.

[00381] The barcoding scheme illustrates in Figures 8 and 9 were used for multiplex detection of 1000 different transcription factors mRNAs with in vitro RNA SPOTs. First, NIH3T3 cells were grown on a 6-well plate until 60% to 80% confluency. Then the cells were lysed and purified according to Qiagen Rneasy Mini Kit. Then, 100ng of total RNA in RNA Binding Buffer consists of 1M LiCl, 40mM pH7.5 Tris-HCl, 2mM EDTA, 0.1% Triton X-100, and 20units of SUPERase Inhibitor(ThermoFischer) was captured on a LNA functionalized coverslips for overnight at room temperature. The capturing time and amount of total RNA can be modified to adjust the dots density on a coverslip in one frame of view. Next, primary probes(1nM/probe) which consists of a gene specific binding region, 4 barcoded secondary readouts regions, spacers, and primers pair for a thousand genes were allowed to hybridize to the mRNA target in 30% hybridization buffer made from 10% Dextran Sulfate, 2x SSC, and 30% formamide at 37 °C overnight. Each gene is targeted by a minimum of 24 probes. After washing, RNA SPOTs began with the first round of hybridization by flowing in 10nM readout probes for each color (3 colors in one round of hybridization) and the hybridization was allowed to happen for 30minutes at room temperature. After a brief wash, anti-bleaching buffer was flowed into the flow cell, and the fluorescent signals were imaged. Then, the fluorescent signals were extinguished by reduction of 50mM TCEP in 2x SSC with 0.1% Triton X-100 at room temperature for 15mins. The process was repeated until the last round of hybridization.

[00382] In this implementation, 16 rounds of imaging was implemented with a total of 48 unique secondary readout adapters conjugated to one of the 3 fluorophores. The coding space consists of one round of error correction for up to 1728 different targets. In this experiment, a thousand different mRNAs were encoded with the scheme described in Figure 10. The target mRNAs were chosen from a list of transcription factors (master regulators of gene expression) that are conserved between mouse and humans.

[00383] Figure 12 depicts an exemplary a raw image from a barcoding experiment. The raw image shows the results from one of the hybridization round in the 1000 transcription factor genes experiment. The images of each serial hybridizations were first aligned to obtain the 12 'color' barcodes. Then the dots were decoded based on the error correction barcode scheme.

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[00384] Figure 13 depicts exemplary results from data analysis, showing decoded 1000 different RNA counts with RNA SPOTs on coverslip and correlate with bulk RNA-Seq Fragments per Kilobase Million (FPKM) for NIH3T3 cells. 753 genes which have a non-zero value of FPKM and a non-zero value of SPOTs are chosen for this correlation. Note that genes with low FPKM values are well detected by RNA SPOTs.

[00385] Figure 14 depicts exemplary results from data analysis, showing correlation between RNA counts from different field of views(FOV)s. Each dot is a single gene with its x value the total number of transcript counted for that gene in FOV 1-5, the y value the counts for FOV 6-10. A high Pearson's correlation coefficient of 0.9131 is observed. This shows that a few FOV is sufficient to accurately quantify transcript abundance.

Imaging-based translational profiling, the RiboCounter

[00386] Intact ribosomes contain 28S rRNA and 18S rRNA. By probing against these rRNA after the initial RNA SPOTs experiment to decode the mRNA identity, one can estimate the number of ribosomes on each mRNA molecule and infers the translational state of the mRNA molecules. By combining this method and RNA SPOTs, we develop the imaging-based polysome profiling to infer the translational state of each detected mRNA molecules by probing against the 28S and 18S rRNA followed by decoding their identity through RNA SPOTs.

[00387] Experimental Design: Cells were lysed and captured on LNA coverslips. Then, by using fluorescence probes either with direct 20mer smFISH or using primary probes, followed by secondary readouts to target the 18S and/or 28S rRNA before RNA SPOTs, one can obtain the intensity for the ribosomes on each mRNA molecules. The identity of each RNA molecules were decoded by RNA SPOTs as described above.

[00388] Results: Figure 15 depicts exemplary results from translational profiling analysis showing the 28S rRNA median intensity extracted from each mRNA molecules of the thousand RNA species. The 28S rRNA intensity for all the 1000 transcription factor genes were all obtained.

[00389] Figure 16 depicts exemplary results from translational profiling analysis, showing the distribution of 28S rRNA median intensity of the gene Myh9 on each mRNA transcripts. The total number of Myh9 transcripts detected are 1169.

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[00390] RNA SPOTs of the transcriptome To implement RNA SPOTs at the transcriptome level, probes were designed targeting the coding regions of 10,212 mRNAs with 28 to 32 probes each gene for a total of 323,156 probes. Probes were stringently designed to avoid off-target and cross hybridization (Supplementary information). The primary probes directly hybridize to the mRNAs captured on a Locked Nucleic Acid(LNA) poly(dT) functionalized coverslip and contains a set of overhang sequences that specifies the barcode unique to each transcript (Figure 4A). A 12 "pseudo-color" based scheme was used such that 4 rounds of barcoding are sufficient to cover the transcriptome (12^4=20,736), with an additional round of error correction to compensate for one drop in any round of barcoding [Shah et al, 2016].

[00391] This design minimizes the barcode length to avoid errors from using long barcodes. The 12 base "pseudo-colors" in each round of barcoding is encoded by a set of 12 readout oligos. Three of the readout oligos were hybridized at a time, imaged in the Cy3b, Alexa 594 and Alexa 647 fluorescence channels, and repeated 4 times to iterate through all 12 readout sequences. After each round of hybridization and imaging, the fluorophores are removed by di-sulfide cleavage and followed by the next round of hybridization. A total of 60 readout oligos in 20 rounds of hybridization were used to decode the 10,212 genes targeted. Every four rounds of hybridization were collapsed onto a single image with 12 pseudo-colors. The barcodes were determined from aligning the 12-color images. A common sequence is present in all primary probes and targeted by an oligo labeled with Alexa488 to serve as an alignment marker through all 20 rounds of hybridization. The switching and rehybridization time is fast, with the overall speed limited by imaging speed. Typically, 100 fields of view containing 106 mRNAs can be imaged with 20 rounds of hybridization in a 14-hour period through an automated fluidics system.

[00392] To determine the accuracy of the transcriptome level measurements, we compare the decoded RNA SPOTs data with RNAseq data in fibroblasts and mESCs, and found that they correlated with R=0.86 and R=0.9 respectively. In addition, RNA SPOTs correlated with the gold standard smFISH quantitation with a correlation of R=0.86 in mESCs (23 genes) [Singer 2014] and R= 0.88 in fibroblasts (7 genes). Between two replicates of RNA SPOTs in fibroblasts, the results agree with R=0.94, indicating that RNA SPOTs is a highly robust and

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reproducible measurement method. Lastly, comparing genes that were differentially expressed in fibroblasts versus mESCs, we observed the same trend as those detected by RNAseq. For example, pluripotency factors such as Rex1, Esrrb and Sox2 are highly expressed in mESCs but not expressed in fibroblasts as determined by RNA SPOTs (Figure 19), similar to the differences observed by RNAseq.

[00393] Several genes were observed to be outliers in the RNA SPOTs to RNAseq comparison (Figure 19). When we examined the genes in cells directly by single molecule FISH, we observed that their expression levels matched those determined with RNA SPOTs. This indicates that either hybridization efficiency on these genes were low or they were over-detected by RNAseq.

[00394] An advantage of RNA SPOTs is that specific sets of genes can be profiled selectively simply by designing probe sets targeting those genes. In this fashion, ribosomal RNA and highly expressed housekeeping genes can be avoided simply by eliminating those probes from the gene set. As each dot detected in our assay corresponds to a single mRNA, RNA SPOTs is more efficient in term of imaging compared to RNAseq, where many sequencing reads are needed to determine the abundance of a transcript.

[00395] RNA SPOTs can be scaled down to single cell in combination with microfluidics tools to trap and lyse cells [Bose 2015] or with split-pool molecular indexing methods [Cao 2017, Rosenberg 2017]. With targeted RNA SPOTs, we can choose to probe only for the 2000 transcription factors [Fulton 2009] or 1000 landmark informative genes [Donner 2012, Duan 2016] in single cells, instead of profiling the transcriptome, to capture the essential information in cells and to increase the number of cells sampled.

[00396] RNA SPOTs can enable many additional methods beyond expression profiling to study RNA variants [Levesque 2013], modifications [Mellis 2017], RNA binding proteins [Buenrostro 2014] and profile ribosomes occupancy [Ingolia 2009] directly on the captured RNA. Lastly, noncoding RNAs and other RNAs without polyA tails can be captured in hydrogels rather than with dT oligos. As cost of sequencing is a major limiting factor in genomics experiments, SPOTs enable an accurate and low-cost alternative to sequencing with many further applications beyond RNA to DNA and proteins.

EXAMPLE 2

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Readout Probes and Rehybridization in Mouse Embryonic Stem Cells

Synthesis of DNA Probes-Disulfide-Dye Conjugates

[00397] An exemplary scheme for synthesizing readout probes-dye conjugates connected by a disulfide bond. Thiol-modified DNA probes were ordered from Integrated DNA Technologies in their oxidized form. 10nmoles of thiol-modified DNA probes was treated with 10mM TCEP at 37°C for 30 minutes. After reduction step and gel column purified, the DNA probes were mixed with 50 equivalents of 3-(2-Pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP) linker in 1x PBS solution containing 10mM EDTA. The mixture was allowed to react at room temperature for at least 2hours. Immediately after the reaction, the mixture was spin column purified and was re-suspended in 60uL of 1x PBS containing 100ug of cadaverine dyes. The reaction was allowed to proceed at room temperature for at least 4 hours before subjected to ethanol-precipitation purification and HPLC purified. The concentration of the final product was determined using Nanodrop.

Rehybridization in mouse embryonic stem cells (mESCs).

[00398] Figure 28 illustrates rehybridization reactions in mouse embryonic stem cells (mESCs). To verify the scheme works in both cells and on coverslips, we perform a proof of concept experiments in mouse embryonic stem cells (mESCs) targeting introns of pgk1 gene. During first round of hybridization, tertiary readout probes conjugated with A647 were applied to identify specific target sequences in mRNAs via secondary bridge binding sites. Real fluorescent spots are shown in red dashed box (28A). As a control, fluorescence presence in channel 594 was also measured during the first hybridization: no fluorescent spots were observed (28B). After TCEP treatment and washing steps, channel 647 was reimaged. The observed dim dots are non-specific binding of dyes which does not interfere with subsequent real fluorescent spots identification (28C). During a second round of hybridization, unique readout probes bearing 594 dye were used to hybridize with the secondary bridge binding sites to give real fluorescent spots (28D). These spots confirmed the positions observed previous when readout probes with A647 were used (28A).

In vitro rehybridization with readout probes

[00399] Transcribed polyA-tailed dCAS9-EGFP mRNA were captured on a dT20 Locked Nucleic Acid (LNA) surface-modified coverslips to show the rehybridization scheme works *in*

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vitro on the coverslips. Once again, specific binding was observed. When tertiary probes conjugated with A647 were used: specific signals were observed in channel 647 while there were few signals observed in channel 594 (Figure 29, top row). After TCEP treatment and wash of the first set of tertiary probes, a second set of tertiary probes conjugated with 594 dye were applied during the set round of hybridization reaction. Consequently, specific signals were observed in channel 594 while there were few signals observed in channel 647 (Figure 29, bottom row).

[00400] Technically, any heterobifunctional cross-linking reagent that can connect between the dye and thiol-modified DNA probes will work for this rehybridization scheme. DNA probes-disulfide-dye conjugates were synthesized using 3-(2-pyridyldithio) propionyl hydrazide (PDPH) linker and NHS ester dyes which work equally well as former conjugates.

EXAMPLE 3

Brain Slice Analysis

[00401] As an illustration, barcodes generated using the error correction mechanisms disclosed herein are used for *in situ* transcription profiling of single cells reveals spatial organization of cells in the mouse hippocampus.

[00402] Identifying the spatial organization of tissues at cellular resolution from single cell gene expression profiles is essential to understanding many biological systems. In particular, there exist conflicting evidence on whether the hippocampus is organized into transcriptionally distinct subregions. Here, a generalizable *in situ* 3D multiplexed imaging method was applied to quantify hundreds of genes with single cell resolution via Sequential barcoded Fluorescence *in situ* hybridization (seqFISH) (Lubeck et al., 2014). seqFISH was used to identify unique transcriptional states by quantifying and clustering up to 249 genes in 16,958 cells. By visualizing these clustered cells *in situ*, we identified distinct layers in the dentate gyrus corresponding to the granule cell layer, composed of predominantly a single cell class, and the subgranular zone, which contains cells involved in adult neurogenesis. Furthermore, it was discovered that distinct subregions within the CA1 and CA3 are composed of unique combinations of cells in different transcriptional states, instead of a single state in each sub-region as previously proposed. In addition, it was revealed that while the dorsal region of the CA1 is relatively homogenous at the single cell level, the ventral part of the CA1 has a high

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degree of cellular heterogeneity. These structures and patterns are observed in sections from different mice, as well as in seqFISH experiments with different sets of genes. Together, these results demonstrate the power of seqFISH in transcriptional profiling of complex tissues.

[00403] The mouse brain contains about 10^8 cells arranged into distinct anatomical structures. While cells in these complex structures have been traditionally classified by morphology and electrophysiology, their characterization has been recently aided by gene expression studies. In particular, the Allen Brain Atlas (ABA) provides a systematic gene expression database using in situ hybridization (ISH) of the entire mouse brain one gene at a time (Dong et al., 2009; Fanselow and Dong, 2010; Thompson et al., 2008). This comprehensive reference provides regional gene expression information, but lacks the ability to correlate the expression of different genes in the same cell. More recently, single cell RNA sequencing (RNA-seq) has identified many cell types based on gene expression profiles (Darmanis et al., 2015; Tasic et al., 2016; Zeisel et al., 2015). However, while single cell RNA-seq provides useful information on multiple genes in individual cells, it has relatively low detection efficiencies and requires cells to be removed from their native environment resulting in the loss of spatial information. These different approaches can lead to contradictory descriptions of cellular organization in the brain and other biological systems.

[00404] In the hippocampus, recent RNA-seq data suggests that CA1 is composed of cells with a continuum of expression states (Cembrowski et al., 2016, Zeisel et al 2015), while ABA analysis indicates that sub-regions within the CA1 have distinct expression profiles (Thompson et al, 2008). To resolve the two conflicting descriptions of hippocampal organization, a method to profile transcription *in situ* in the hippocampus with single cell resolution is needed. Here, we demonstrate a general method that enables the mapping of cells and their transcription profiles with single molecule resolution in tissue, allowing an unprecedented resolution of cellular transcription states for molecular neuroscience (Figure 32A).

[00405] A great deal of progress has been made recently in developing highly quantitative methods to profile the transcriptome of single cells. Building upon single molecule fluorescence *in situ* hybridization (smFISH) (Femino et al., 1998; Raj et al., 2006;) , Lubeck et al. devised a general method to highly multiplex single molecule *in situ* mRNA imaging irrespective of transcript density using super-resolution microscopy (Betzig et al., 2006; Rust et al., 2006;

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Lubeck and Cai, 2012). However, the spectral barcoding methods used in these previous works is difficult to scale up beyond 20-30 genes because of limited number of fluorophores (Fan et al., 2001; Lubeck and Cai, 2012).

[00406] To overcome the scalability problem, a temporal barcoding scheme was developed that uses a limited set of fluorophores and scales exponentially with time (Lubeck et al., 2014). Specifically, by using sequential rounds of probe hybridizations on the mRNAs in fixed cells to impart a unique pre-defined temporal sequence of colors, different mRNAs can be uniquely identified *in situ*. The multiplex capacity scales as F^N, where F is the number of fluorophores and N is the number of rounds of hybridization. Thus, one can increase the multiplex capacity by increasing the number of rounds of hybridization with a limited pool of fluorophores. This approach is called Sequential barcoded Fluorescence i*n situ* Hybridization (seqFISH) (Lubeck et al., 2014). In parallel, *in situ* sequencing methods were developed to directly sequence transcripts in tissue sections, but these methods suffer from low detection efficiency (<1%) (Ke et al., 2013; Lee et al., 2014). Recently, Chen et al. expanded the error correction method in the original seqFISH demonstration by using a Hamming distance 2 based error correcting barcode system, called merFISH. However, this implementation requires larger transcripts (>6kb) and many more rounds of hybridization than the method described here (Chen et al., 2015b). Furthermore, seqFISH and its variants have only been applied in cell culture systems due to the difficulty of smFISH detection in tissue. Here, an improved version of seqFISH in complex tissues by including signal amplification and a time-efficient error correction scheme (Figures 32A-D, Figure 40) were demonstrated to resolve the structural organization of the hippocampus with single cell resolution.

EXAMPLE 4

Brain Slice Analysis with Error Correction

[00407] *Signal amplification and error correction enable robust detection of mRNAs in tissues.*

[00408] To overcome the autofluorescence and scattering inherent to brain tissues, we used an amplified version of smFISH, called single molecule Hybridization Chain Reaction (smHCR) (Figure 32E) (Shah et al., 2016). Single molecule HCR amplified signal 22.1 ± 11.5 (mean ± s.d., n=1288, Figure 38B) fold compared to smFISH, enabling robust and rapid

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detection of individual mRNA molecules in tissues and facile alignment of spots between hybridizations (Figure 38A). Single transcripts can be detected and localized in 3D with just 24 probes in tissues, enabling detection of transcripts <1kb in size,with a fidelity comparable to the smFISH gold standard (Figures 41C-D) but with signals 20-fold brighter (Shah et al., 2016). Single molecule HCR DNA polymers can also be digested by DNAse and re-hybridized in brain slices, allowing HCR-seqFISH to be robustly implemented (Figure 33A). We note the smHCR enables true 3D imaging in tissues, whereas the previous sequential FISH demonstrations (Lubeck et al., 2014, Chen et al., 2015) were performed only in flat cell cultures.

[00409] Furthermore, we improved upon our existing barcode system by implementing a time-efficient error correction scheme. The major source of error in seqFISH is the loss of signal due to mis-hybridization, which increases with the numbers of hybridization. We introduced an extra round of hybridization to correct loss of signal during any round of hybridization (Figure 32D). By minimizing the number of hybridizations, this error correction scheme is efficient in practical implementation. For example, using 5 fluorophores and 4 rounds (instead of 3 rounds) of hybridization to code for 125 genes, we can still uniquely assign barcodes to genes even when signal from any single round of hybridization is missing. Although merFISH can tolerate 2 errors in the barcodes, it requires 16 rounds of hybridization to code 140 genes (Chen et al. 2015). As increasing the number of hybridizations can potentially lead to more experimental error and analysis complexity, our simple error correction method corrects for the most common error, dropped signal. Also, the fewer rounds of hybridizations decreases the total imaging time, which is rate-limiting for tissue experiments. HCR-seqFISH with simpler error-correction scheme allows efficient and accurate quantification of transcription profiles in tissues.

[00410] Using this HCR-seqFISH method, we surveyed the regional and sub-regional transcriptional heterogeneity within the temporal and parietal cortex and hippocampus of the mouse brain by imaging similar coronal sections collected from 3 different animals. Two similar sections from separate mice were profiled with probes for 125 genes, while one additional brain slice was imaged for 249 genes. In each of the coronal slices, between 60-80 fields of view were imaged, each 216μm x 216μm x 15μm, in the cortex and hippocampus (Figure 32A and Figure 41E). For the 125 gene set, 56 of the genes (Figure 32D, Figure 40) were selected because they showed spatially heterogeneous expression based on the ABA (Lein et al., 2007), another 44

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were selected from a list of transcription factors, and 25 marker genes were selected from single cell RNA-seq datasets (Zeisel et al., 2015). One hundred of these genes were barcoded by 4 rounds of hybridization (Figure 32B). The remaining 25 high abundance genes were measured individually using 5-color smHCR in 5 serial rounds of hybridizations (Figure 32C). This hybrid approach of measuring medium expression genes with barcoding seqFISH and high copy number genes serially in subsequent hybridizations allows a large dynamic range of transcripts to be profiled in the same cell.

[00411] *seqFISH is an accurate and efficient method to multiplex RNA in situ.*

[00412] To determine the accuracy of the seqFISH method in quantifying mRNA levels in single cells in tissue, we compared the copy number of 5 of the 100 target genes measured by barcoding to the copy number found by colocalized smHCR detection in the same cell (Figure 33B, Figure 42A) in 15μm brain sections. We found that the copy number of the RNAs per cell as measured by barcoding and smHCR agreed with an R-value of 0.85 and a slope of 0.84 (N=3851). As colocalized smHCR matches smFISH transcript quantitation (Shah et al., 2016), the barcoded seqFISH method can quantify mRNA molecules in single cells with 84% efficiency compared to the gold standard of smFISH. In comparison, single cell RNA-seq measurements are 5-20% efficient based on spike-in controls and *in situ* sequencing is less than 1% efficient (Darmanis et al., 2015; Klein et al., 2015; Lee et al., 2014; Macosko et al., 2015; Tasic et al., 2016; Zeisel et al., 2015; Ståhl et al., 2016). This high efficiency of detection results from a low transcript drop rate and a high barcode recovery rate due to the error correction round of hybridization. In our experiment, 78.9% of barcodes (N=2,115,477 barcodes) were found in all 4 hybridization rounds and 21.1% were identified in 3 out of the 4 hybridizations (Figure 33C), indicating that the probability of detecting a given mRNA molecule is 94% in each round of hybridization (Figure 42B).

[00413] To quantify the amount of false positive signal due to misalignment of barcodes and nonspecific binding of probes, the amount of off-target barcodes that were detected was measured. With four rounds of hybridizations and 5 fluorophores, there were 5^4=625 unique codes. 100 of these barcodes were assigned to measure mRNAs detected at 914.8 ± 570.5 counts per cell (mean ± s.d., N=3439). In comparison, the 525 remaining off-target barcodes that were not used were detected at 4.6 ± 4.7 (mean ± s.d., N =3439) counts per cell (Figure 33D). False

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positives, due to chance alignment of nonspecifically bound spots, contributed minimally to the barcode readouts because of this three order of magnitude difference in detected barcodes (on target vs. off target). The false positives we observe fall only on barcodes hamming distance one away from on-target barcodes, yet minimally contribute to undercounting on-target barcodes (Figure 33E). Furthermore, even the most frequent off-target barcode was observed 65.57 times less frequently than the most infrequent mRNA coding barcode (Figure 33E, Figure 42). Even though during each round of hybridization, 24.8 ± 0.4% (mean ± s.e., N=4 rounds of hybridization) of the spots were nonspecifically bound probes, barcode miss-assignments did not occur frequently because non-specifically bound probes do not reappear in the same location after digestion with DNAse and re-hybridization (Figure 33A). Together the quantifications of false positive and false negative barcodes demonstrate that this method is highly efficient and accurate at detecting RNAs *in situ* in single cells within tissues.

[00414] *Cell clusters are based on combinatorial expression profiles.*

[00415] We imaged the expression of 125 genes in coronal sections from two mice for a total of 14,908 cells (Figure 41E). Cortical and hippocampal cells were segmented based on DAPI and Nissl staining. A tessellation algorithm was developed to accurately segment densely packed cells in the hippocampus. To avoid capturing mRNA from neighboring cells, we contracted by 10% the borders of cells determined by the segmentation algorithm.

[00416] To group the single cell data into distinct transcriptional states, we Z-score normalized the copy number of each transcript in every cell (Figure 34A) and hierarchically clustered the cells to identify cells with similar expression patterns (Figure 43). Many of these clusters, based on overall expression patterns, contain clear transcriptional markers of known cell types previously identified by single cell RNA-seq (Figure 34B) (Zeisel et al., 2015, Tasic et al 2016). Cell clusters 12 and 13 contained clear expression of *Gja1* which marks out astrocytes (Zeisel et al., 2015, Tasic et al 2016). Cluster 12 also expresses *Mfge8* while cluster 13 did not, indicating two distinct population of astrocytes (Figure 34B). There are further subclusters within each of the astrocyte populations with different spatial localization patterns (Figure 43C). Cluster 11 cells expressed *Laptm5*, a known microglia marker (Zeisel et al., 2015, Tasic et al 2016). Cluster 3 expressed interneuron genes while cluster 1-2 and 4-5 expressed genes associated with pyramidal neurons (Zeisel et al., 2015, Tasic et al 2016). Some clusters

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contained many distinct subclusters, such as Amigo2 enriched Mural cells (cluster 9.4) or Omg expressing oligodendrocytes (cluster 10.4 and 10.5). The major clusters were robust to down-sampling the number of cells used in clustering (Figure 44), with some of the hippocampal pyramidal and glia clusters robustly defined even with 400 cells. Similarly, principal component analysis (PCA) visualization of the data (Figure 43F) recapitulated the major clusters that corresponded to astrocyte, microglia, cortical pyramidal, hippocampal pyramidal, dentate gyrus (DG) granule, and interneuron cells.

[00417] *Cell clusters show distinct regional localization*

[00418] Many neuronal clusters mapped to distinct regions in the brain (Figure 34B). Several classes of pyramidal cells (cluster 1-2) showed exclusive localization to the hippocampus, while other classes (4-5) showed predominantly cortical localization. There were also a class of cells (cluster 7) that were almost exclusively present in the DG. Interestingly, these clusters segregated based solely on gene expression profiles without adding any spatial information into the clustering algorithm. These differences in transcriptional states of neurons could be due to intrinsic differences in the cells or due to different local environment and activity patterns.

[00419] In contrast, astrocyte, microglia and other non-neuronal cell clusters were generally uniformly present in all areas of the brain (Figure 34B). However, subclusters of astrocytes did localize to different regions of the brain preferentially (Figure 43C), with subcluster 12.3 localized preferentially to the cortex, while 12.1 subcluster was uniformly distributed. Similarly, cluster 9 cells contain subclusters (9.3, 9.5 and 9.6) that localize exclusively to the DG, while other subcluster (9.1) localize almost exclusively to the cortex. The regional localization of neurons are especially pronounced with cluster 1 and 2 localized almost exclusively to the hippocampus, with some of the subclusters localized predominantly to the CA3. Furthermore, while pyramidal cell clusters 4 and 5 are preferentially cortically localized, the few hippocampal cells in these clusters form their own subclusters (4.4 and 5.4) (Figure 43C). In cluster 6 cells, many subclusters with distinct expression profiles are localized almost exclusively in the CA1, CA3 or the DG (Figures 34C, 43C). In contrast, cluster 7 cells show a relatively homogenous regionalization pattern, but further subdivide based on combinatorial expression patterns (Figure 34D). Subclusters of cluster 9 also show significant regionalization where subclusters 9.1, 9.3, 9.5, and 9.6 show localization to the SGZ (Figure 43C). Overall, cell

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clusters with similar expression profiles exhibited similar spatial localizations across the brain with a correlation coefficient of 0.67 (Figure 43E), indicating the existence of archetypal regional expression patterns and potential spatial markers in the brain. These results show that the tissue-optimized HCR seqFISH approach can directly identify a variety of transcriptional states and quantify broad spatial patterns of expression.

[00420] *Combinatorial expression patterns define fine clusters.*

[00421] While certain cell clusters contain strong expression of marker genes, not all clusters are defined based on a few genes. How much power do individual genes or groups of genes have in explaining the observed cell clusters? To understand this, we examined whether subsets of genes can recapitulate the observed clusters (Figure 34E). We found that any set of 25 genes recovers about half of the correlation structure in the cell-to-cell correlation map (Figures 34E, 45B-C, and 44, N=10 bootstrap replicates). The fact that the selection of any 25 genes can explain the gross patterns in the data is likely due to the high correlations amongst the expression patterns of genes, as shown in the gene-to-gene correlation map (Figure 45A). Thus, a small subset of the measured genes can provides sufficient information to infer the gross transcriptional states of the cells. Interestingly, this may be the same reason why low-coverage single cell sequencing methods such as drop-seq and inDrop (Klein et al., 2015; Macosko et al., 2015) can capture the large distinction of cell types, because many highly expressed genes are correlated to other genes that collectively define cell types.

[00422] At the same time, the finer correlation structure in the data, required to define the cell clusters accurately, can only be captured with more genes (Figures 34F, 45B-C). Consistent with this, using a "random-forest" machine learning algorithm (Breiman, 2001) to classify cell clusters, we found that 75 genes are needed to classify cells with 50% accuracy, indicating that correct cluster assignment requires more detailed information from many genes (Figure 34E). Supporting this view, the first 10 principal components (PC) explained 59.5% of the variation in the data, while the rest of the variation required the remaining 115 PCs (Figures 34F, 43D). The "random forest" algorithm required 10 PCs to predict the cell cluster assignments with 50% accuracy (Figure 34F), but accuracy steadily increased with more PCs. These observation indicated two levels of information in the data: a coarse level, where large distinctions in cell

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clusters are observable by a few genes, and a fine level, where subtle distinctions require many more genes.

[00423] These results suggest two points experimentally. First, multiplexing at the level of 20 genes by seqFISH can give broad cell cluster identification that is not available with 2-3 gene smFISH experiments. Although single marker genes are useful for inference, we find that they frequently are not sufficient for cell classification. For example, all DG specific granule cells (clusters 7) have *Gpc4* and *Vps13c* as their enriched marker genes (Figure 34B); yet, *Gpc4* and *Vps13c* are also strongly expressed in other hippocampal cells outside of the DG, as seen in both our experiments and the ABA. Thus, smFISH against *Gpc4* and *Vps13c* alone would not be sufficient to uniquely identify the DG granule cells. Furthermore, even the strongly bimodal markers that are known to define cell types (i.e. *Mgfe8*, *Gja1*, etc.) are correlated enough to overall expression profiles that cells fall into the appropriate cluster even when these genes are excluded. This point suggests that while marker genes can be essential in assigning a cell to a known cell type, they are not necessary to identify unique clusters in the dataset provided enough measurements are made. Second, accurate measurement of combinatorial expression of many genes enabled by seqFISH can allow for more specific cell cluster identification. As a comparison, in single cell RNAseq data, CA1 pyramidal cells are clustered into a single cluster (Zeisel et. al, 2015; Habib et. al 2016) potentially because of the relatively lower detection efficiency of the method. In our seqFISH experiments, measuring hundreds of genes quantitatively, we can resolve several clusters and subclusters with robust regionalization within the CA1 (Figures 34B, 43C).

[00424] *Cells are patterned in the dentate gyrus.*

[00425] To further visualize the spatial organization of cells, we mapped cluster definitions of cells back into the images. In the DG, we observed a striking lamina layering of cell classes. The two blades of the DG (Figures 35A-B) showed mirror arrangements of cells, with cluster 9 cells, forming the subgranular zone (SGZ), leading into a granule cell layer (GCL) dominated by a single cluster of granule cells (cluster 7) (Figure 34B). In the 125 gene data set, the cells of the GCL were found to be dominated by expression of Gpc4 and Vps13c matching ISH data from the ABA (Figure 48B). Cluster 7 was found to be further subdivided into 6 subclusters (Figure 40C). These subclusters were found to have varying levels of calbindin D-

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28K (*Calb1*) expression which is known to increase with granule cell maturation (Figure 34D)(Yang et al., 2015). On the other hand, the cells of the SGZ were found to be significantly enriched in astrocyte markers such as *Mfge8* and *Mertk*, which has been also been observed previously (Miller et al, 2013) and in the ABA data. However, these cells do not cluster with typical astrocytes (cluster 12 and 13) because their combinatorial expression patterns are different from astrocytes, consistent with their classification as a completely different population of cells.

[00426] In the fork region of the DG, the layer of cluster 9 cells appeared on the interior surface of the fork, followed by a layer of granule cells (cluster 7) (Figure 35C). A different layering pattern is seen at the crest of the DG, where astrocytes, microglia, and some other glial cells line the exterior of the crest ensheathing the GCL (Figure 35D). In both brains of the 125 gene experiments, the same cell clusters and spatial arrangements are observed. Furthermore, because the mRNAs are imaged in 3D in the 10-15um brain slices, we can obtain a 3D view of the expression profiles, shown in the fork regions of the DG (Figure 35F).

[00427] *Distinct regions of CA1 and CA3 are composed of different combination of cell clusters.*

[00428] While each region of the DG contains similar compositions of cells, distinct subregions within the CA1 and CA3 contained different combinations of cell classes (Figures 36, 46F). In the CA1, there were 3 distinct regions defined by their individual cellular compositions. In the dorsal region of CA1 (CA1d), neuron cluster 6 (enriched in *Nell1*, a protein kinase C binding protein) was the major cell type in the pyramidal layer, with astrocyte, microglia and other cells (clusters 10-13) intercalating into the stratum pyramidale (SP) (Figures 36A-C). Transitioning into the CA1 intermediate region (CA1i) (Figure 36D), pyramidal cell cluster 4 displaced cell cluster 6 as the dominant cell, with the co-appearance of cluster 1 and 2 pyramidal cells.

[00429] As the middle of the CA1i region was reached, a small amount of cluster 4 pyramidal cells remain, while cluster 1 and 2 pyramidal cells dominate (Figures 36E-F). Cluster 1 and 2 are enriched in *Nell1* (EGF like protein), *Npy2r* (neuropeptide Y receptor), *Slc4a8* (sodium bicarbonate transporter) and *B3gat2* (glucuronosyltransferase). The CA1i region displayed a characteristic spatial organization where glial cells line the outermost regions, while

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pyramidal cell cluster 1 and 2 longitudinally partitioned the pyramidal layer. This separation of the inner versus the outer layers of CA1 matches those observed in previously (Dong et al., 2008). Furthermore, interneurons (cluster 3) were found to preferentially line the inner edge of the pyramidal layer in the CA1i region (Figures 36E-F). This patterning of interneurons, particularly subcluster 3.1 cells which were enriched in Slc5a7, a choline transporter, was consistent with the patterning of cholinergic interneurons observed with ChAT-GFP labeling (Yi et al., 2015). Finally, the largest amount of heterogeneity in the CA1 was seen in the ventral CA1 region (CA1v), where cell clusters 3, 5, and 10 began to mix in with clusters 1 and 2 (Figures 36G-I).

[00430] Similarly, the CA3 was found to have four transcriptionally distinct regions with different pyramidal cell compositions and abrupt transitions. The ventral most region of CA3 contained a high level of heterogeneity of pyramidal cell clusters (Figures 36J-K), while the intermediate region of CA3 contain a mixture of cell clusters 1 and 2 (Figures 36L-M). As the CA3 progressed towards the hilus of the DG, the cell types transitioned first to primarily cluster 4 neurons (enriched in *dcx*, doublecortin, and *Col5a1*, a collagen), and then to almost exclusively cluster 6 neurons in the region most proximal to the DG hilus (Figures 36O-P). It is interesting to note that while cluster 6 cells appear in both the CA1 (subcluster 6.8) and CA3 (subclusters 6.1 and 6.4), sub-clusters of 6 show distant regional localization (Figure 43C), suggesting that the gene expression differences in CA1 and CA3 cells are captured in the seqFISH data.

[00431] The regionalized expression patterns we observed in the hippocampus match closely to those observed in previous literature (Thompson et al Neuron 2008 and Dong et al PNAS 2009). For example, CA1d, CA1i, CA1v boundaries correspond to the boundaries shown in Fig 2B in Dong et al. In CA3, the subregions observed in our experiment match the CA3 subregion 4-7 in Thompson et al. (Thompson et al., 2008).

[00432] Lastly, we note that the two slices from two different mice in the 125 gene experiment show not only the same subregional structure (Figures 35-37), but also the same clusters of cells (Figures 36 and 37) in the different subregions of the hippocampus (Figure 46). In both brains, the CA1d consists of relatively homogenous population of cluster 6 cells, which transition to a mixture of 1 and 2 cells in CA1i, and finally to a mixture of 1-6 and 10 cells in the CA1v (Figure 46F). These results together show that the sub-regions of the hippocampus are a

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robust feature in the organization of CA1 and CA3, consisting of cells classes with distinct expression profiles. The stereotypical nature of the spatial arrangement of these structures suggest further experiments with seqFISH and other functional assays to probe the distinct functions of the different cell clusters in the CA1 and CA3.

[00433] *249 gene multiplex experiments show the same hippocampal subregions*

[00434] To further show that the sub-regional structure of the hippocampus is independent of the target genes, we performed a 249 gene seqFISH experiment on a third coronal section. Of these 249 genes, only 22 genes overlapped with the 125 gene experiment set. For this set of genes, 214 were selected from a list of transcription factors and signaling pathway components and the remaining 35 were selected from cell identity markers from another single cell RNAseq dataset (Tasic et al, 2016). The 214 genes were barcoded by 5 rounds of hybridization, while the remaining genes were imaged in 7 rounds of non-barcoding serial hybridization. To quantify the efficiency of this experiment, 4 genes in the barcoding set (*Smarca4*, *Sin3a*, *Npas3*, and *Neurod4*) were re-probed with smHCR. The barcoding efficiency of the 249 gene probe set was found to be 71% with and R value of 0.80 (Figure 46D). In single cells, we detect on average 2807±1660 (mean±s.d., N=2050 cells) total barcoded barcodes.

[00435] The same arrangement in the DG was observed in the 249 gene experiment, despite different genes used, indicating robust identification of the layering in the DG by seqFISH (Figures 38S-T). In particular, the cells in the SGZ are clustered independently from cells in the GCL, similar to the layers observed in the 125 gene experiment. In the SGZ cells, we observed enrichment of Sox11, a key transcription factor in neurogenesis (Miller et al, 2013). Other transcription factors involved in neurogenesis, NFIA and Tbr1 are also enriched in the SGZ cells as seen in our data and the ABA images (Figure 48A). The observations of this distinct layer in both the 249 and 125 gene experiment and the combined gene enrichment pattern (increased Sox11, Sox9, NFIA, and Tbr1 in the 249 gene experiment and increased Mertk and Mfge8 in the 125 gene experiment) suggests that many cells in this layer are involved in adult neurogenesis in the SGZ.

[00436] In addition, the same regionalized cellular patterns are observed in CA1d, CA1i, and CA1v, where different subregions utilize different cell classes in characteristic ratios (Figure 46F). As seen with the 125 gene experiment, while the CA1d uses only a few cell classes and is

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relatively homogeneous, while the CA1v region is made up of many different cell classes resulting in a high level of cellular heterogeneity. Furthermore, the distinction between CA1 and CA3 cell clusters are more clear in the 249 gene experiment suggesting more resolving power of spatial patterns (Figures 38A-K). The 249 gene experiment also suggests that the CA3 may be composed of 3-4 subregions based on cell cluster composition (Figures 38L-R). The cellular heterogeneity of the CA3 is again shown to mirror that of the CA1, where the cellular heterogeneity increases along the dorsal to ventral axis. Cells with distinctive marker gene expression in the hippocampus are shown in supplementary Figure 38A.

[00437] *Single cell data resolves cellular organizations in the sub-regions of the CA1 and CA3.*

[00438] Two conflicting views of the cell types in the hippocampus have been proposed based on the analysis of the Allen Brain Atlas data (Thompson 2008) as well as recent RNA-seq data (Cembrowski et al., 2016, Zeisel et al 2015). Analysis of the ABA in situ data showed that distinct subregions of the hippocampus expressed different molecular markers, indicating that the CA1 and CA3 are "regionalized" into distinct sub-structures (Fanselow and Dong, 2010; Thompson et al., 2008). However, recent bulk RNA-seq experiments on the CA1 found that gene expression patterns changed gradually along the dorsal to ventral axis, contradicting the sharp boundaries observed in the ABA analysis (Cembrowski et al., 2016). Further supporting this "continuous" cell type view of the hippocampus, analysis of the single cell RNA-seq data (Zeisel et al, 2015) identified a single continuous population of cells in the CA1 region.

[00439] Our data provides a single cell resolution picture of the spatial organization of cells in the hippocampus and reconciles both the RNA-seq and the ABA data. While our data mostly supports a regionalized view of the hippocampus, we observe that a single cell class does not in general define CA1 and CA3 sub-regions. Instead, we observed that different subregions of CA1 and CA3 are composed of distinct combinations of cell clusters (Figures 33-35). For example, CA1d consists primarily of cluster 6 pyramidal cells (Figures 36A-C), in addition to the cluster 1,2, 10, and 12cells, while CA1v consists of a large set of cell classes including cluster 1-6 and 10 cells, but at different relative abundances (Figures 36-37, Figures 46F-G). Due to this intermixing of cell classes in each sub-region, a bulk measurement of transcription profiles would find a lack of regionalization, but single cell analysis with spatial resolution would

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identify these distinct regions based on their unique cell class compositions. Indeed, when we averaged the single cell expression profile within each sub-region of the CA1, we can reproduce the continuous correlation profiles found by bulk RNA-seq between CA1v, CA1i, and CA1d (Figure 39) (Cembrowski et al., 2016). The bulk RNA-seq observation that CA1i lacked specific marker genes can also be explained. This is in fact consistent with our findings that CA1i contained cell classes present in both CA1d and CA1v (Figures 36-38).

[00440] This organization of cell classes is observed in both the 125 gene experiments as well as in the 249 gene experiment. It is worth noting that the complexity of cell populations observed in the CA1d versus the CA1v matches the functional differences in CA1. CA1d is responsible for spatial learning and navigation and contains a higher concentration of place cells and send projections to dorsal subiculum and cortical retrosplenial area (Cenquizca and Swanson, 2007; Jung et al., 1994; Risold et al, 1997; O'Keefe and Dostrovsky, 1971). We observed that CA1d is composed of a relatively homogeneous population of cells, predominantly of cluster 6 cells. In contrast, the ventral region is involved in a variety of cognitive tasks, such as stress response, emotional and social behavior (Cenquizca and Swanson, 2007; Jung et al., 1994; Fanselow and Dong, 2010; Kishi et al., 2006; Muller et al., 1996; Petrovich et al., 2001; Pitkänen et al., 2000; Saunders et al., 1988; Witter and Amaral, 1991; Yi et al., 2015). Correspondingly, we observed a large set of cell classes in the CA1v regions. It is intriguing to hypothesize that the different cell classes identified based on molecular profiles may correspond to neurons with distinct connectivity and functional patterns. This hypothesis can be investigated in future experiments combining anterograde tracing as well as electrophysiological recording followed by seqFISH.

[00441] A list of the 249 genes being analyzed can be found in the following Table 3.

Name of Genes being analyzed
Tal1
Dmbx1
Emx2
Uncx
Paxip1
Ctnnb1
Prdm1
Rybp
Nfkb2

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Name of Genes being analyzed
Tfdp2
Grhl1
Sp8
Irf2
Zfp287
Esr2
Zfp128
Vav1
Sp1
Ppargc1b
Sp7
Pin1
Nfya
Vsx1
Klf1
Vsx2
Mybl1
Mybl2
Rnf2
Blzf1
Topors
Nr3c2
Nfia
Taf6l
Nr4a3
Hoxd12
Hoxd13
Ttf1
Sox9
Nr2e1
Polr2b
H■tf
Sox6
Pbx3
Sox5
Foxa1
Cdc5l
Cebpg
Ciita
Rest
Ets1
Mafk

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Name of Genes being analyzed
Tbx15
Scml2
Myb
Clock
Rbpj
Foxc1
Zfp422
Pias3
Runx1
Ppara
Relb
Vdr
Cdc6
Arid3a
Lhx1
Hoxb8
Hoxb9
Hic1
Lhx6
Six4
Hoxb3
Zfp263
Cbfa2t3
Ehf
Nhlh1
Gata6
Gata4
Gata5
Lpp
Nfe2l3
Nfe2l2
Tmf1
Gli1
Tbx2
En1
En2
Hnf1a
Tbx4
Zfp423
Elf1
Foxb1
Elf2

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Name of Genes being analyzed
Elf4
Mxd1
Wt1
Rfx4
Bhlhe41
Sox13
Taf4b
Rfx2
Sox17
Ahr
Sall4
Med14
Smyd1
Sall3
Arid2
Zfp64
Pgr
Trps1
Hoxa1
Bach2
Bach1
Notch3
Pknox1
Pknox2
Sin3a
Etv3
Smad9
Smad5
Alx1
Egf
Mn1
Nkx3-1
Rbak
Gabpa
Nfkbiz
Zscan21
Trp73
E2f7
Esrrg
Rbpjl
Nfatc4
Nr5a1

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Name of Genes being analyzed
Neurod4
Esrrb
Tbx21
Rorc
Mitf
Pax7
Pax6
Pax1
Pax3
Pax2
Pax9
Zkscan17
Gfi1
Mzf1
Runx3
Smarca4
Foxd4
Foxd3
Creb1
Srebf1
Sox11
Gmeb2
Irx4
Pou3f2
Ikzf1
Tcf23
Mtf2
Npas3
Nfatc3
Nfil3
Phox2b
Plag1
E2f2
Ddx3x
Taf2
Pou4f1
Trim33
Tsc2
Lmx1a
Nr2f2
Eomes
Wwtr1

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Name of Genes being analyzed
Foxo1
Ar
Zfp354a
Elk4
Foxo4
Sall1
Mycn
Maml3
Foxp3
Atrn
Uaca
Tbr1
Pml
Lhx3
Atr
Zbtb33
Ptch1
Lhx5
Barhl1
Irx5
Tfap2b
Tfap2e
Rxra
Rxrb
Gli2
Gli3
Zic4
Zic5
Zic2
Zic3
Satb1
Onecut2
Foxn4
Mnat1
Foxn1
Dlx2
Vezf1
sncg
sst
th
vip
xdh

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Name of Genes being analyzed
slc17a8
slc5a7
slc6a3
slc6a8
smad3
opalin
pdgfra
palvb
reln
slc17a7
lyve
mfge8
mog
myl14
ndnf
ctss
foxj1
gad1
htr3a
igtp
acta2
alldh1l1
camk2
chat
cldn5
ngef
tiam1
slc1a2
gja1
fbll1

[00442] *seqFISH provides a generalized method to multiplex mRNA imaging in tissues*

[00443] seqFISH with amplification and error correction provides a highly quantitative method to profile hundreds of mRNA species directly in single cells within their native anatomical context. Our method of stripping the probes from the RNA has many advantages. DNAse digestion of probes allows false positives to be rejected as nonspecifically bound probes do not colocalize between different rounds of hybridization (Figure 33A). In addition, the same region of the transcript can be hybridized in every round, allowing seqFISH to efficiently target

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mRNAs shorter than 1kb, enabling targeting of most genes. Lastly, seqFISH allows exponential scaling of barcode numbers, thus 4-5 rounds of hybridization can code for hundreds of transcripts with a simple error correction scheme. Theoretically, the entire transcriptome can be coded for with error correction by using 8-9 rounds of hybridization with seqFISH. These advantages of HCR seqFISH allows robust multiplexed RNA detection in tissues, shown here in the mouse brain.

[00444] Ultimately, the multiplexing capability of seqFISH is limited by the amount of optical space within a cell, and not by the coding capacity of the method (supplementary text). We showed previously that super-resolution microscopy can significantly increase the optical space available in the cell for transcription profile imaging, but super-resolution microscopy experiments proved difficult to image in samples thicker than 1μm, and were experimentally cumbersome and time consuming to image (Lubeck and Cai, 2012). A recent development in expansion microscopy as well as correlation methods (Coskun et al., 2016) however offers promise for multiplexing to levels of high transcript density (Chen et al., 2015a; Treweek et al., 2015, Chen et al., 2016). In addition, by labeling subcellular components (i.e., dendrites and axons) with antibodies, the local transcriptome in compartments of the cell can be measured.

[00445] It was observed that, because expression patterns amongst genes are highly correlated, the distinction between large classes of cells can be determined from 10-20 genes, while a finer classification of cell clusters depends on the quantitative measurement of the combinatorial expression patterns of many genes (Figures 34E and F). This correlation amongst genes can be used to "stitch" our seqFISH data with single cell RNAseq data, similar to the approach explored with single cell RNAseq and ISH in Satija et al (Satija et al., 2015) . By correlating seqFISH data to single cell RNA-seq expression data, cells types identified based on RNA-seq can be "mapped" back into our seqFISH data.

[00446] As shown here, seqFISH with hundreds of genes in tissues can become a general and widely used tool to answer a wide range of fundamental questions in biology and medicine. For neuroscience, by combining the insights into the spatial organization of transcription provided by seqFISH with connectomics and electrophysiological measurements, we can obtain a comprehensive understanding of the molecular basis of the neuroanatomy of the brain.

EXAMPLE 5

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Supplementary Experimental Procedure for Brain Slide Analysis

[00447] **Probe Design.** Genes were selected from the Allen Brain Atlas database. We identified genes that are heterogeneously expressed in coronal sections containing the hippocampus at Bregma coordinates -2.68 mm anterior. Using the ABA region definitions, we break down the voxels representing the ABA data in those brain sections into 160 distinct regions and average the expression values within each region. We selected 100 genes that had high variances across these distinct regions and that also had low-medium expression levels. These genes included transcription factors and signaling pathways components as well as ion channels and other functional genes. Lastly, we chose 25 genes from single cell RNA-seq data that were enriched in certain cell types. Briefly, the design criteria used were 1) constant regions of all spliced isoforms were identified, 2) Masked regions of UCSC genome were removed from possible probe design, 3) 35mer sequences were tiled 4nt apart, 4) sets of non-overlapping probes with tightest GC range around 55% were found, 5) probes were blasted for off-target hits. Any probe with an expected total off-target copy number of more than 5000 was dropped. Once all possible probes for every target gene was acquired, the probe set oligo-pool was optimized using the following criteria: 1) Expected # of off-target hits for entire probe pool was calculated, 2) probes were sequentially dropped from genes until any off-target gene was hit by no more than 6 probes from entire pool, 3) HCR adapters were added to designed probes and 10nt in either direction of the adapter junction was blasted and screened for off-target hits, 4) probe pools were searched for regions of 18mer complementary, 5) the probe sets for a given transcript was refined down to 24 probes by dropping probes in order of the expected number of off-target hits, 6) Cutting sites and hybridization specific primers were added to probes.

[00448] **Probe Generation.** All oligoarray pools were purchased as 92k synthesis from Customarray Inc. Probes were amplified from array-synthesized oligo pool), with the following modifications: (i) a 35nt RNA-targeting sequence for in situ hybridization, (ii) a 35nt HCR initiator sequence designed to initiate one color of 5 possible HCR polymers, (iii) two hybridization specific flanking primer sequences to allow PCR amplification of the probe set and (iv) EcoRI (5'-GAATTC—3') and KpnI (5'-GGTACC-3') sites for cutting out flanking primers to reduce probe size. Ethanol precipitation was used to purify the final digested probes.

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[00449] **Brain extraction and sample mounting.** C57BL/6 with Ai6 Cre-reporter (uncrossed) (Jackson Labs, SN: 007906) female mice aged 50-80 days were anesthetized with isoflurane according to institute protocols (protocol #1701-14). No randomization of mice was used and blinding was not necessary as the study was exploratory. Mice were perfused for 8 minutes with perfusion buffer (10U/ml heparin, 0.5% $NaNO_2$ (w/v) in 0.1M PBS at 4°C). Mice were then perfused with fresh 4% PFA\0.1M PBS buffer at 4C for 8 minutes. The mouse brain was dissected out of the skull and immediately placed in a 4% PFA buffer for 2 hours at room temperature under gentle mixing. The brain was then immersed in 4C 30% RNAse-free Sucrose (Amresco 0335-2.5KG)\1x PBS until the brain sank. After the brain sank, the brain was frozen in an dry ice\isopropanol bath in OCT media and stored at -80C. Fifteen micron sections were cut using a cryotome and immediately placed on an aminosilane modified coverslip.

[00450] **Sample permeabilization, hybridization, and Imaging.** Brain sections mounted to coverslips were permeabilized in 4C 70% EtOH for 12-18 hours. Brains were further permeabilized by the addition of rnase-free 8% SDS (Ambion AM9822) for 10 minutes. Samples were rinsed to remove SDS, desiccated and a hybridization chamber (Grace Bio-Labs 621505) was adhered around the brain section. Samples were hybridized overnight at 37C with Split Color PGK1 Probes in Hybridization Buffer (2X SSC (Invitrogen 15557-036), 10% Formaldehyde (v/v) (Ambion AM9344), 10% Dextran Sulfate (Sigma D8906), 2mM Vanadyl Ribonucleoside Complex (VRC; NEB S1402S) in Ultrapure water (Invitrogen 10977-015)). Samples were washed in 30% Wash Buffer (WBT: 2X SSC, 30% Formaldehyde (v/v)] 10% Dextran Sulfate, 0.1% Triton-X 100 (Sigma X-100), 2mM VRC in Ultrapure water) for 30 minutes. While washing aliquoted HCR hairpins (Molecular Instruments Inc) were heated to 95C for 1.5 minutes and allowed to cool to RT for 30 minutes. HCR hairpins were diluted to a concentration of 120nM per hairpin in amplification buffer (2X SSC, 10% Dextran Sulfate) and added to washed tissue for 45 minutes. Following amplification, samples were washed in the same 30% WBT for at least 10 minutes to remove excess hairpins. Samples were stained with DAPI and submerged in pyranose oxidase antibleaching buffer. Sample port covers were closed with a glass coverslip or a transparent polycarbonate sheet to exclude oxygen.

[00451] Samples were imaged using a standard epifluorescence microscope (Nikon Ti Eclipse with custom built laser assembly) for the 125-gene experiment. Exposures times were

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200 ms for cy7 and alexa 488 channels and 100 ms for alexa 647, alexa 594, and cy3b channels. For the 249-gene experiment, a Yokogawa CSU-W1 spinning disk confocal unit attached to an Olympus IX-81 base was used for imaging. The exposure times were 500 ms for each channel. At this stage, intact and accessible mRNA should always appear in two channels. If the RNA was deemed to be intact, DAPI data was collected in this hybridization. Samples were digested with DNAse I (Roche 04716728001) for 4 hours at room temperature on the scope. Following DNAse I the sample was washed several times with 30% WBT and hybridized overnight with 70% Formamide HB and the experiment probes at 1 nM concentration per probe sequence at room temperature. Samples were again washed and amplified as before. Barcode digits were developed by repeating this cycle with the appropriate probes for each hybridization. Fluorescent Nissl stain (ThermoFisher N-21480) was collected at the end of the experiment along with images of multispectral beads to aid chromatic aberration corrections.

[00452] **Image Processing.** To remove the effects of chromatic aberration, the multispectral beads were first used to create geometric transforms to align all fluorescence channels. Next, the background illumination profile of every fluorescence channel was mapped using a morphological image opening with a large structuring element. These illumination profile maps were used to flatten the illumination in post-processing resulting in relatively uniform background intensity and preservation of the intensity profile of fluorescent points. The background signal was then subtracted using the imagej rolling ball background subtraction algorithm with a radius of 3 pixels. Finally, the calculated geometric transforms were applied to each channel respectively. The 150 pixel border region around the image was ignored in all analysis to avoid errors from edge effects of illumination.

[00453] **Image Registration.** The processed images were then registered by first taking a maximum intensity projection along the z direction in each channel. All of the maximum projections of the channels of a single hybridization were then collapsed resulting in 4 composite images containing all the points in a particular round of hybridization. Each of these composite images of hybridization 1-3 were then cross-correlated individually with the composite image of hybridization 4 and the position of the maxima of the cross-correlation was used as the translation factor to align hybridizations 1-3 to hybridization 4.

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[00454] **Cell Segmentation.** For cells in the cortex, the cells were segmented manually using the DAPI images taken in the first round of hybridization and the fluorescent nissl stain taken at the end of the experiment. Furthermore, the density of the point cloud surrounding a cell was taken into account when forming cell boundaries, especially in cells that did not stain with the nissl stain. For the hippocampus, the cells were segmented by first manually selecting the centroid in 3D of each DAPI signal of every cell. Transcripts were first assigned based on nearest centroids. These point clouds were then used to refine the centroid estimate and create a 3D voronoi tessellation with a 10% boundary-shrinking factor to eliminate ambiguous mRNA assignments from neighboring cells.

[00455] **Barcode calling.** The potential mRNA signals were then found by LOG filtering the registered images and finding points of local maxima above a specified threshold value. Once all potential points in all channels of all hybridizations were obtained, dots were matched to potential barcode partners in all other channels of all other hybridizations using a 1 pixel search radius to find symmetric nearest neighbors. Point combinations that constructed only a single barcode were immediately matched to the on-target barcode set. For points that matched to construct multiple barcodes, first the point sets were filtered by calculating the residual spatial distance of each potential barcode point set and only the point sets giving the minimum residuals were used to match to a barcode. If multiple barcodes were still possible, the point was matched to its closest on-target barcode with a hamming distance of 1. If multiple on target barcodes were still possible, then the point was dropped from the analysis as an ambiguous barcode. This procedure was repeated using each hybridization as a seed for barcode finding and only barcodes that were called similarly in at least 3 out of 4 rounds were used in the analysis. The number of each barcode was then counted in each of the assigned cell volumes and transcript numbers were assigned based on the number of on-target barcodes present in the cell volume. All image processing and image analysis code can be obtained upon request.

[00456] **Clustering.** To cluster the dataset with 14,908 cells and 125 genes profiled, we first z-score normalized the data based on gene expression. Once the single cell gene expression data is converted into z-scores, we compute a matrix of cell-to-cell correlations using Pearson correlation coefficients. Then hierarchical clustering with Ward linkage is performed on the cell-to-cell correlation data with cells in the center field of view. The cluster definitions are then

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propagated to the remaining cells using a random forest machine learning algorithm. To analyze the robustness of individual clusters, a random forest model was trained using varying subsets of the data and used to predict the cluster assignment of the remaining cells. A bootstrap analysis by dropping different sets of cells was performed in increments (Figure 42). To determine the effect of dropping out genes on the accuracy of the clustering analysis, we used a random forest decision tree to learn the cluster definition based on the 125 gene data. Then we ask the decision tree to re-compute the cluster assignment on cell-to-cell correlation matrices with fewer and fewer genes (Figure 34F, green line). Bootstrap resampling was also performed with this analysis (Figure 34F, bluelines). The PCA and tSNE analysis were performed using the same cell-to-cell z-scored Pearson correlation matrix. The cell-to-cell correlation in Figure 34E was calculated with increasing number of principal components dropped (have their eigenvalues set to zero). The cluster assignment accuracy is again computed through the random forest decision tree.

[00457] Optical Space for Barcodes in Cells. The theoretical upper limit for the number of barcodes that can be identified accurately within a cells primarily depends on the volume of the cell. As mRNA spots are diffraction limited, if a microscope is configured to have sub-diffraction limited pixel size, the ability to identify smFISH signal without any super-resolution would require no two mRNA signals to be immediately adjacent to each other in x, y or z dimension. These minimum required voxels are called "coding voxels." The absolute upper limit of the number of transcripts that can be coded unambiguously without any super-resolution methods is solely a function of the number of coding voxels present in a cell. Assuming a diffraction limit of λ um and a resolution of z um in the z direction, there exists $\frac{V}{(3\lambda)^2 z}$ coding voxels per cell, where V is the volume of the cell in microns. In the seqFISH method, we use 5 or more channels to hold mRNA spots which would increase the total number of coding voxels by a multiplicative factor equal to the number of channels used for barcoding. Therefore,

$$\#B = \frac{F\,V}{(3\lambda)^2 z}$$

where #B is the maximum number of unambiguous barcodes a cell can hold, and F is the number of channels used. As mammalian cells range from about 500 – 4000 microns in volume, these cells can accommodate roughly between 6100 – 49,000 barcodes assuming 5 fluorescence

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channels are being used, the diffraction limit is 0.3 um, and the z resolution is 0.5 um. In principle, this calculation would provide the total number of perfectly discernible spots a cell can accommodate. In our actual experimental data, we have some amount of dropped barcodes due to ambiguity in barcode assignment due to spot overlaps. This is one of the main factors that reduces the efficiency of seqFISH as compared to single transcript detection (i.e., smFISH or smHCR). Expansion microscopy could further increase the number of coding voxels in a cell by the expansion factor leading to fewer drops and imaging of denser transcripts.

[00458] Additional background information can be found in the following references, each of which is hereby incorporated by reference in its entirety.

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EQUIVALENTS

[00523] Having described some illustrative embodiments of the invention, it should be apparent to those skilled in the art that the foregoing is merely illustrative and not limiting, having been presented by way of example only. Numerous modifications and other illustrative embodiments are within the scope of one of ordinary skill in the art and are contemplated as falling within the scope of the invention. In particular, although many of the examples presented herein involve specific combinations of method acts or system elements, it should be understood that those acts and those elements may be combined in other ways to accomplish the same objectives. Acts, elements, and features discussed only in connection with one embodiment are not intended to be excluded from a similar role in other embodiments. Further, for the one or more means-plus-function limitations recited in the following claims, the means are not intended to be limited to the means disclosed herein for performing the recited function, but are intended to cover in scope any means, known now or later developed, for performing the recited function.

[00524] Use of ordinal terms such as "first", "second", "third", *etc.*, in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim

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element over another or the temporal order in which acts of a method are performed, but are used merely as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements. Similarly, use of a), b), *etc.*, or i), ii), *etc.* does not by itself connote any priority, precedence, or order of steps in the claims. Similarly, the use of these terms in the specification does not by itself connote any required priority, precedence, or order.

[00525] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

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WE CLAIM:

1. A method of barcoding molecular targets, comprising:

identifying N molecular targets in a biological sample, wherein the N molecular targets are immobilized;

associating a unique barcode to each molecular target via n sequential barcoding rounds (where $n \geq 2$), wherein each barcoding round comprises m serial hybridizations of probes collectively bound to the N molecular targets (where $m \geq 2$), wherein each serial hybridization comprises:

contacting one or more groups of probes to a subset of the N molecular targets, the total number of groups of probes corresponding to the number of molecular targets in the subset, wherein probes in each group comprise one or more binding sequences specifically targeting a molecular target in the subset, wherein each probe is capable of generating at least one detectable visual signal representing binding of the probe to a molecular target in the subset, and wherein probes in the one or more groups generate one or more different detectable visual signals corresponding to the number of molecular targets in the subset;

detecting the detectable visual signals that reflect the binding between the one or more groups of probes and the subset of the N molecular targets; and

removing the visual signals, when applicable, prior to the next serial hybridization;

wherein the unique barcode to each molecular target consists of n components, each component is assigned from S unique symbols, where S is an integer that equal to or greater than $\sqrt[n]{N}$; and

optionally removing probes between two barcoding rounds.

2. The method of claim 1, wherein the detecting the detectable visual signals comprises:

capturing, for each serial hybridization round, an image of the detectable visual signals that reflect the binding between the one or more groups of probes and the subset of the N molecular targets.

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3. The method of claim 1 or claim 2, further comprising:

generating, for each barcoding round, a composite image by superimposing m images corresponding to the m serial hybridizations, wherein the m images are aligned based on one or more alignment references whose positions remain constant relative to the biological sample.

4. The method of any one of claims 1-3, further comprising:

applying Gaussian analysis to super-localize the detectable visual signals in an image.

5. The method of any one of claims 1-4, further comprising:

decoding the detectable visual signals in each composite image based on the unique barcodes for the N molecular targets and the S unique symbols.

6. The method of any one of claims 1-5, further comprising:

detecting reference visual signals associated with the one or more alignment reference.

7. The method of any one of claims 1-6, wherein the one or more alignment references comprise one or more selected from the group consisting of an oligonucleotide sequence immobilized on the coverslips and detected by a complementary oligo, a common sequence embedded in all probes, a microscopic object, a metal bead, a gold bead, a polystyrene bead, a PCR handle sequence on a primary binding probe, and combinations thereof.

8. The method of any one of claims 1-7,wherein the n sequential barcoding rounds includes x round for error correction, where x is an integer equal or greater than 1; and wherein assigning unique barcodes for each of N molecular targets requires S unique symbols, where S is an integer equal or greater than $\sqrt[n-x]{N}$.

9. The method of any one of claims 1-8, wherein the biological sample comprises a tissue sample, a cell sample, a cell extract sample, a nucleic acid sample, an RNA transcript sample, a

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protein sample, an mRNA sample, DNA molecules, protein molecules, RNA and DNA isoform molecules, single nucleotide polymorphism molecules, or combinations thereof.

10. The method of any one of claims 1-9, further comprising:

determining a secondary molecular target that are associated with the *N* molecular targets by contacting the biological sample with molecules specifically binding to the secondary molecular target.

11. The method of any one of claims 1-10, wherein the secondary molecular target comprises one selected from the group consisting of a RNA binding protein molecule, ribosome, a DNA binding protein molecule, a transcription factor, a chromatin binding protein, a protein binding molecule, a scaffold protein, and combinations thereof.

12. The method of any one of claims 1-11, wherein probes in the one or more groups of probes further comprise:

one or more binding sequences each specifically targeting one or more sites within a molecular target in the biological sample; and

n unique readout sequences associated with the one or more binding sequences, wherein, in each barcoding round, only one unique readout sequence is associated with a detectable visual signal for a particular molecular target.

13. The method of any one of claims 1-12, wherein the one or more binding sequences target multiple different sites within the same molecular target.

14. The method of any one of claims 1-13, wherein the one or more binding sequences target multiple different sites within different molecular targets.

15. The method of any one of claims 1-14, wherein each probe comprises one or more of the *n* unique readout sequences.

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16. The method of any one of claims 1-15, wherein at least one of the *n* unique readout sequences is located in an overhang sequence directly connected to the binding sequence of a probe.

17. The method of any one of claims 1-16, wherein at least one of the *n* unique readout sequences is indirectly connected to the binding sequence of a probe via one or more intermediate molecules.

18. The method of any one of claims 1-17, wherein the one or more intermediate molecules comprise an RNA bridge probe, a DNA bridge probe, a protein bridge probe, a probe for hybridization chain reaction (HCR), a hairpin nucleic acid probe, an HCR initiator, an HCR polymer, or combinations thereof.

19. The method of claim any one of claims 1-18, wherein the one or more binding sequences specifically target one or more non-nucleic acid sites in the molecular target, and wherein the *n* unique readout sequences comprising nucleic acid sequences that are directly or indirectly connected to the binding sequences of the probes.

20. The method of any one of claims 1-19, wherein the detectable visual signal is connected to the binding sequence of a probe or an intermediate molecule via a cleavable linker.

21. The method of any one of claims 1-20, wherein the one or more binding sequences comprises a peptide sequence binding to a specific antigen within a particular molecular target, an aptmer, or click chemistry group.

22. The method of any one of claims 1-21, wherein the *S* unique symbols comprise colors, numbers, letters, shapes, or combinations thereof.

23. The method of any one of claims 1-22, wherein, for each serial hybridization, the one or more groups of probes to a non-overlapping subset of the *N* molecular targets.

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24. A method of hybridization analysis of binding between labeled probes and molecular targets in a biological sample, comprising:

generating multiple composite images of labeled probes bound to a plurality of molecular targets in the biological sample, wherein each composite image is generated from a plurality of images of labeled probes collectively bound to the plurality of molecular targets, wherein the plurality of molecular targets are immobilized within the biological sample, and wherein each image of the plurality of images reveals:

labeled probes bound to a subset of molecular targets within the plurality of molecular targets, wherein the labeled probes comprise one or more groups of probes, the total number of groups of probes corresponding to the number of molecular targets in the subset, wherein probes in each group comprise one or more binding sequences specifically targeting a molecular target in the subset, and wherein each labeled probe is capable of generating a visual signal representing binding of the probe to a molecular target; and

one or more reference targets whose positions remain constant in the biological sample for aligning the plurality of images.

25. The method of claim 24, wherein the biological sample comprises a tissue sample, a cell sample, a cell extract sample, a nucleic acid sample, an RNA transcript sample, a protein sample, an mRNA sample, DNA molecules, protein molecules, RNA and DNA isoform molecules, single nucleotide polymorphism molecules, or combinations thereof.

26. The method of claim 24 or claim 25, wherein, in each image, the labelled probes bind to a non-overlapping subset of molecular targets within the plurality of molecular targets.

27. The method of any one of claims 24-26, further comprising:

contacting the one or more groups of probes with the subset of molecular targets of the plurality of molecular targets;

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detecting visual signals that reflect the binding between the one or more groups of probes and molecular targets in the subset; and

removing the visual signals, when applicable, prior to a next round of hybridization of labeled probes binding to a new subset of molecular targets within the plurality of molecular targets.

28. The method of any one of claims 24-27, further comprising:

detecting reference visual signals associated with the one or more alignment references.

29. The method of any one of claims 24-28, further comprising:

aligning the plurality of images based on the positions of the one or more alignment references.

30. A sequential hybridization method, comprising:

identifying a plurality of target genes; and

associating, via sequential hybridization of binding probes to the plurality of target genes, a first plurality of unique codes with the plurality of target genes, wherein each target gene in the plurality of target genes is represented by a unique code in the first plurality of unique codes, wherein the sequential hybridization comprises n rounds of hybridization (where $n \geq 2$), and wherein each round of hybridization in n rounds of hybridization comprises:

contacting the plurality of target genes with a plurality of binding probes, wherein each probe in the plurality of binding probes comprises:

a binding sequence that specifically binds a target sequence in a gene in the plurality of target genes, wherein target genes from the plurality of target genes are spatially transfixed from each other, and wherein each probe is capable of emitting a detectable visual signal upon binding of the probe to a target sequence;

detecting visual signals that reflect the binding between the plurality of binding probes and the plurality of target genes; and

NanoString EX2011.404

removing the visual signals, when applicable, prior to the next round of hybridization;

wherein probes used in the n rounds of hybridization are capable of emitting at least F types of detectable visual signals (where $F \geq 2$ and F^n is greater than the number of target genes in the plurality of target genes),

wherein a unique code in the first plurality of unique codes for a target gene consists of n components,

wherein each component is determined by visual signals that reflect the binding between binding probes and the target gene during one of the n rounds of hybridization,

wherein the n rounds of hybridization include m error correction round ($m \geq 1$),

wherein a second plurality of unique codes for the plurality of target genes is generated after the m error correction round is removed from the n rounds of hybridization, and

wherein each unique code in the second plurality of unique codes consists of $(n - m)$ components and uniquely represents a target gene in the plurality of target genes.

31. A hybridization method, comprising:

identifying a plurality of target genes;

performing sequential hybridization of binding probes to the plurality of target genes, wherein the sequential hybridization comprises n rounds of hybridization (where $n \geq 2$), and wherein each round of hybridization in n rounds of hybridization comprises:

contacting the plurality of target genes with a plurality of binding probes, wherein each probe in the plurality of binding probes comprises:

a binding sequence that specifically binds a target sequence in a gene in the plurality of target genes, wherein target genes from the plurality of target genes are spatially transfixed from each other, and wherein each probe is capable of emitting a detectable visual signal upon binding of the probe to a target sequence;

detecting visual signals that reflect the binding between the plurality of binding probes and the plurality of target genes, wherein each target gene in the plurality of target genes is represented by visual signals that are unique for the target gene, and wherein

NanoString EX2011.405

probes used in the n rounds of hybridization are capable of emitting at least F types of detectable visual signals (where $F \geq 2$, and F^n is greater than the number of target genes in the plurality of target genes); and

removing the visual signals, when applicable, prior to the next round of hybridization; and

performing serial hybridizations against one or more serial target genes, wherein the expression level of each serial target gene is above a predetermined threshold value, wherein each serial hybridization comprises:

contacting the one or more serial target genes with a plurality of binding probes, wherein each probe in the plurality of binding probes comprises:

a binding sequence that specifically binds a target sequence in a serial target gene in the one or more serial target genes, wherein one or more serial target genes are spatially transfixed from each other,

wherein each probe is capable of emitting a detectable visual signal upon binding of the probe to the target sequence, and wherein probes binding to target sequences in the same serial target gene emit the same detectable visual signals; and

detecting visual signals that reflect the binding between the plurality of binding probes and the one or more serial target gene.

32. The sequential hybridization method of claim 30 or claim 31, wherein each component of a n-component unique code in the first plurality of unique codes is assigned a numerical value that corresponds to one of the at least F types of detectable visual signals; and wherein at least one component of the n-component unique code is determined based on the numerical values of all or some of the other n-1 components.

33. The sequential hybridization method of any one of claims 30-32, wherein the n-component unique code is determined as:

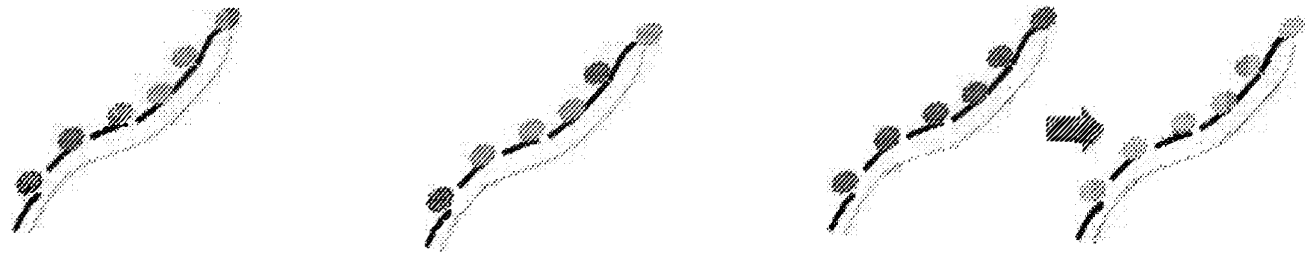
$\{j_1, j_2, \ldots (a_1*j_1 + a_2*j_2 \ldots + a_n*j_n + C) \text{mod } F, \ldots, j_n\}$,

NanoString EX2011.406

wherein j_1 is a numerical value that corresponds the detectable visual signals used in the first round of hybridization, j_2 is a numerical value that corresponds the detectable visual signals used in the second round of hybridization, and j_n is a numerical value that corresponds the detectable visual signals used in the *n*th round of hybridization; and

wherein $j_1, j_2, \ldots j_n, a_1, a_2, \ldots a_n$ and n are none zero integers and C is an integer.

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Scales:	${}_{F}P_{N}$	${}_{F}C_{N}$	seqFISH F^{N}
Max capacity: (assuming 5 dyes)	720	31	5^{7}=78,125 5^{3}=125
Coding scheme			4-5 colors
Probes needed	20-40	20-40	10-20 rehybs probes

FIG. 1

NanoString EX2011.408

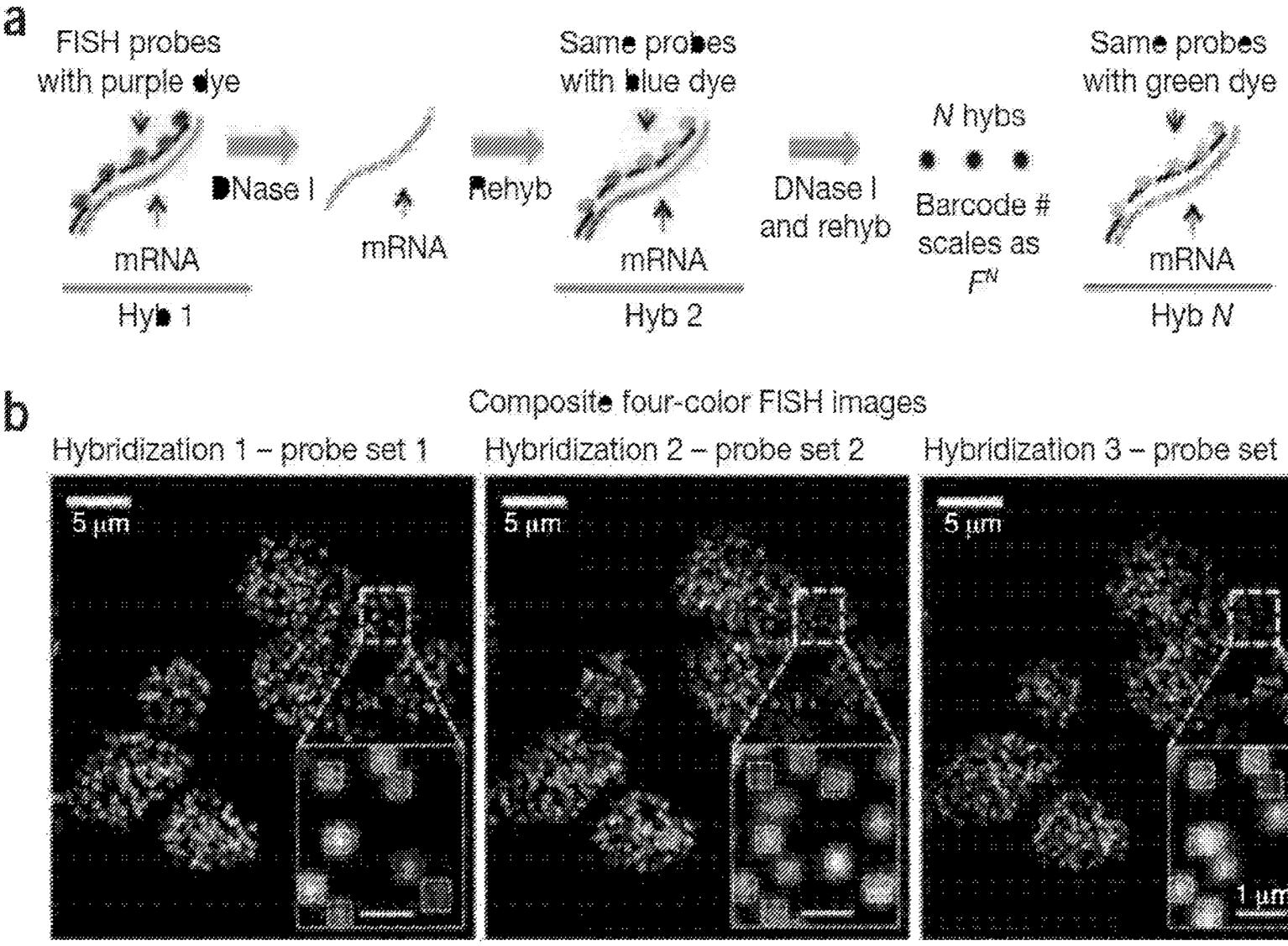


FIG. 2

NanoString EX2011.409

C

FIG. 2 (Continued)

NanoString EX2011.410

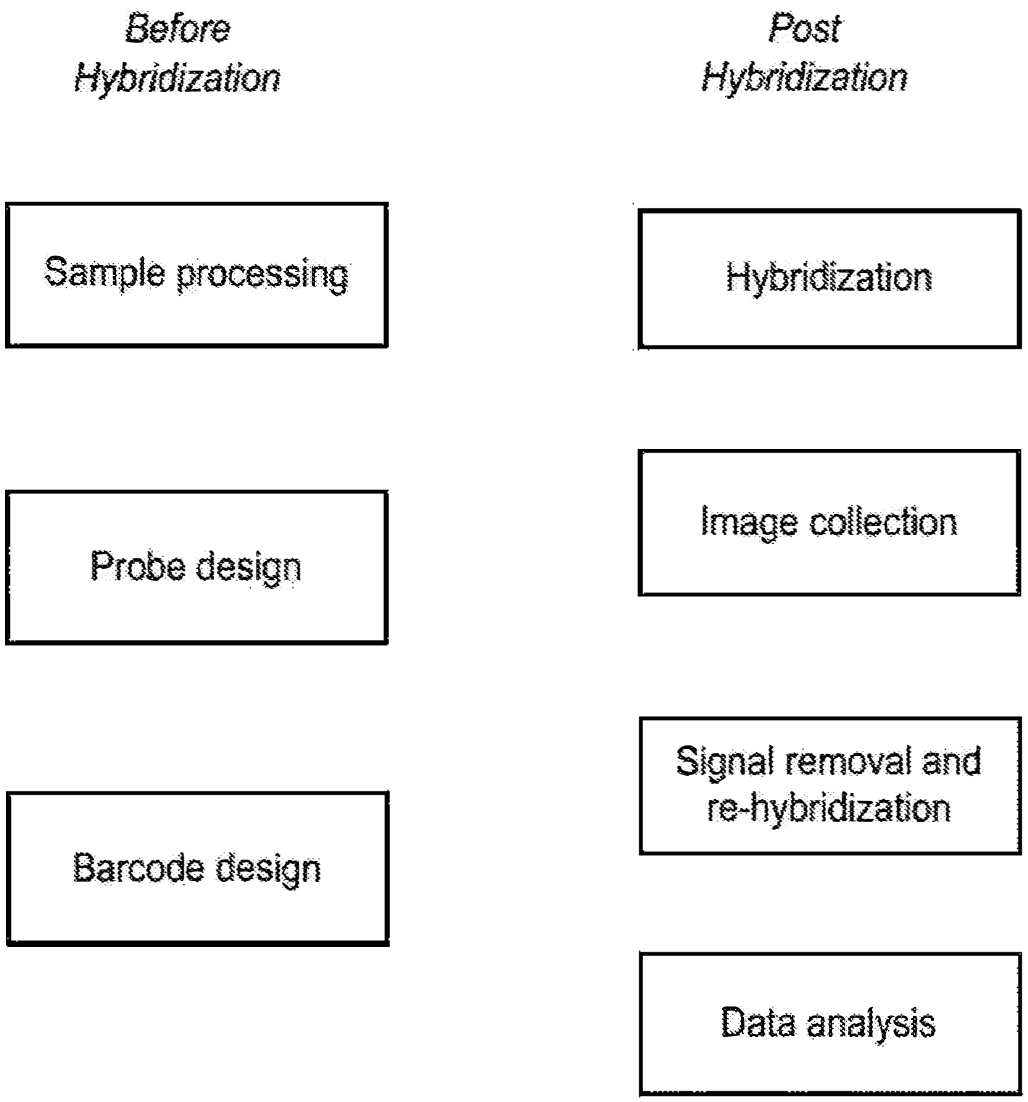

Before Hybridization
Post Hybridization
Sample processing
Probe design
Barcode design
Hybridization
Image collection
Signal removal and re-hybridization
Data analysis

FIG. 3A

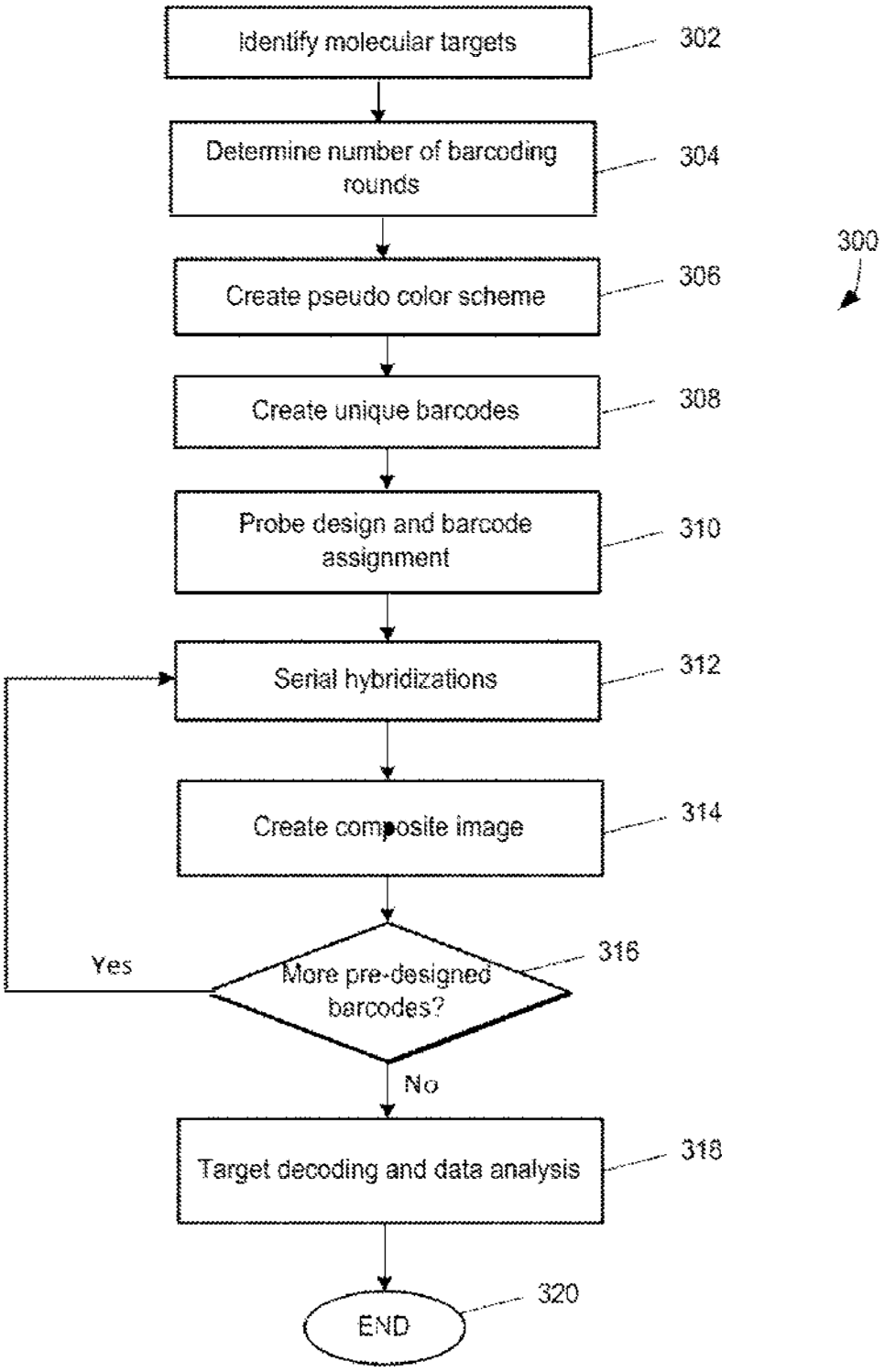

Identify molecular targets
302
Determine number of barcoding rounds
304
300
Create pseudo color scheme
306
Create unique barcodes
308
Probe design and barcode assignment
310
Serial hybridizations
312
Create composite image
314
More pre-designed barcodes?
316
Yes
No
Target decoding and data analysis
318
END
320

FIG. 3B

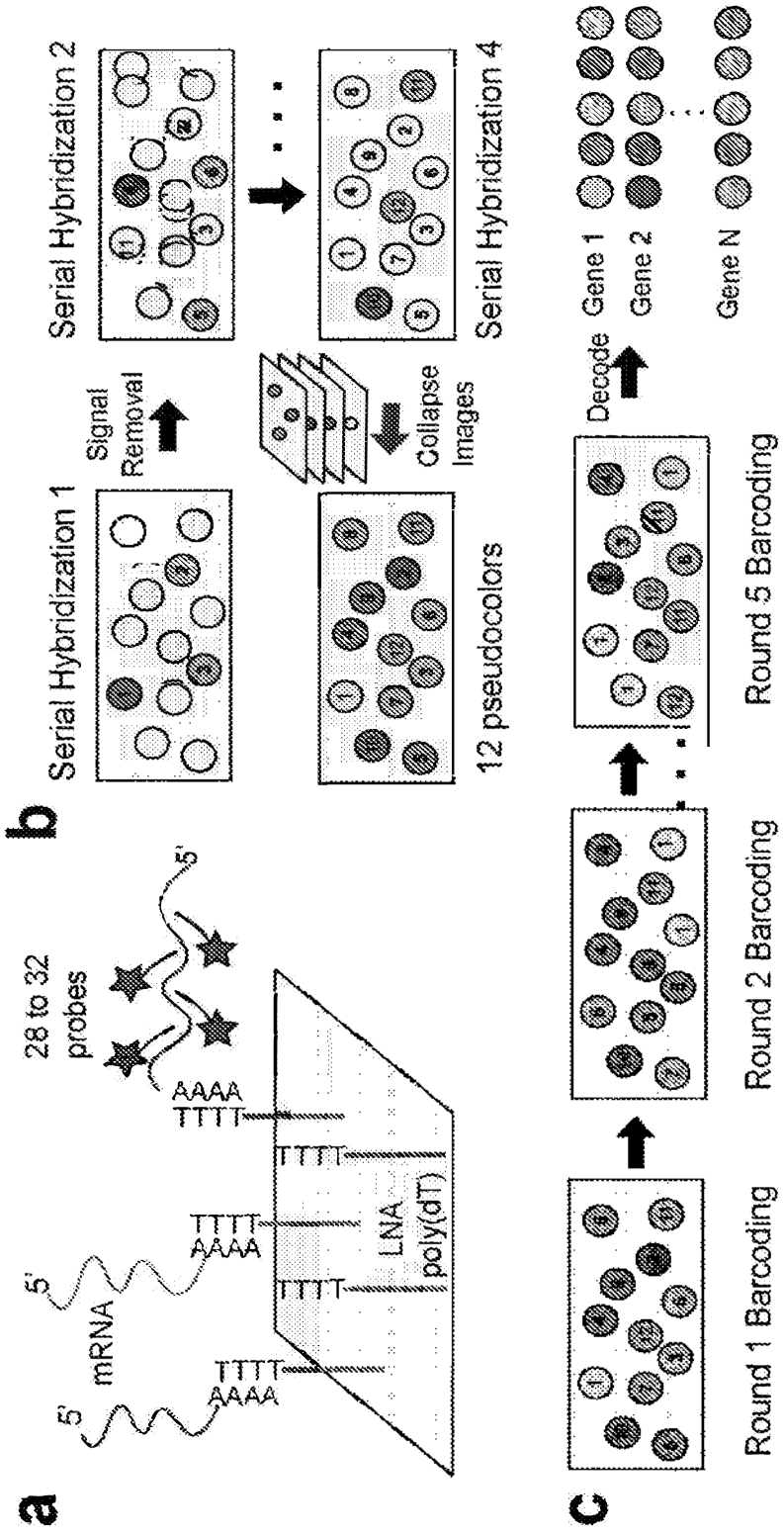


FIG. 4

NanoString EX2011.413

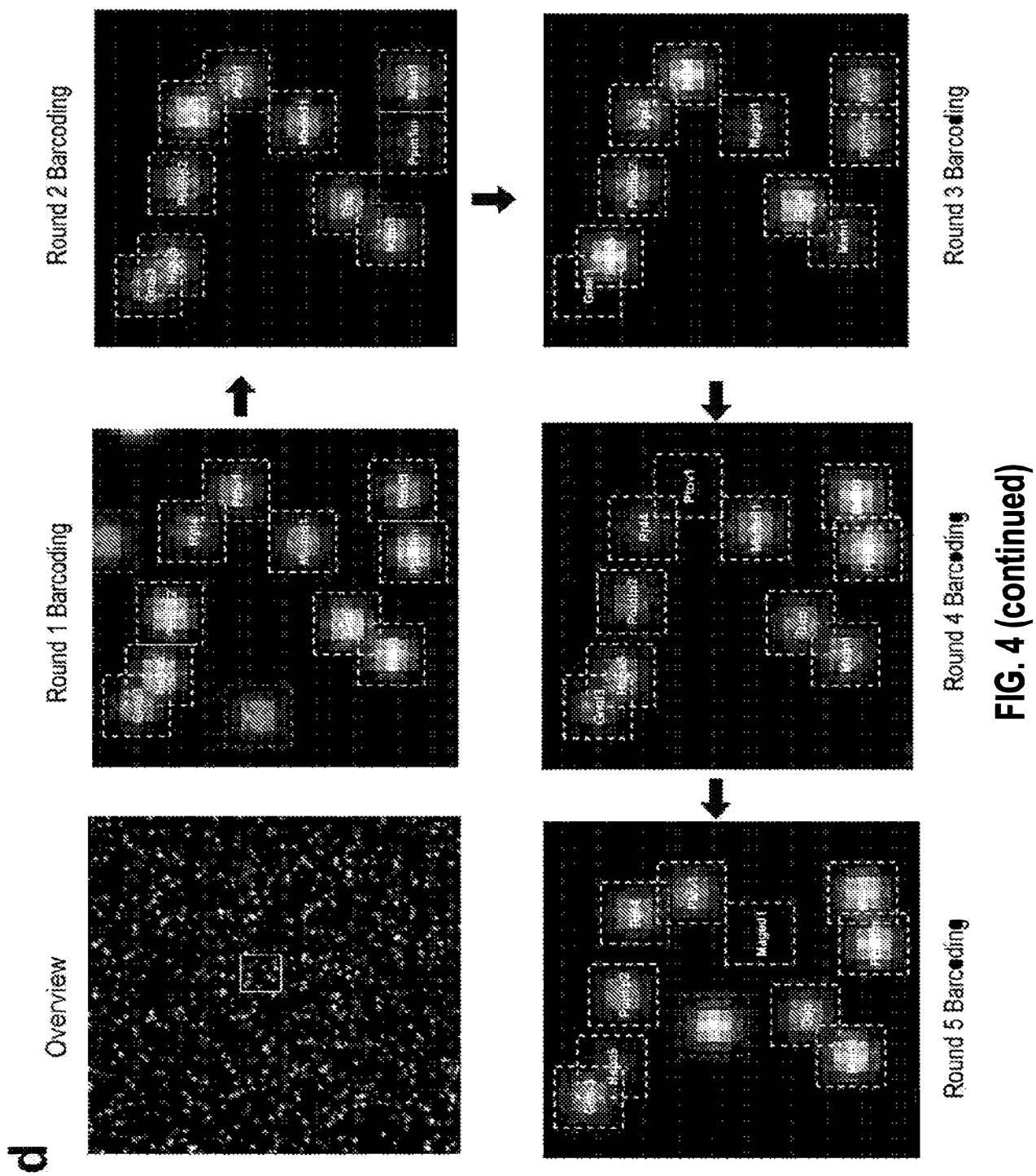


FIG. 4 (continued)

NanoString EX2011.414

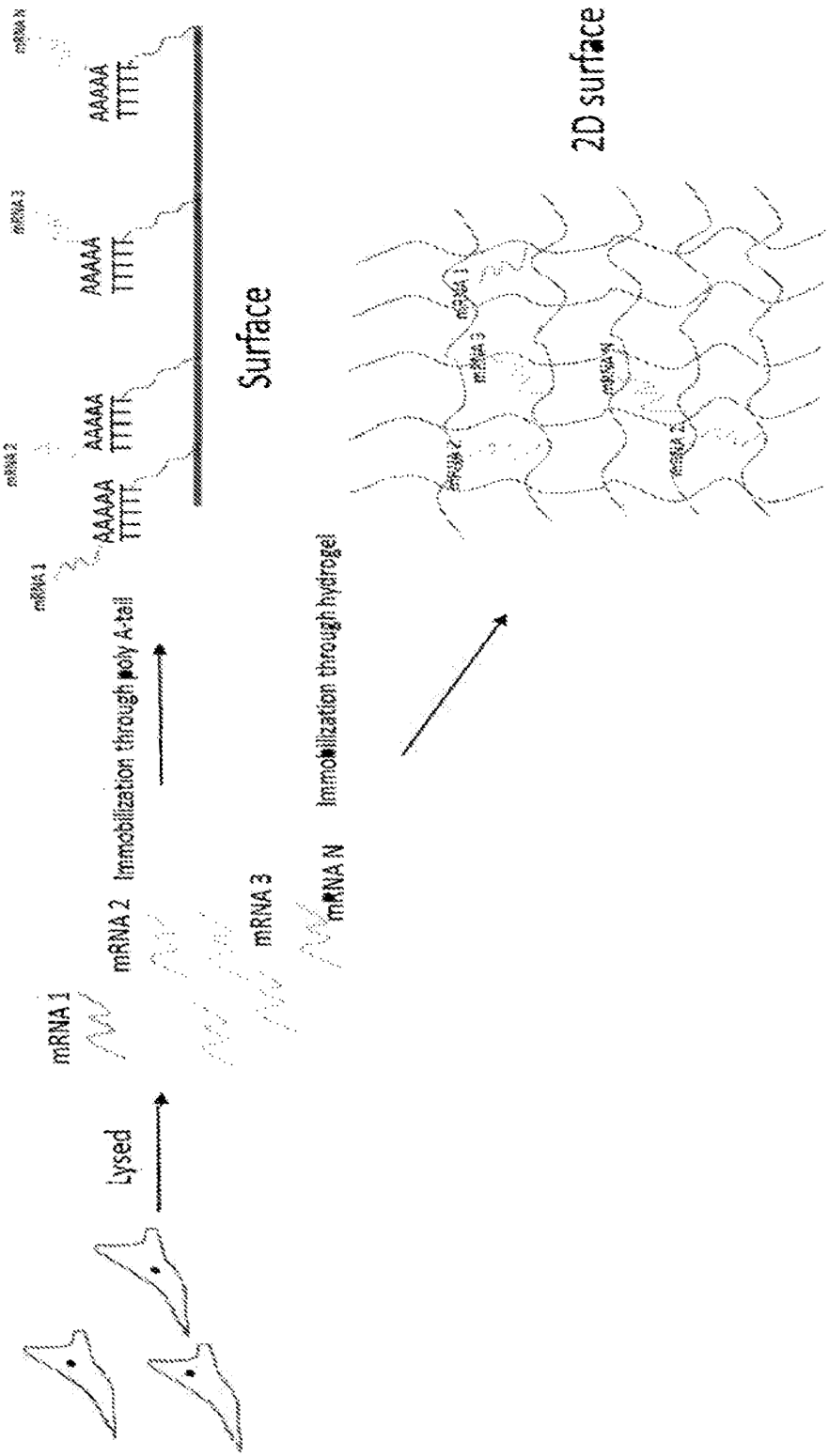


FIG. 5

NanoString EX2011.415

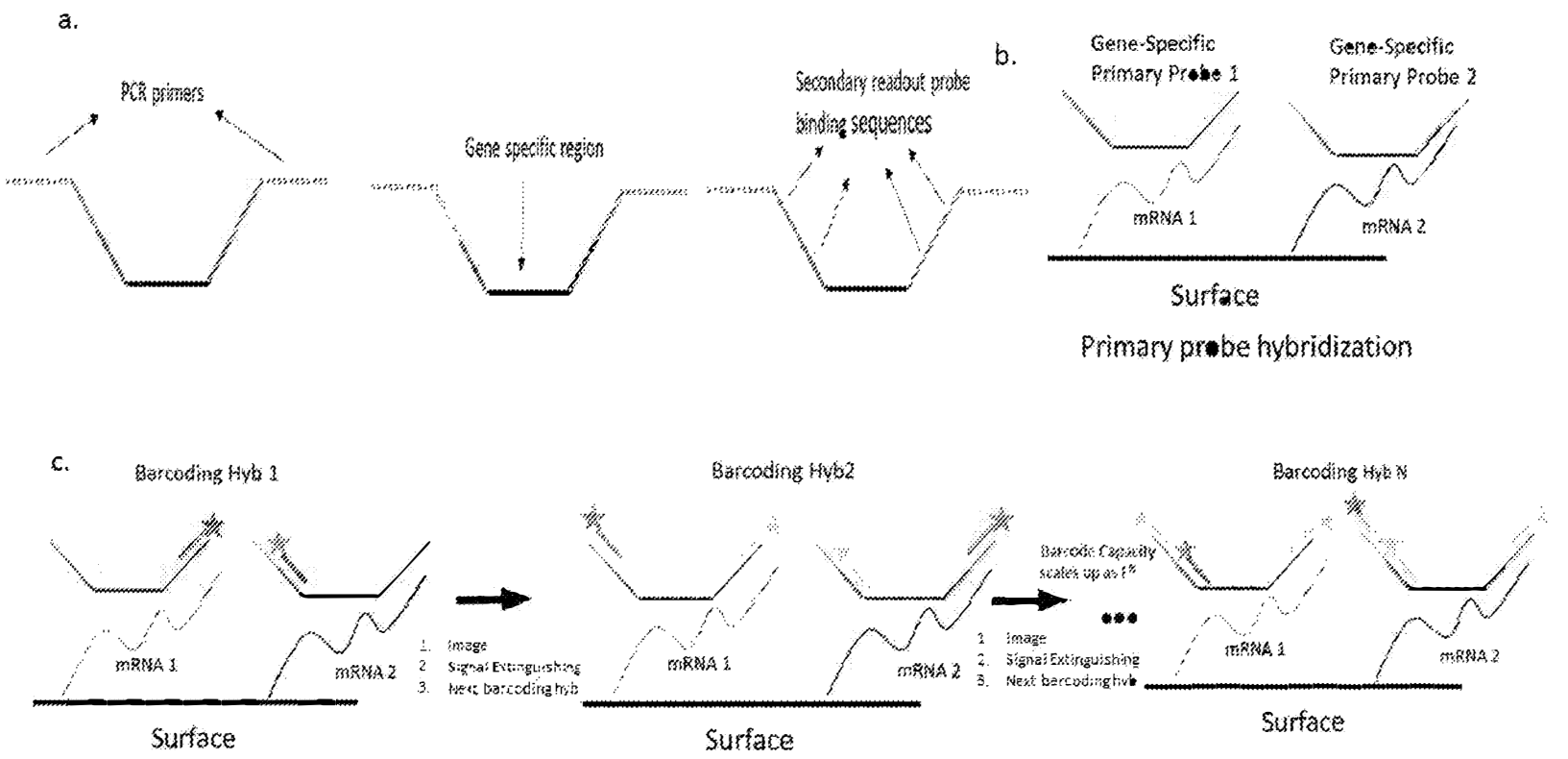


FIG. 6

NanoString EX2011.416

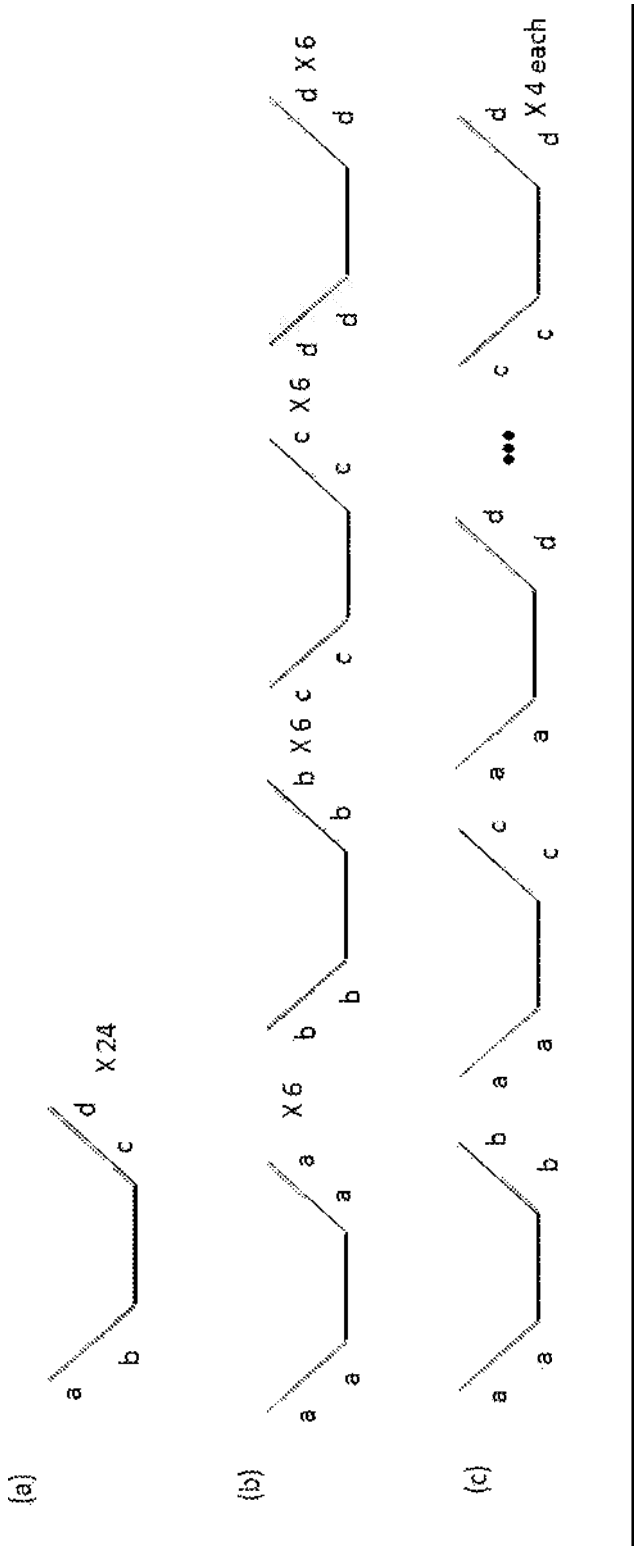


FIG. 7

NanoString EX2011.417

FIG. 8

NanoString EX2011.418

FIG. 9

NanoString EX2011.419

Spot (row)	Barcoding Hyb1	Barcoding Hyb2	Barcoding Hyb3	Barcoding Hyb4
1	Readout 1 (Red)	Readout 13 (Red)	Readout 25 (Red)	Readout 37 (Red)
	Readout 2 (Green)	Readout 14 (Green)	Readout 26 (Green)	Readout 38 (Green)
	Readout 3 (Blue)	Readout 15 (Blue)	Readout 27 (Blue)	Readout 39 (Blue)
2	Readout 4 (Red)	Readout 16 (Red)	Readout 28 (Red)	Readout 40 (Red)
	Readout 5 (Green)	Readout 17 (Green)	Readout 29 (Green)	Readout 41 (Green)
	Readout 6 (Blue)	Readout 18 (Blue)	Readout 30 (Blue)	Readout 42 (Blue)
3	Readout 7 (Red)	Readout 19 (Red)	Readout 31 (Red)	Readout 43 (Red)
	Readout 8 (Green)	Readout 20 (Green)	Readout 32 (Green)	Readout 44 (Green)
	Readout 9 (Blue)	Readout 21 (Blue)	Readout 33 (Blue)	Readout 45 (Blue)
4	Readout 10 (Red)	Readout 22 (Red)	Readout 34 (Red)	Readout 46 (Red)
	Readout 11 (Green)	Readout 23 (Green)	Readout 35 (Green)	Readout 47 (Green)
	Readout 12 (Blue)	Readout 24 (Blue)	Readout 36 (Blue)	Readout 48 (Blue)

FIG. 10

NanoString EX2011.421

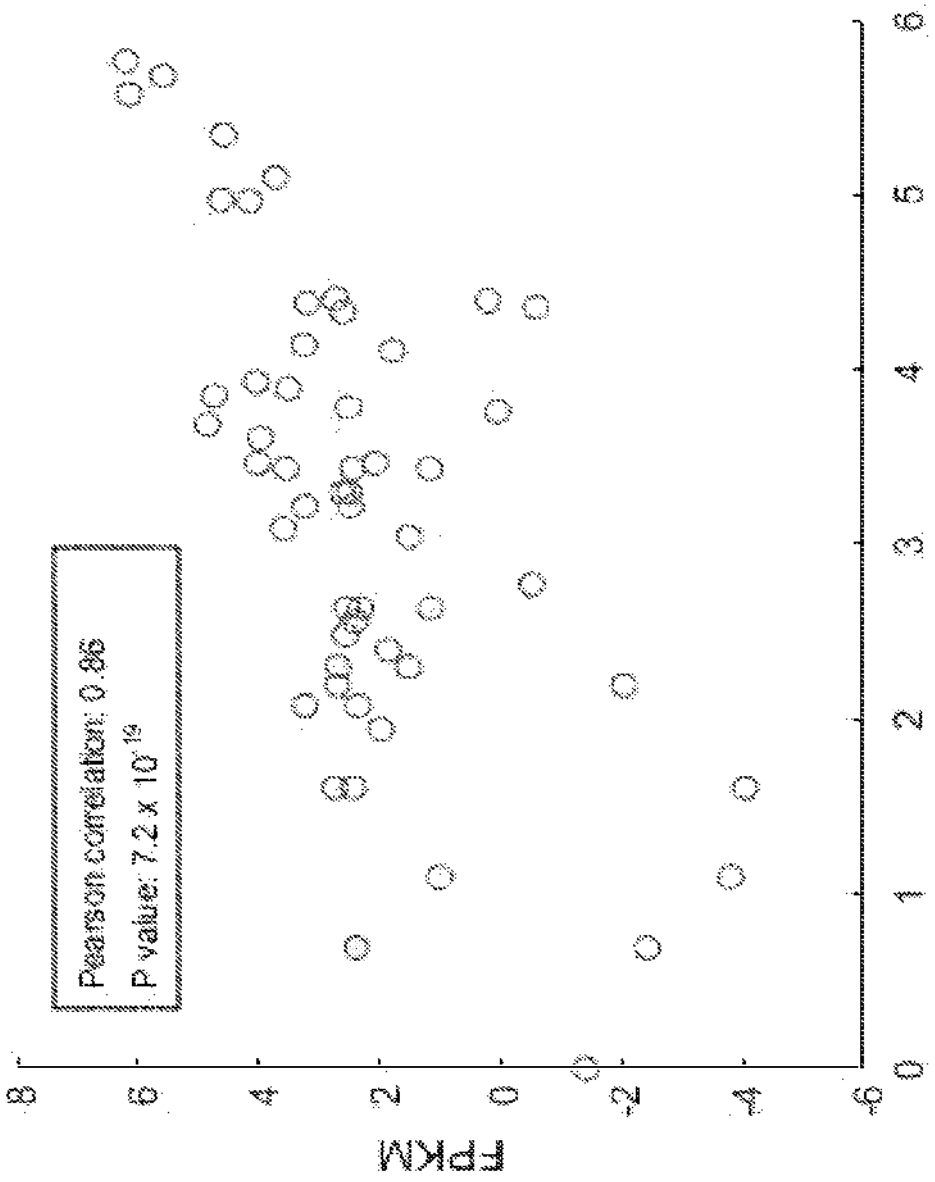


FIG. 11

NanoString EX2011.422

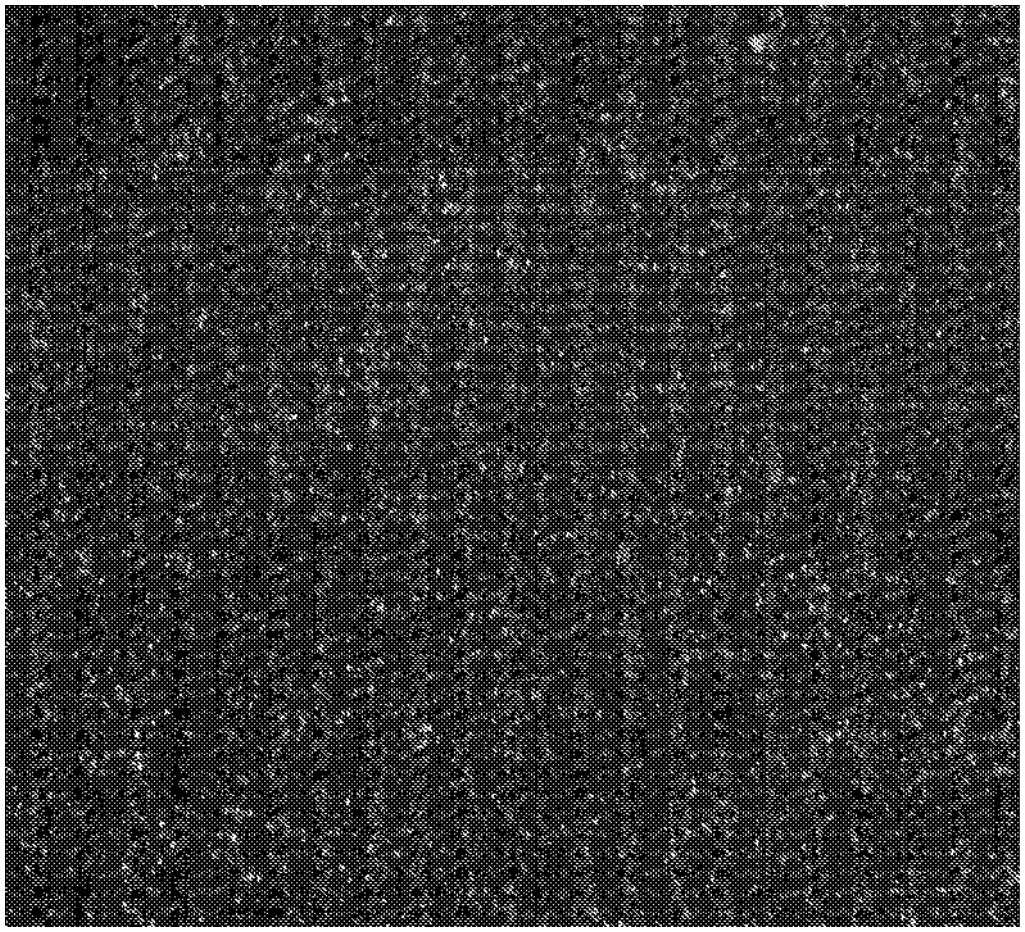


FIG. 12

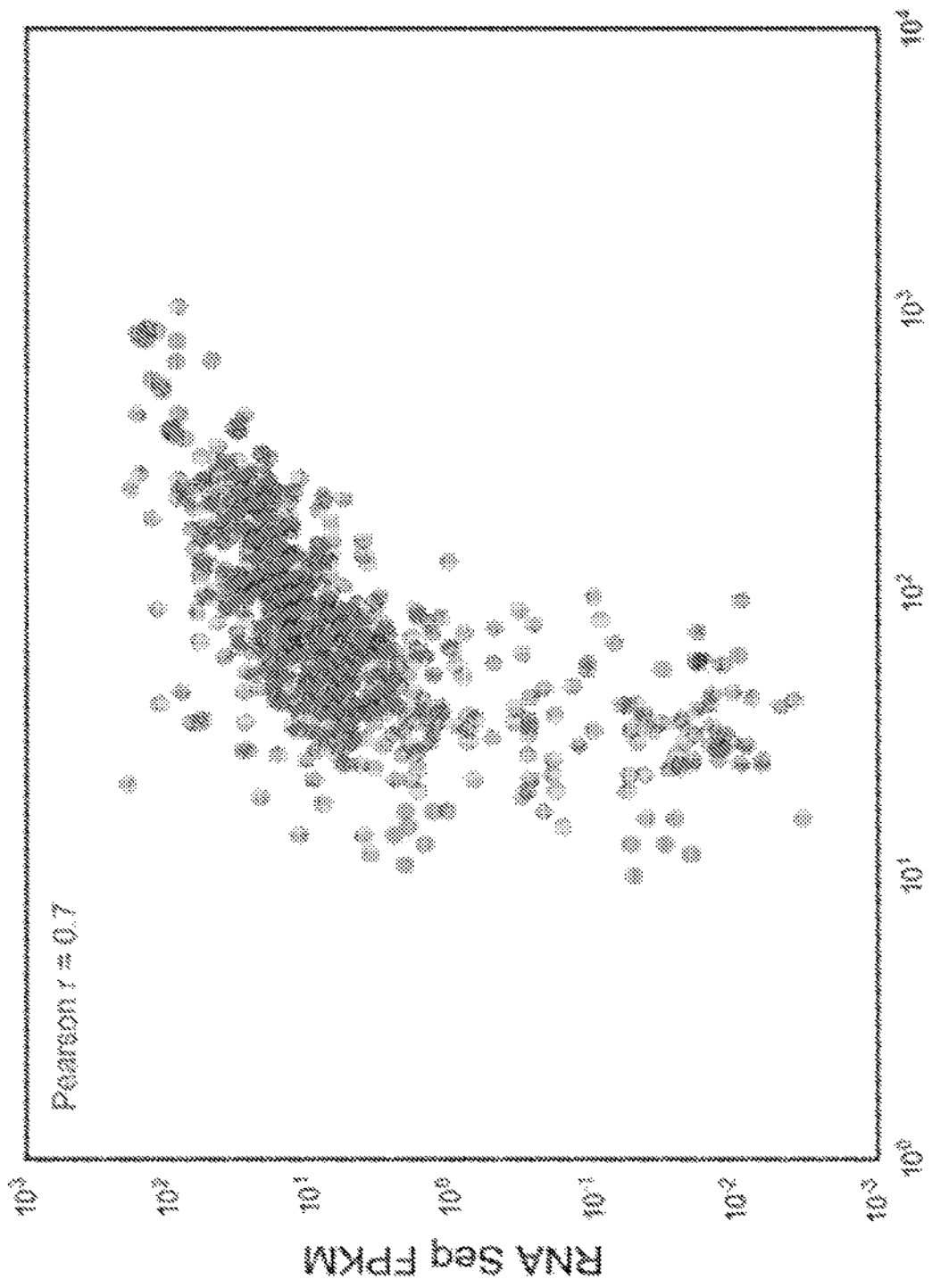


FIG. 13

NanoString EX2011.423

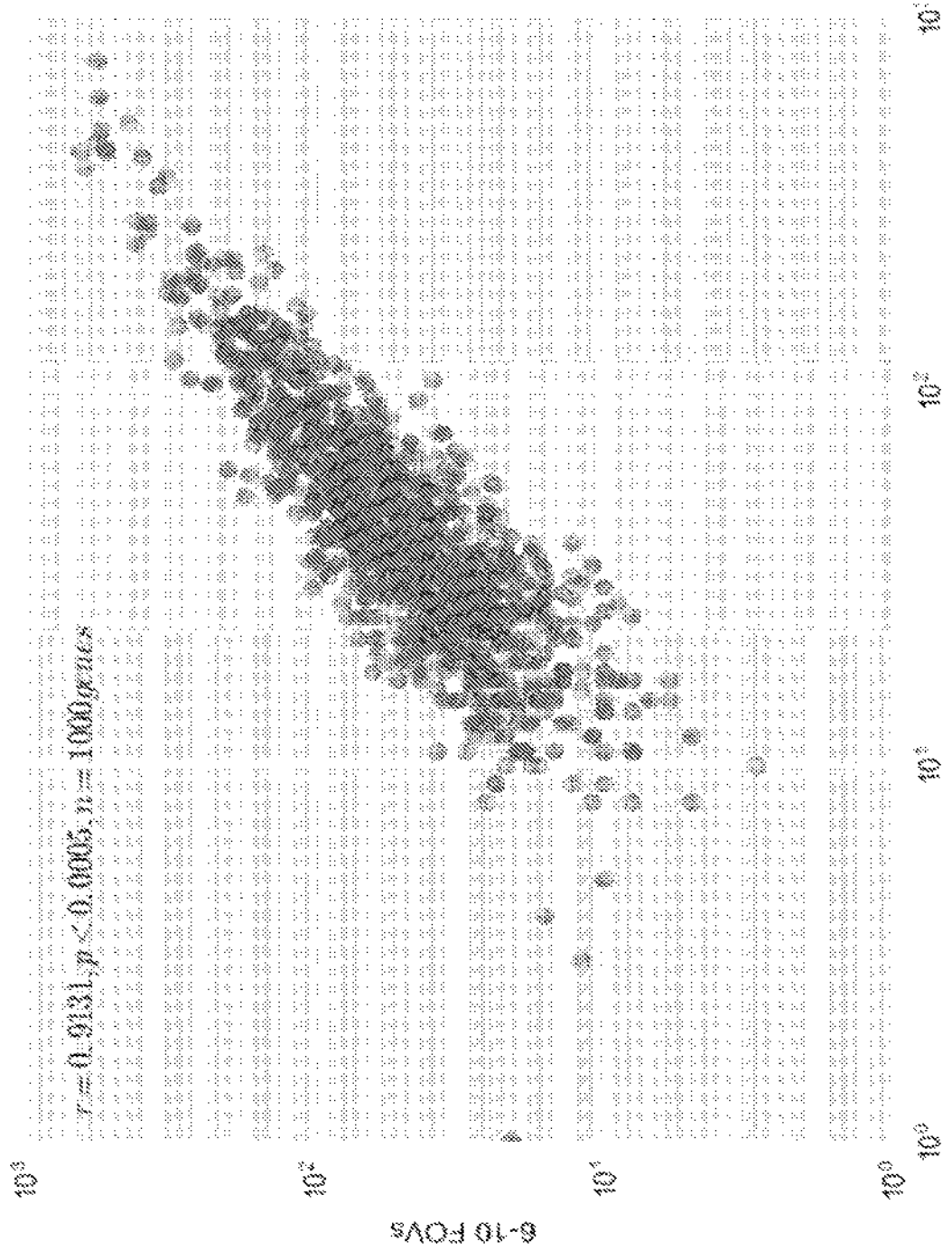


FIG. 14

NanoString EX2011.424

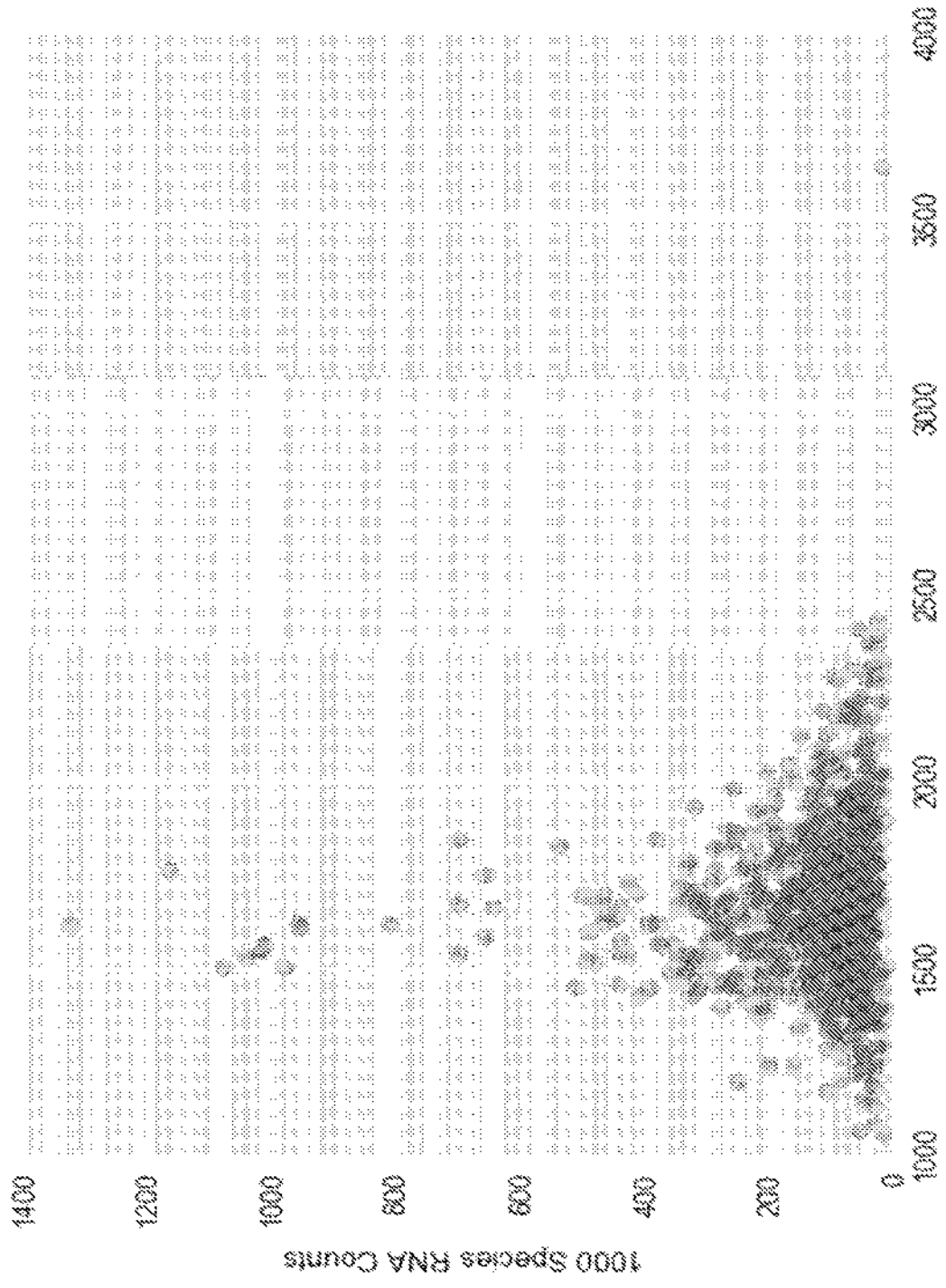


FIG. 15

NanoString EX2011.425

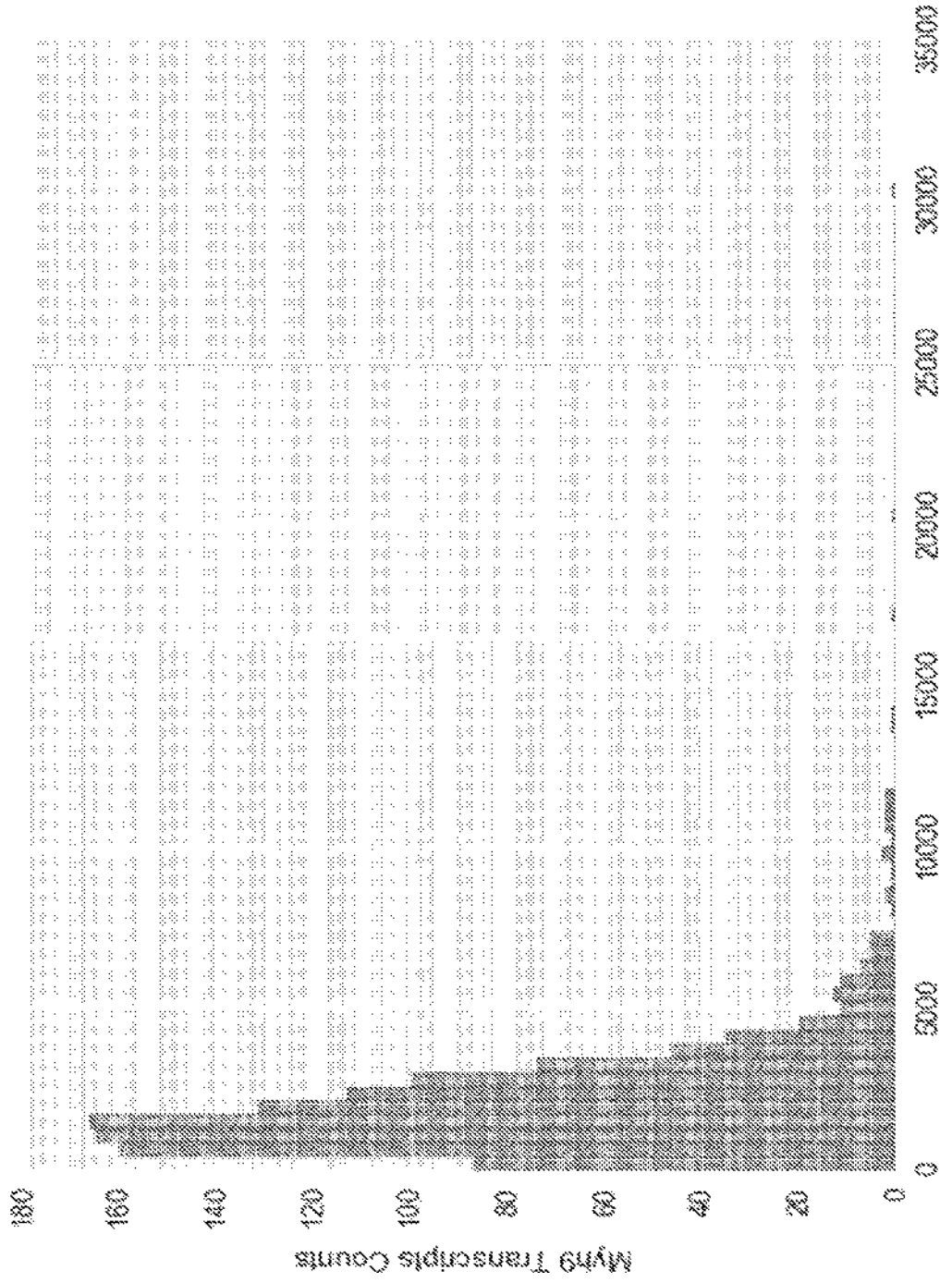


FIG. 16

NanoString EX2011.426

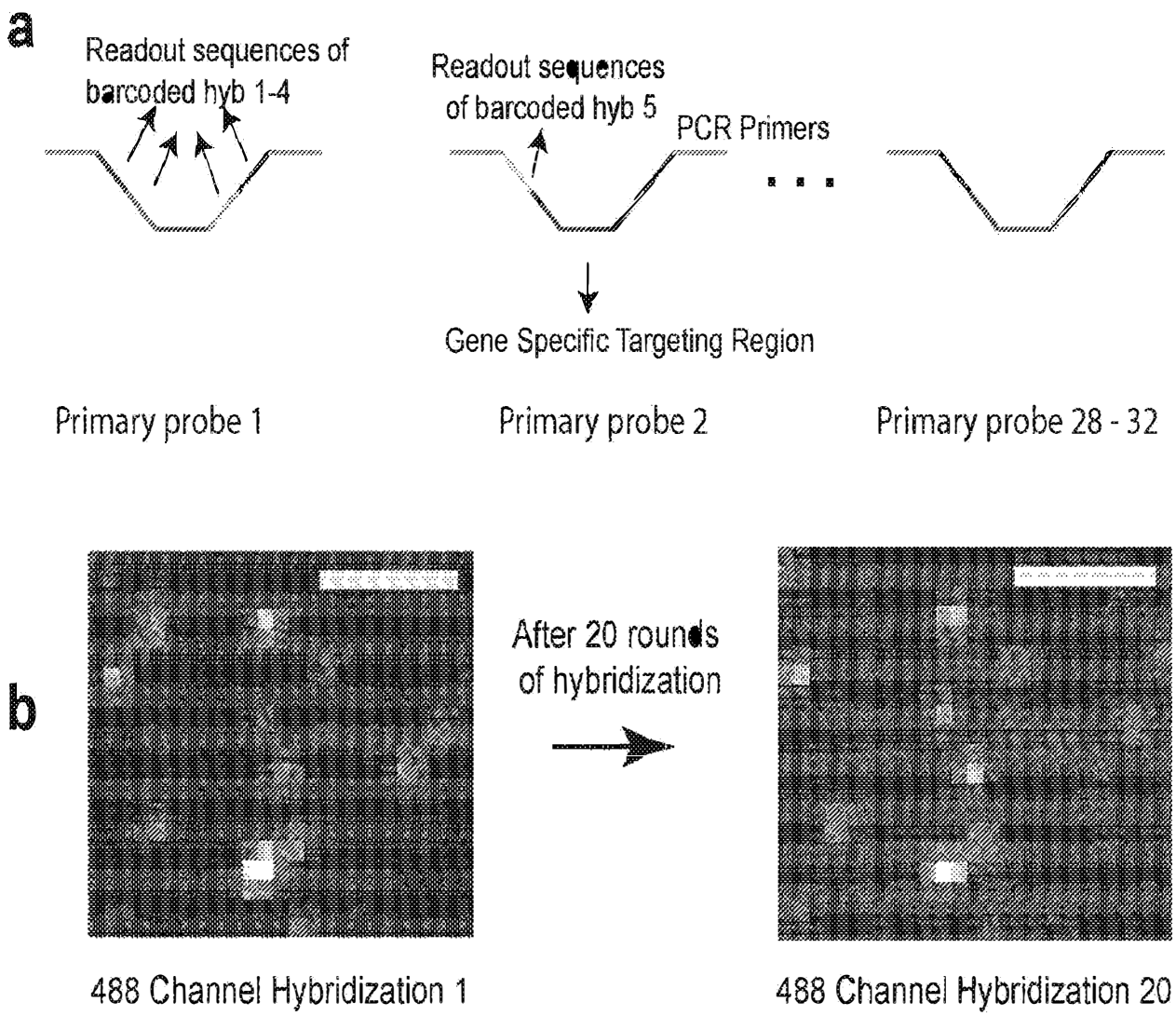

a
Readout sequences of barcoded hyb 1-4
Readout sequences of barcoded hyb 5
PCR Primers
Gene Specific Targeting Region
Primary probe 1
Primary probe 2
Primary probe 28 - 32
b
After 20 rounds of hybridization
488 Channel Hybridization 1
488 Channel Hybridization 20

FIG. 17


a
readout probe
TCEP
SH
registration marker
Round 1 barcoding hyb
Round 2 barcoding hyb
Round 5 barcoding hyb

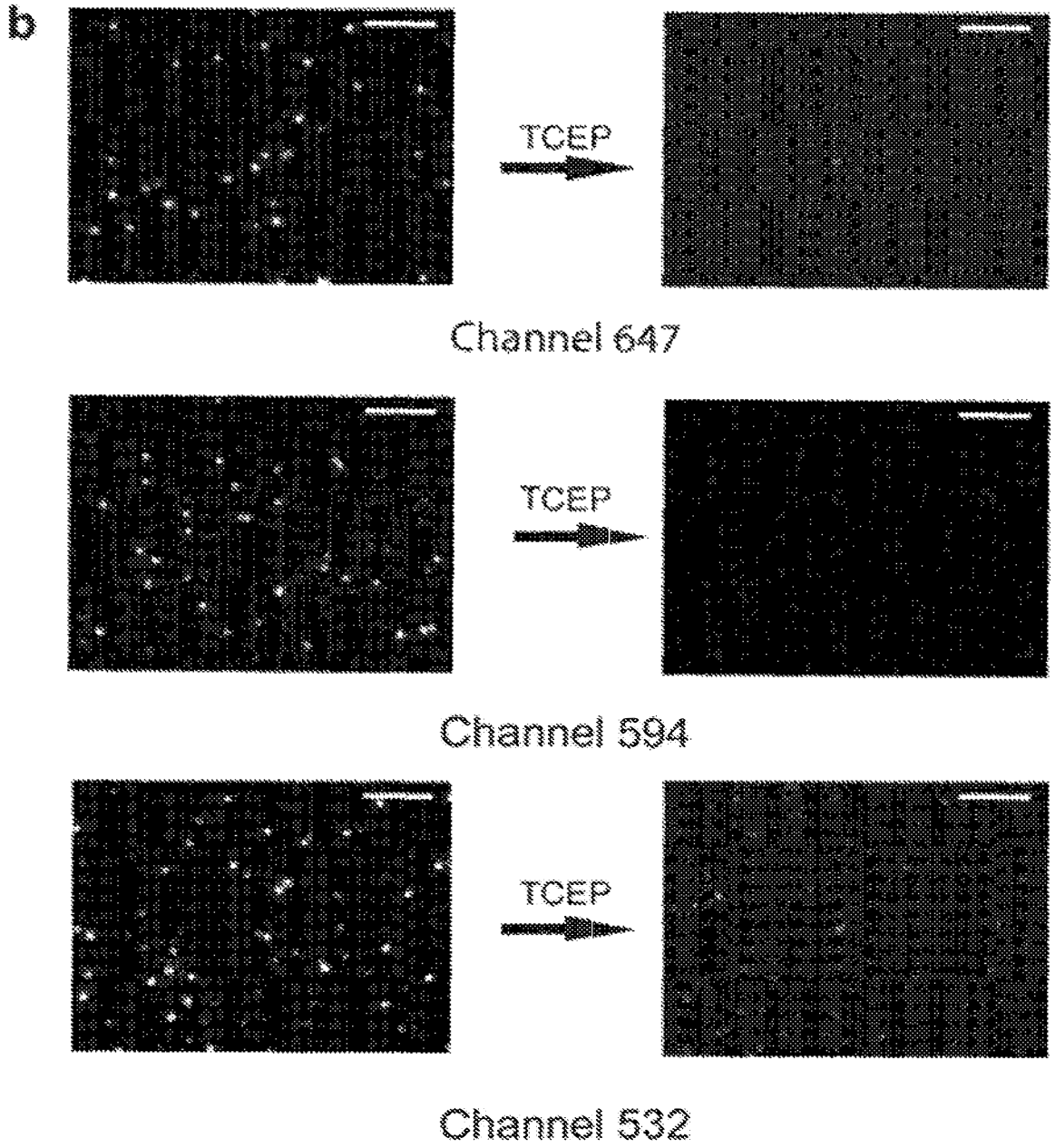

b
TCEP
Channel 647
TCEP
Channel 594
TCEP
Channel 532

FIG. 18

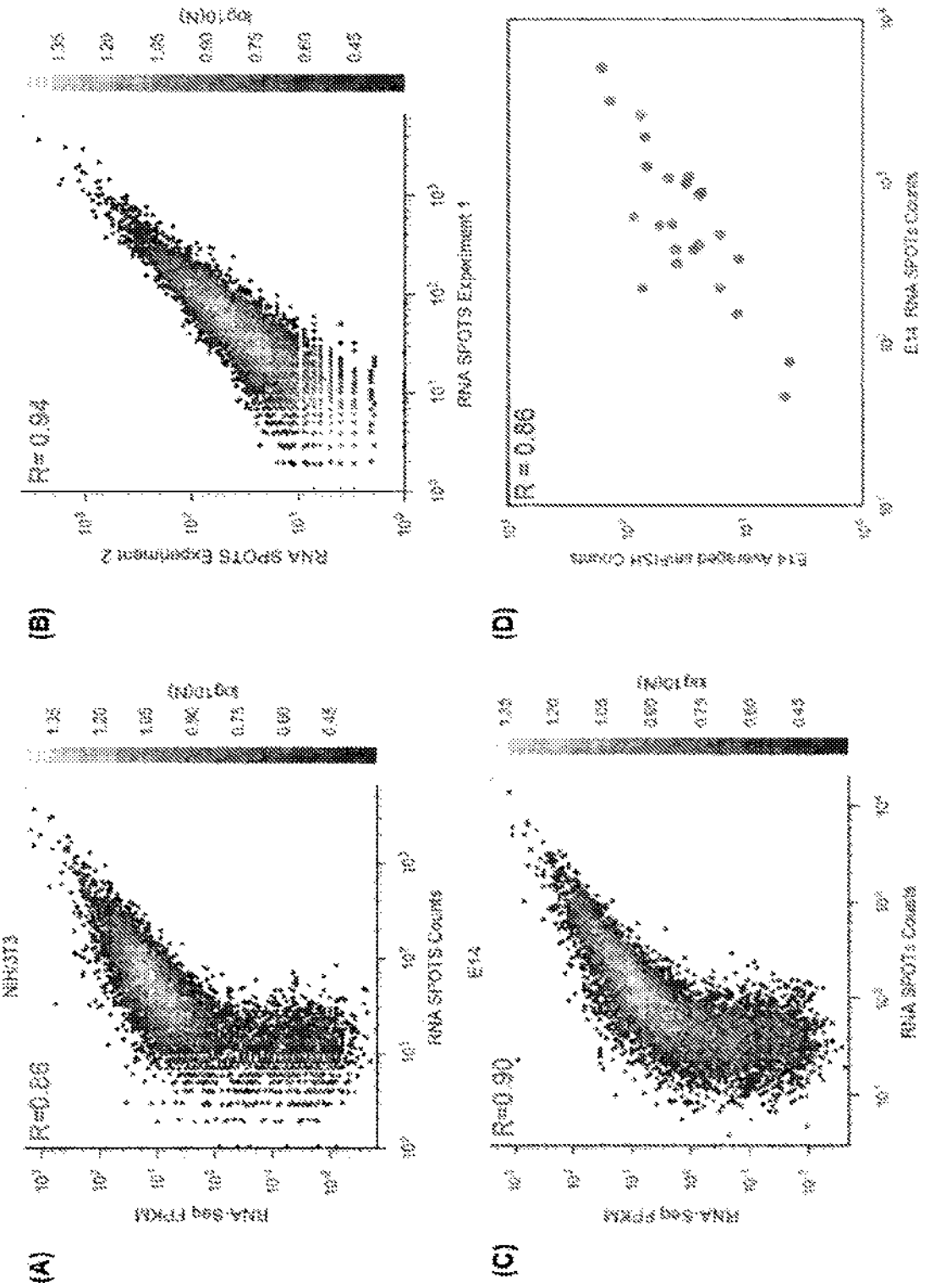


FIG. 19

NanoString EX2011.429

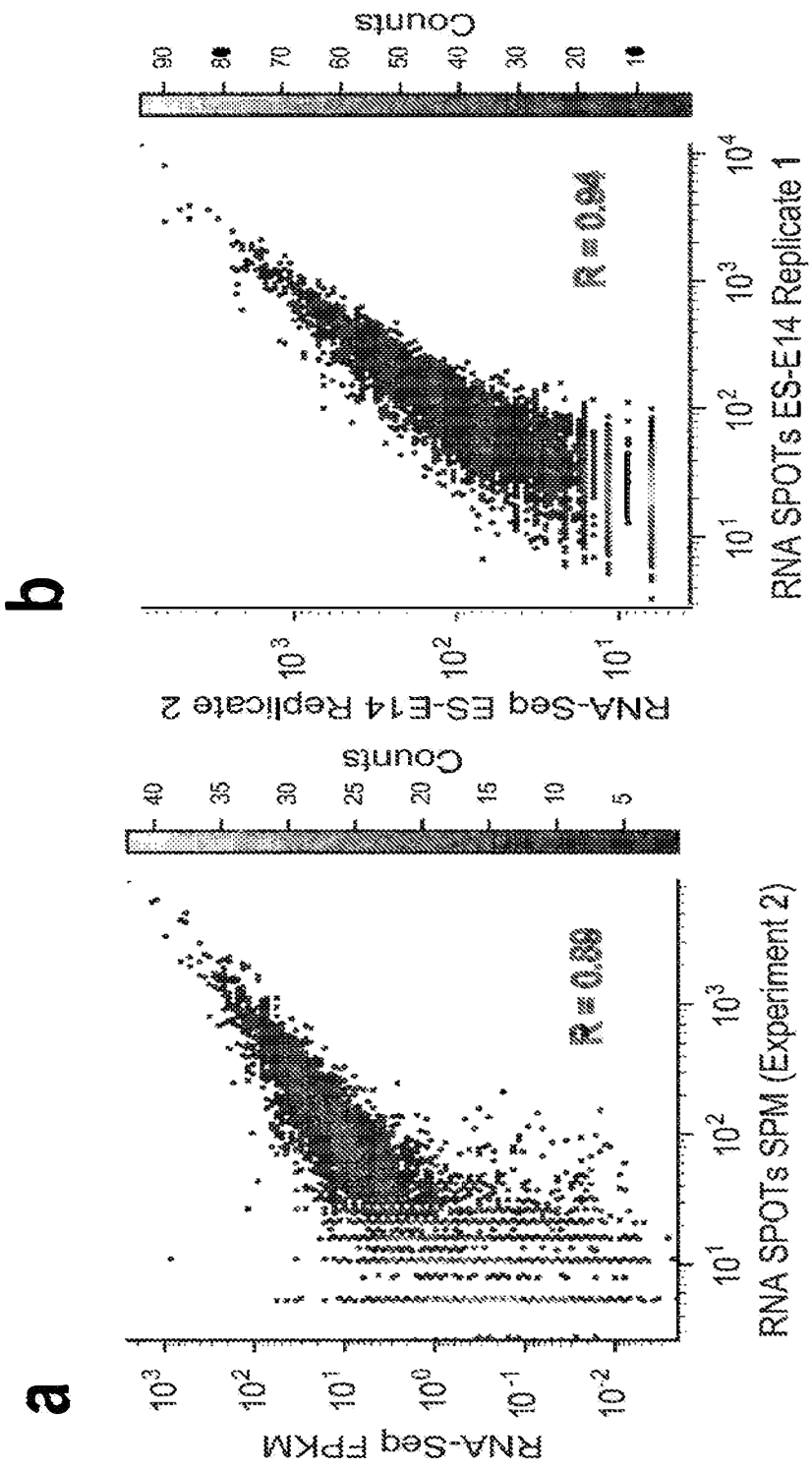


FIG. 20

NanoString EX2011.430

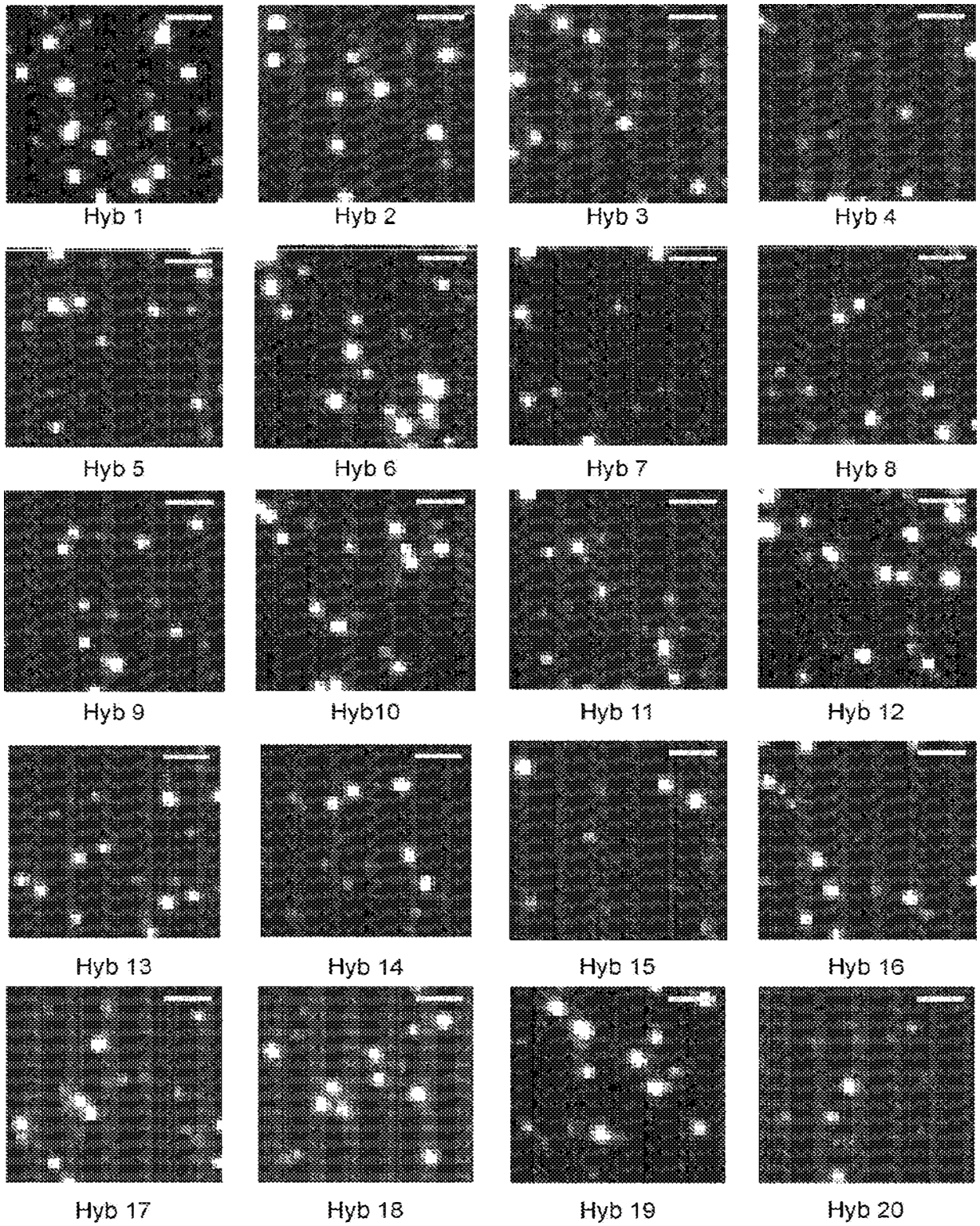

Hyb 1
Hyb 2
Hyb 3
Hyb 4
Hyb 5
Hyb 6
Hyb 7
Hyb 8
Hyb 9
Hyb10
Hyb 11
Hyb 12
Hyb 13
Hyb 14
Hyb 15
Hyb 16
Hyb 17
Hyb 18
Hyb 19
Hyb 20

FIG. 21

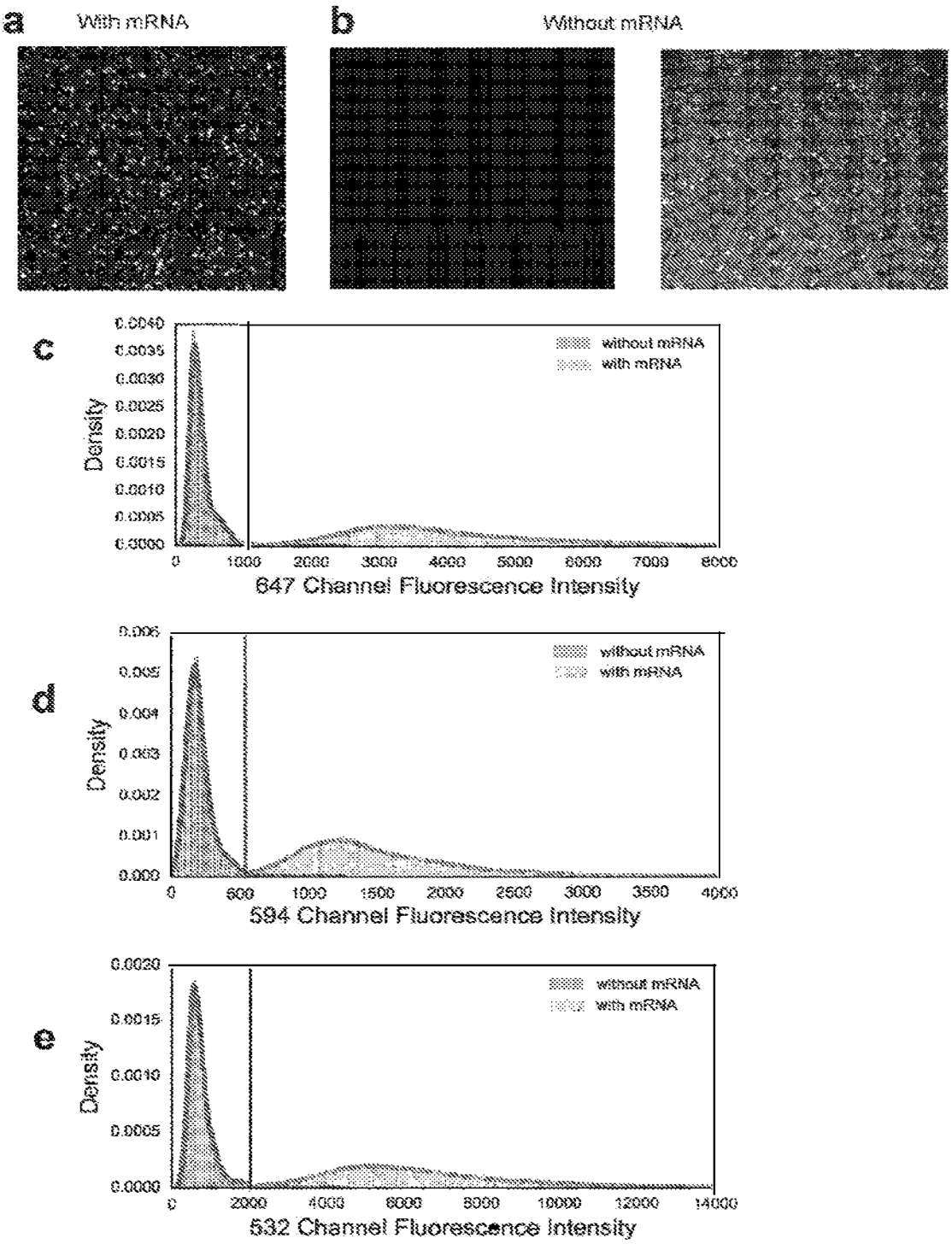

a
With mRNA
b
Without mRNA
c
Density
without mRNA
with mRNA
647 Channel Fluorescence Intensity
d
Density
without mRNA
with mRNA
594 Channel Fluorescence Intensity
e
Density
without mRNA
with mRNA
532 Channel Fluorescence Intensity

FIG. 22

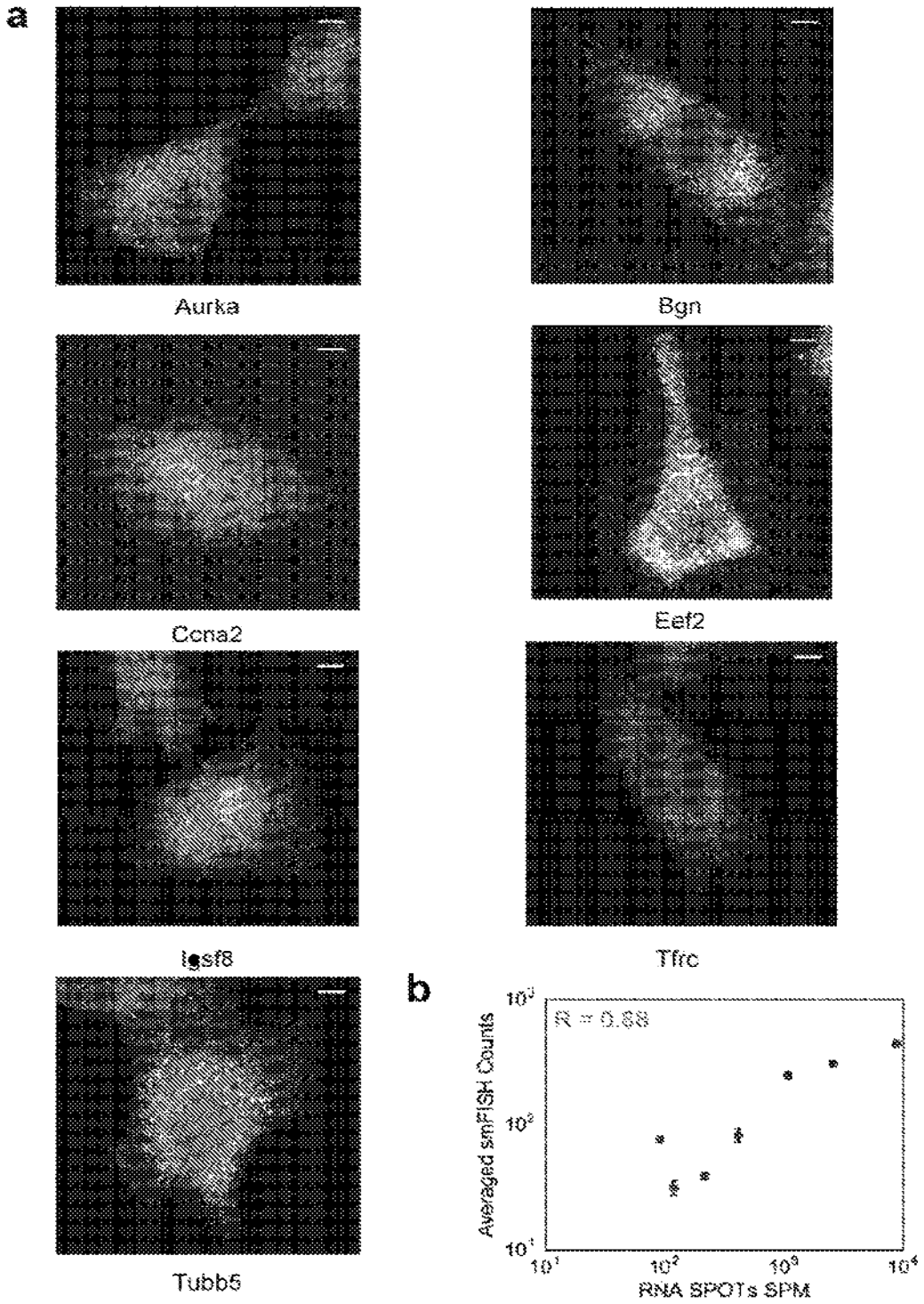
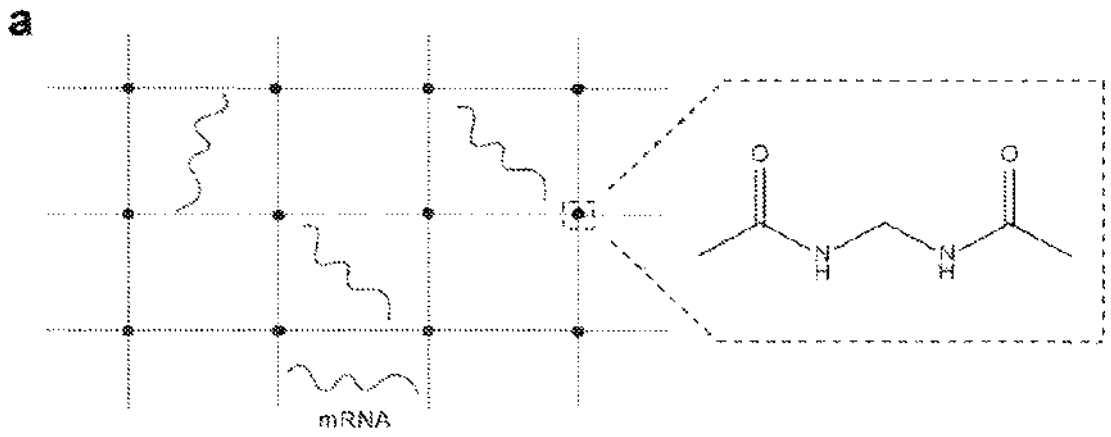

a
Aurka
Bgn
Ccna2
Eef2
Igsf8
Tfrc
Tubb5
b
R = 0.88
Averaged smFISH Counts
RNA SPOTs SPM

FIG. 23


a
mRNA

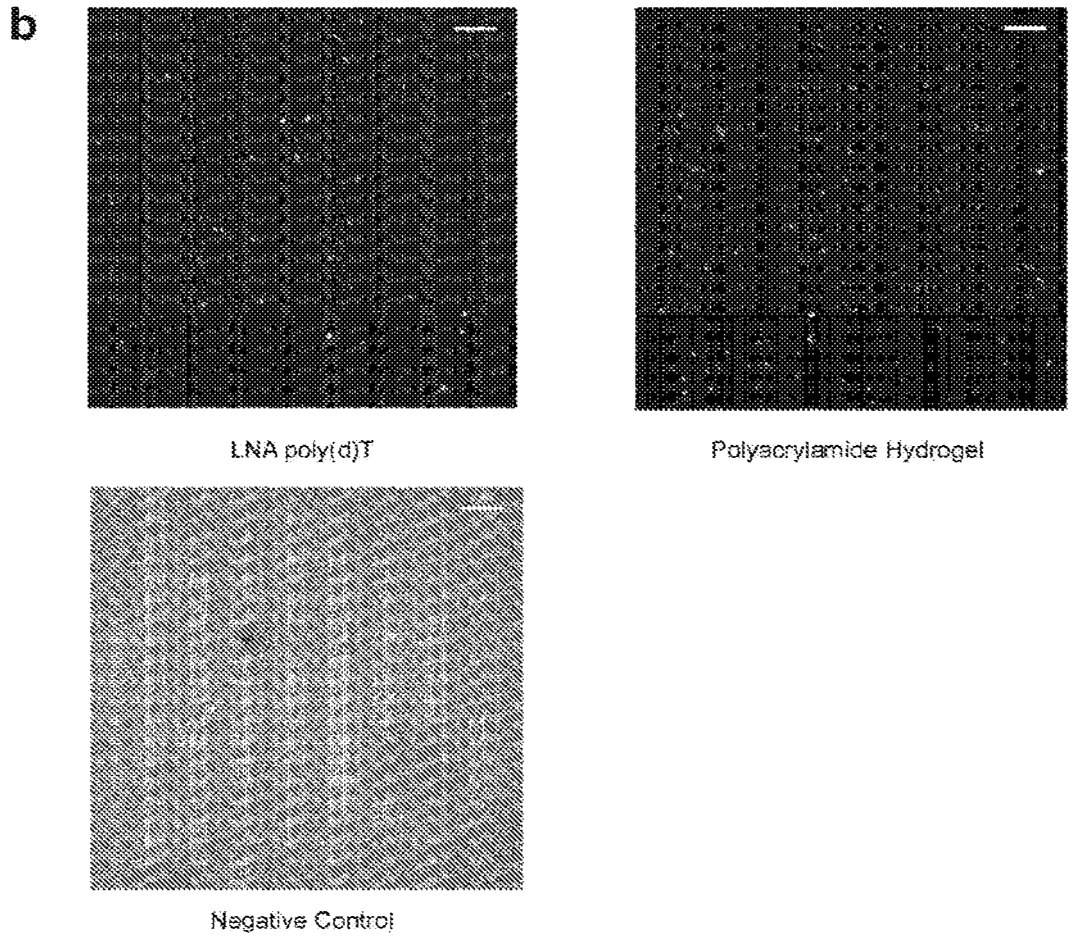

b
LNA poly(d)T
Polyacrylamide Hydrogel
Negative Control

FIG. 24

FIG. 25

NanoString EX2011.435

FIG. 26

NanoString EX2011.436

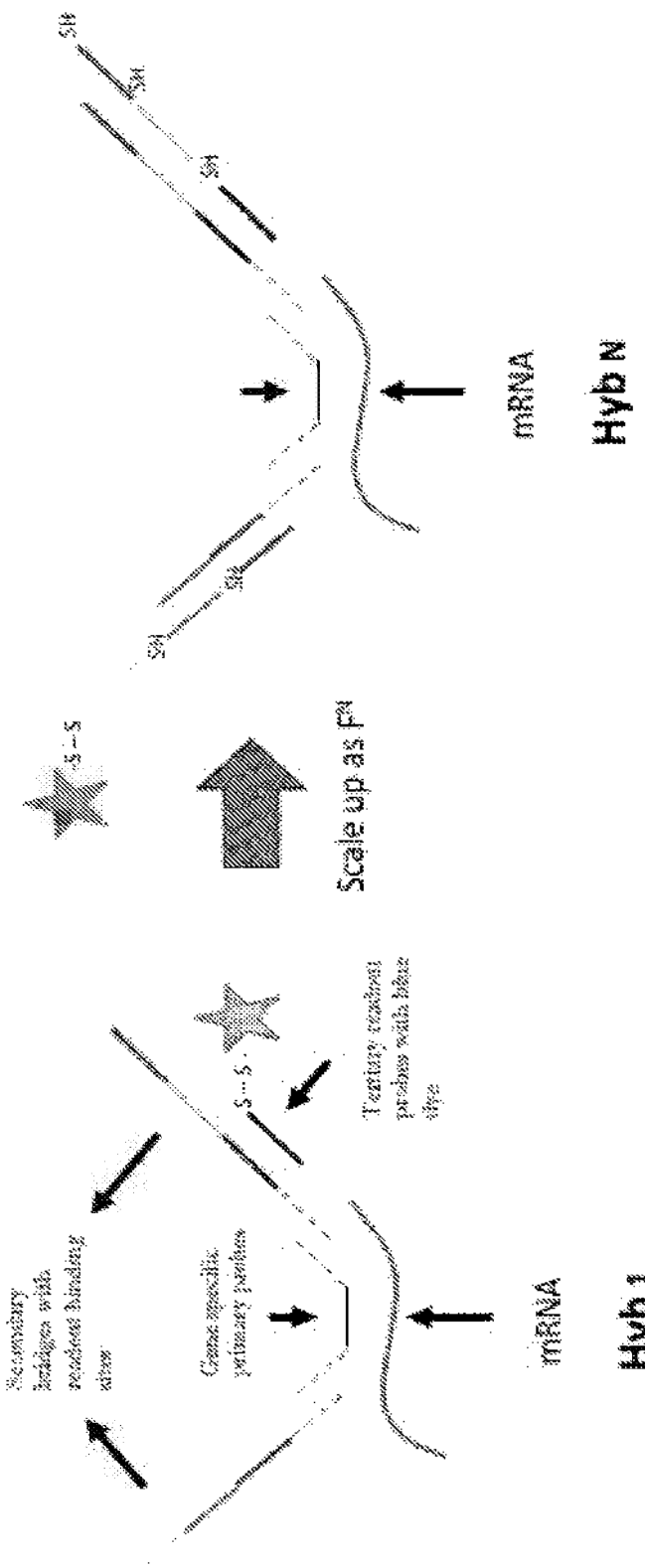


FIG. 26 (continued)

NanoString EX2011.437

FIG. 27A (Prior Art)

One implementation of cleavable amplification scheme

FIG. 27B

NanoString EX2011.439

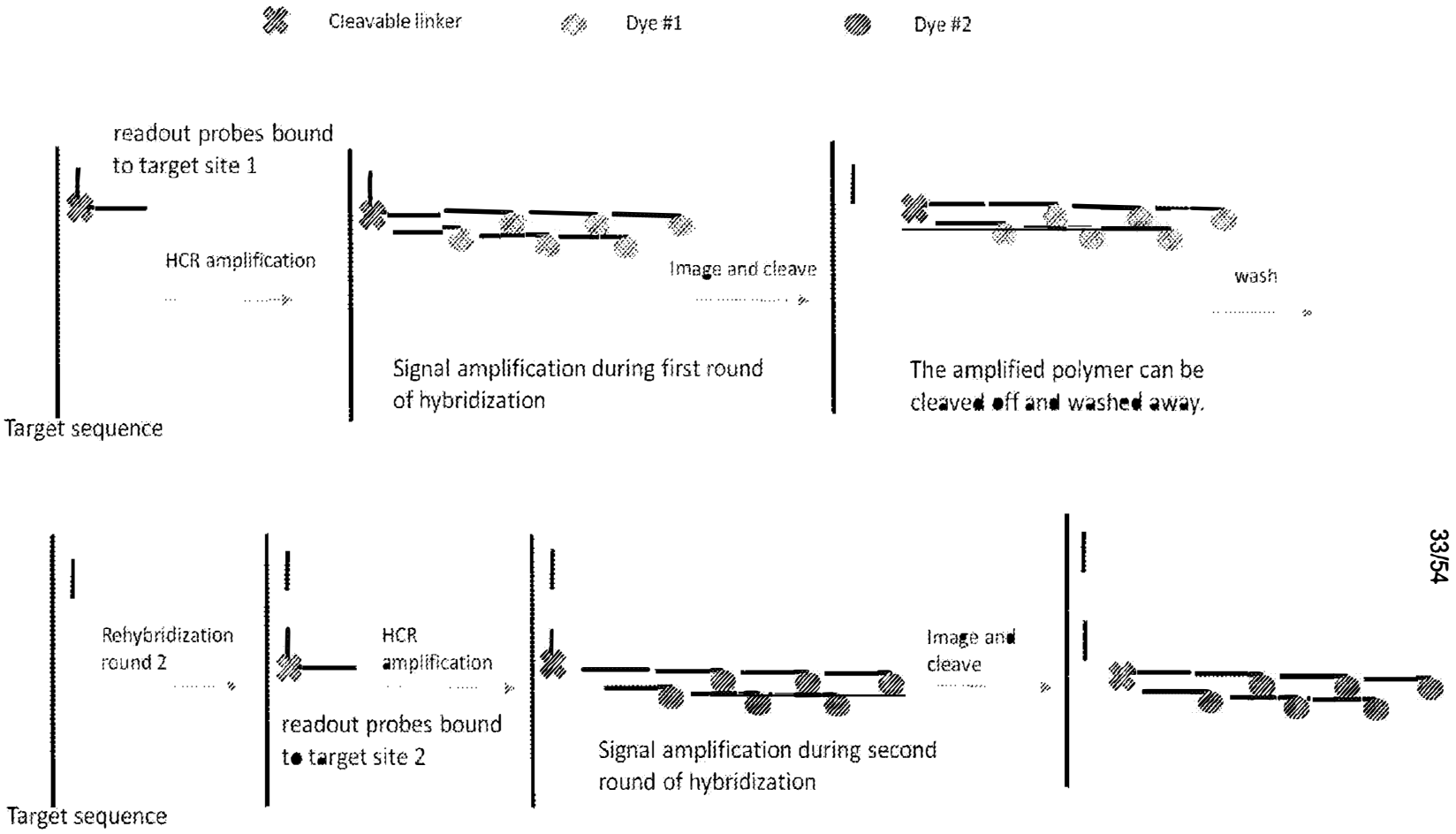


FIG. 27C

NanoString EX2011.440

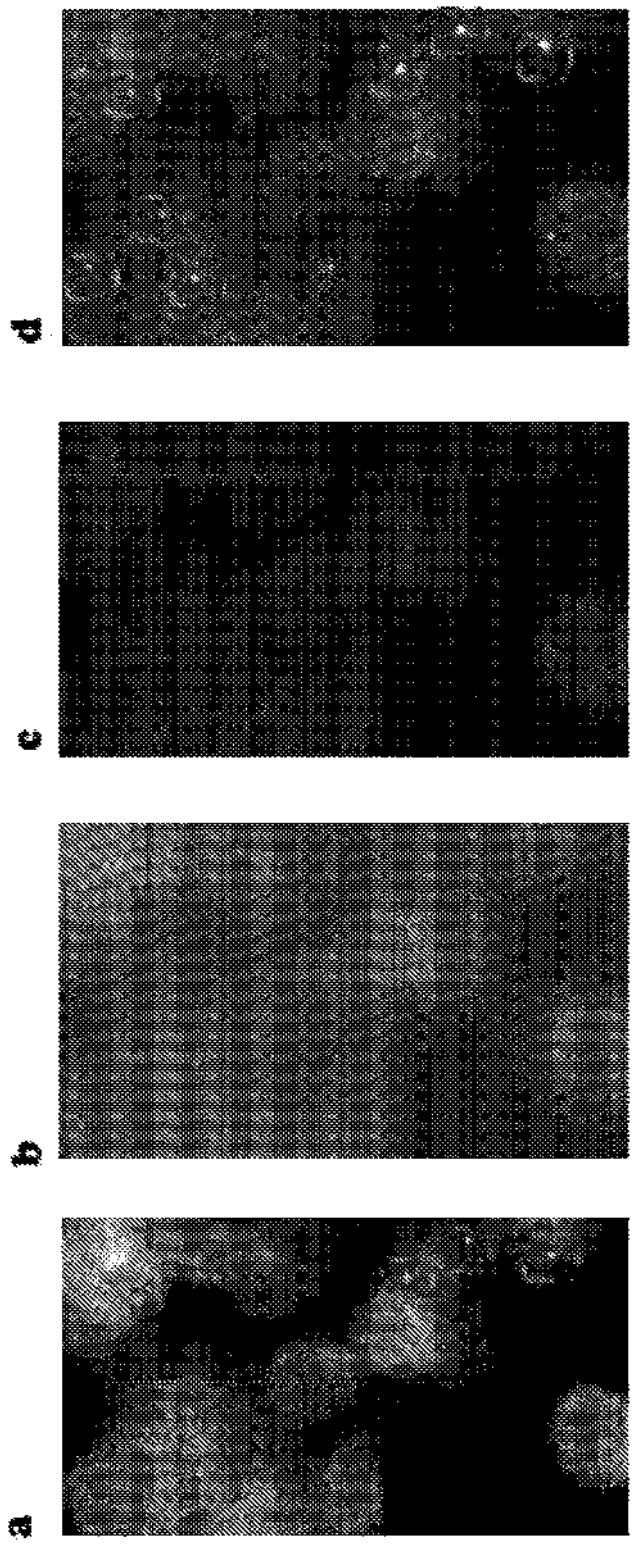


FIG. 28

NanoString EX2011.441

NanoString EX2011.442

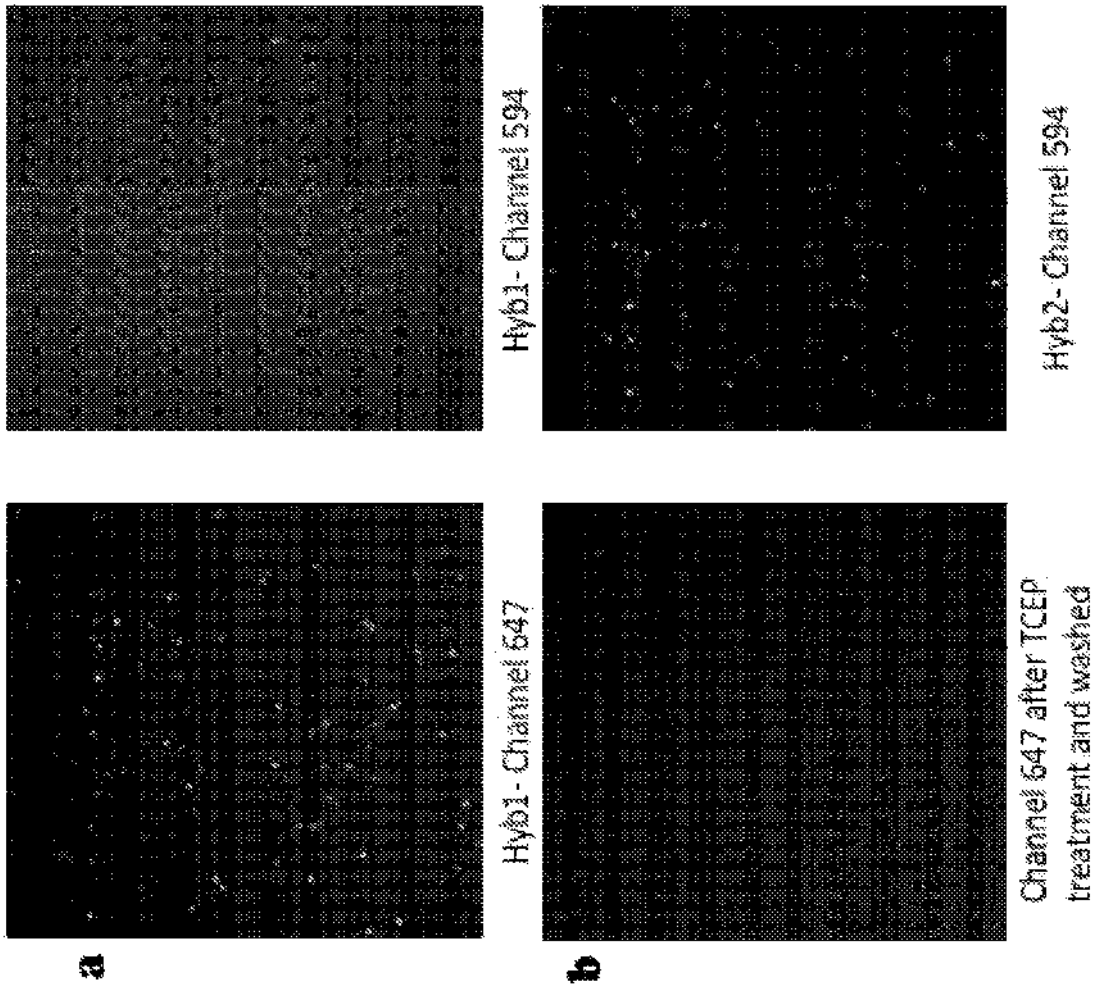


FIG. 29

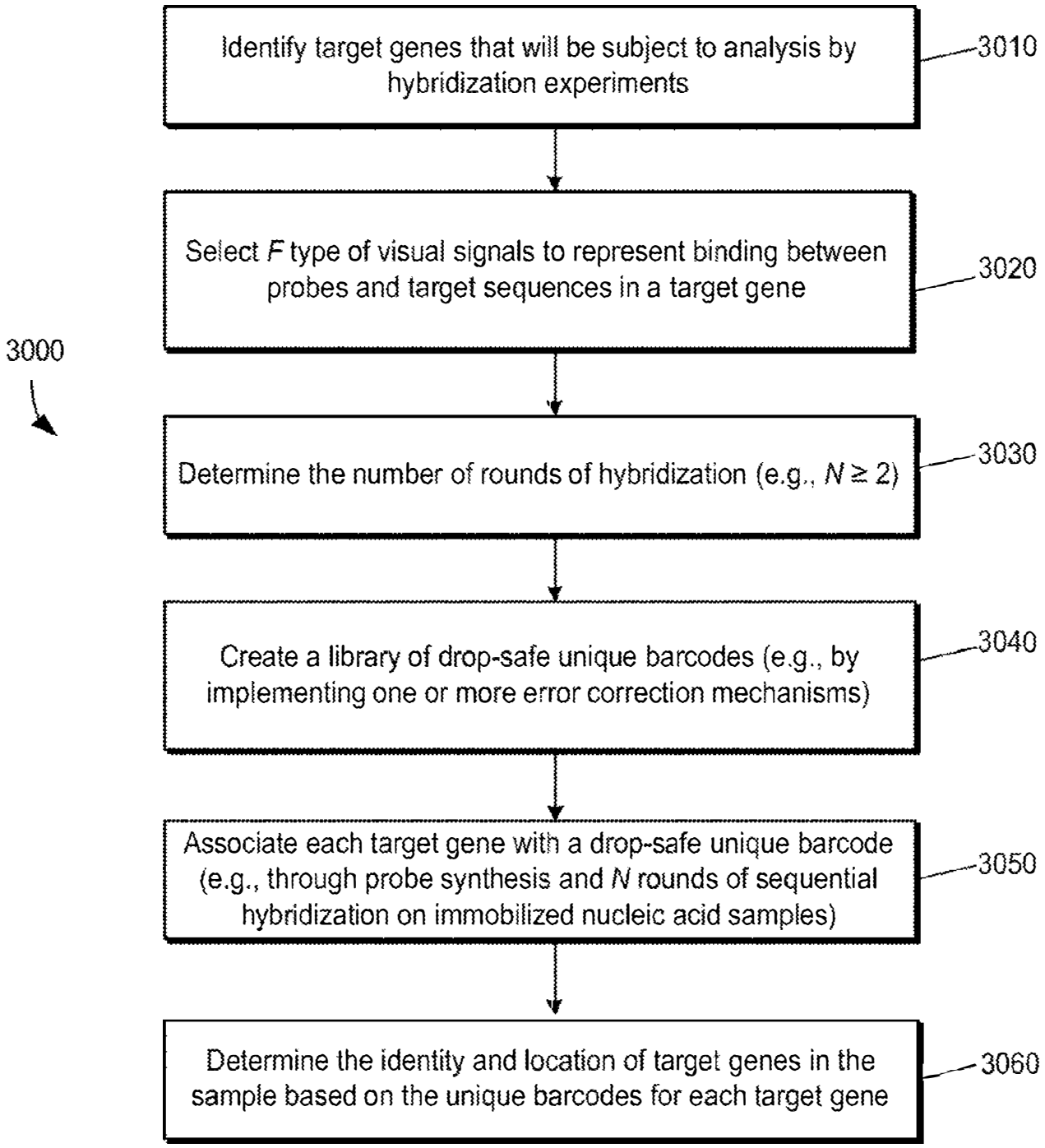

3000
Identify target genes that will be subject to analysis by hybridization experiments
3010
Select F type of visual signals to represent binding between probes and target sequences in a target gene
3020
Determine the number of rounds of hybridization (e.g., N ≥ 2)
3030
Create a library of drop-safe unique barcodes (e.g., by implementing one or more error correction mechanisms)
3040
Associate each target gene with a drop-safe unique barcode (e.g., through probe synthesis and N rounds of sequential hybridization on immobilized nucleic acid samples)
3050
Determine the identity and location of target genes in the sample based on the unique barcodes for each target gene
3060

FIG. 30

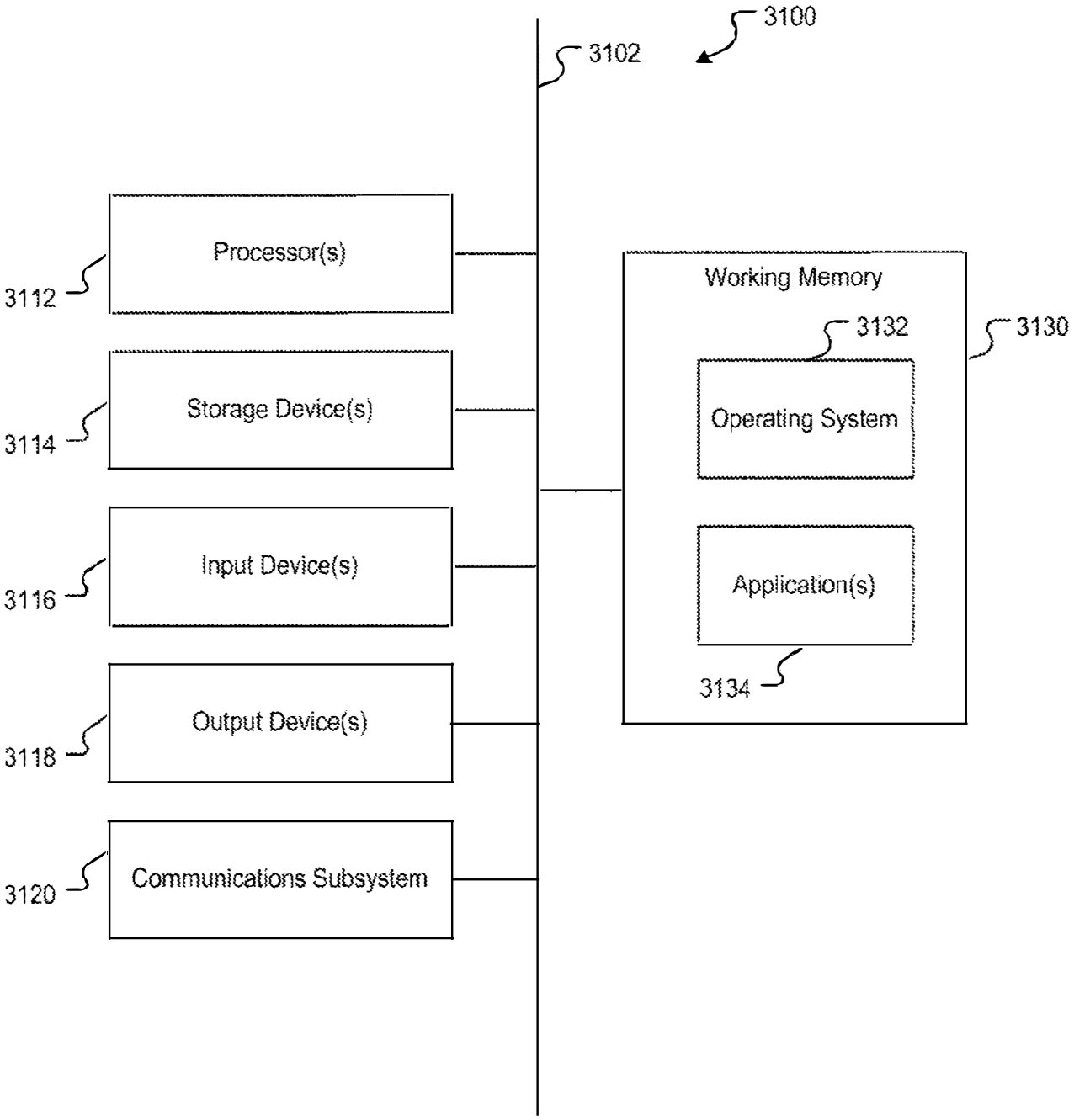

3100
3102
Processor(s)
3112
Storage Device(s)
3114
Input Device(s)
3116
Output Device(s)
3118
Communications Subsystem
3120
Working Memory
3130
3132
Operating System
Application(s)
3134

FIG. 31

Fig. 32

NanoString EX2011.445

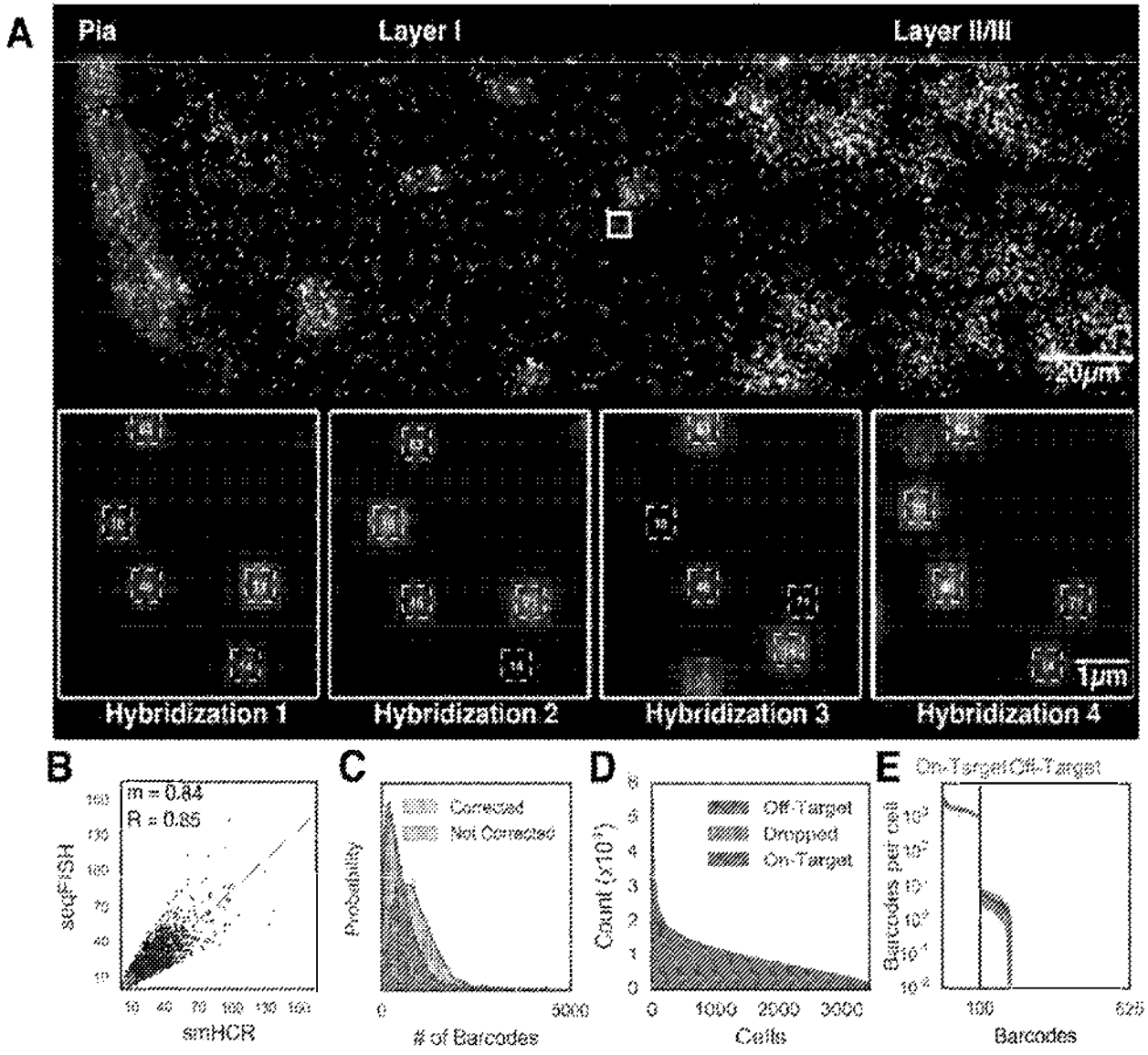

A
Pia
Layer I
Layer II/III
20μm
1μm
Hybridization 1
Hybridization 2
Hybridization 3
Hybridization 4
B
m = 0.84
R = 0.85
seqFISH
smHCR
C
Corrected
Not Corrected
Probability
of Barcodes
D
Off-Target
Dropped
On-Target
Count (x10³)
Cells
E
On-Target
Off-Target
Barcodes per cell
Barcodes

Fig. 33

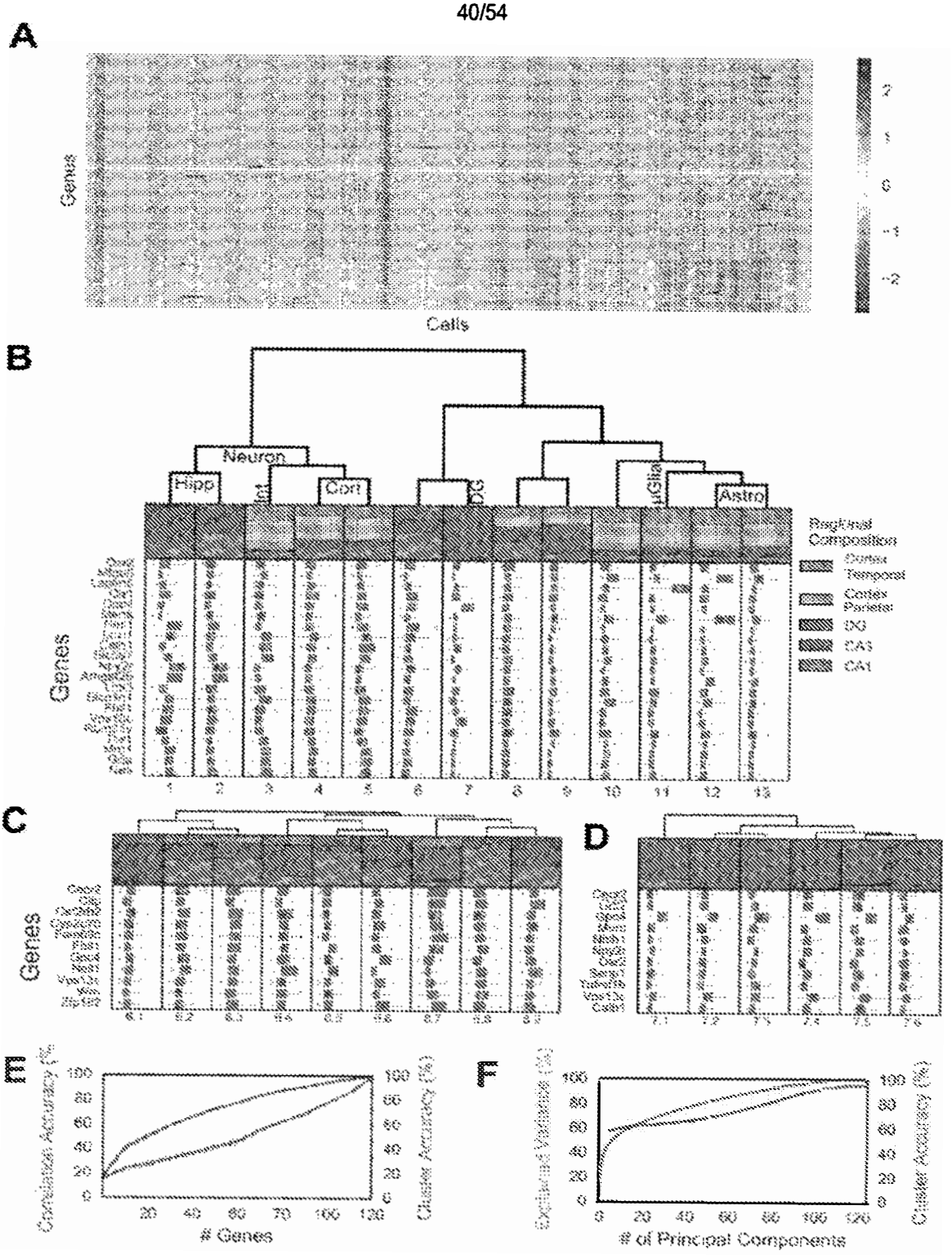

A
Genes
Cells
B
Neuron
Hipp
Int
Cort
DG
µGlia
Astro
Regional Composition
Cortex Temporal
Cortex Parietal
DG
CA3
CA1
Genes
C
D
E
Correlation Accuracy (%)
Cluster Accuracy (%)
Genes
F
Explained Variance (%)
Cluster Accuracy (%)
of Principal Components

Fig. 34

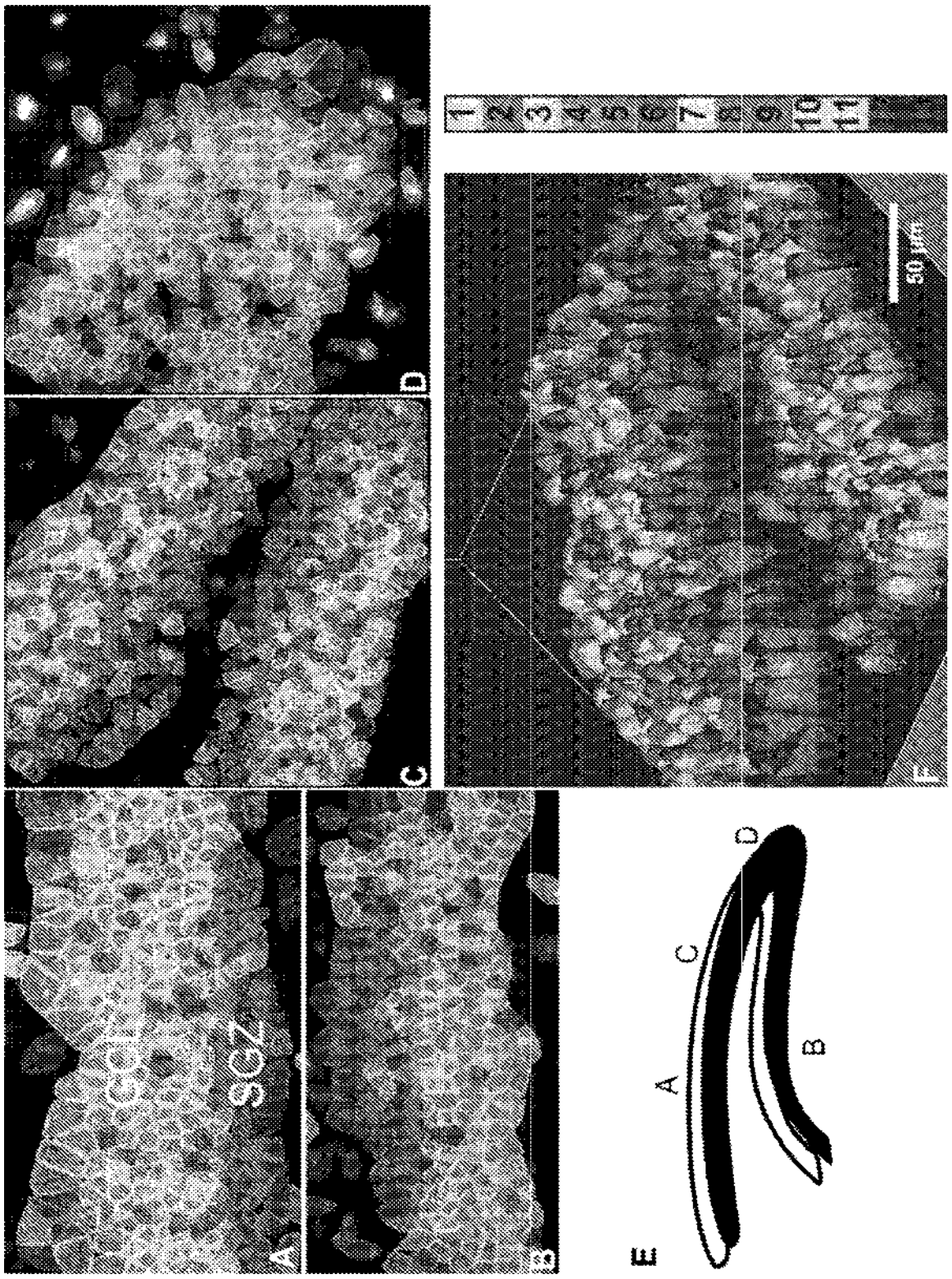


Fig. 35

NanoString EX2011.448

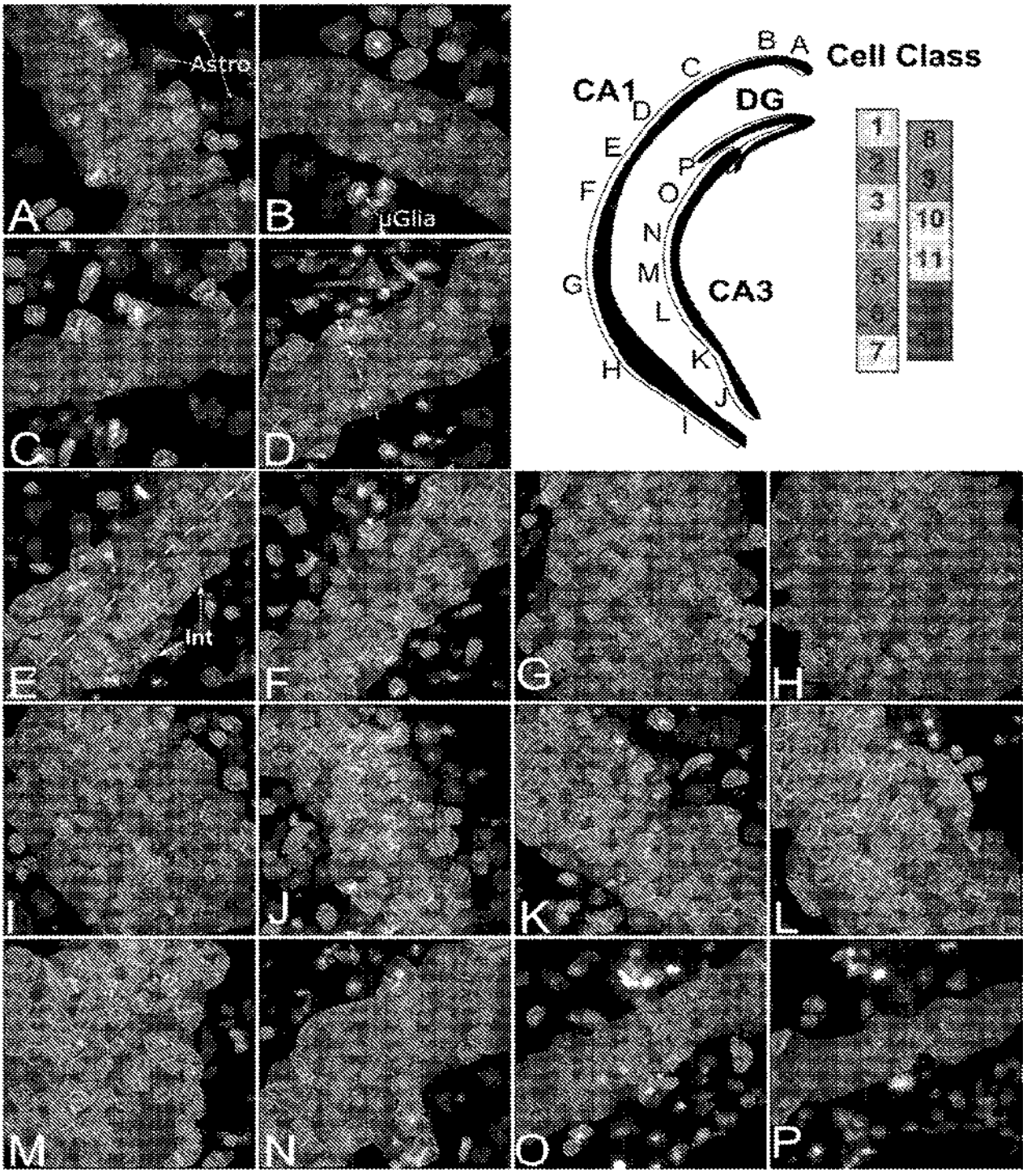


Fig. 36

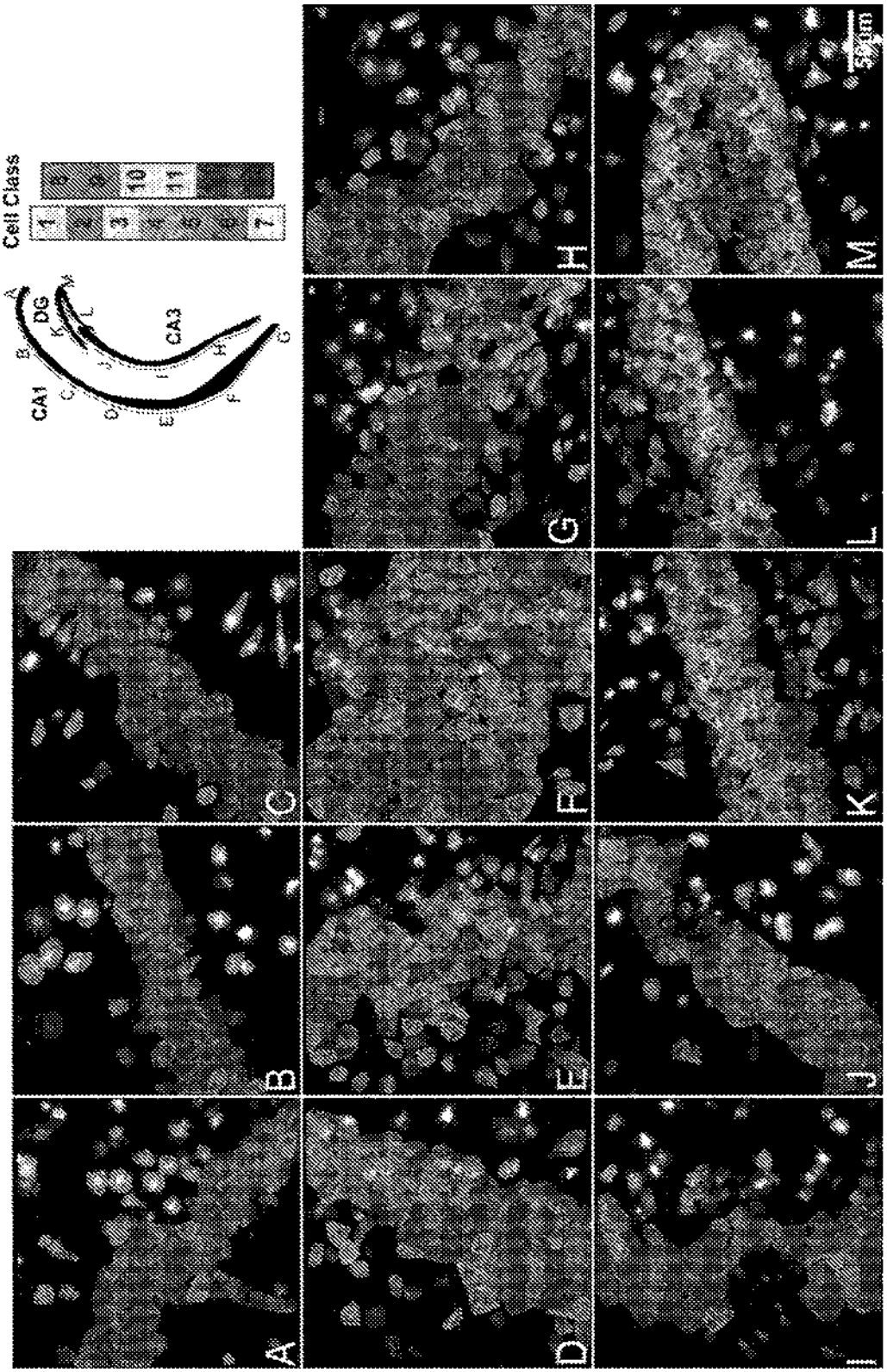


Fig. 37

NanoString EX2011.450

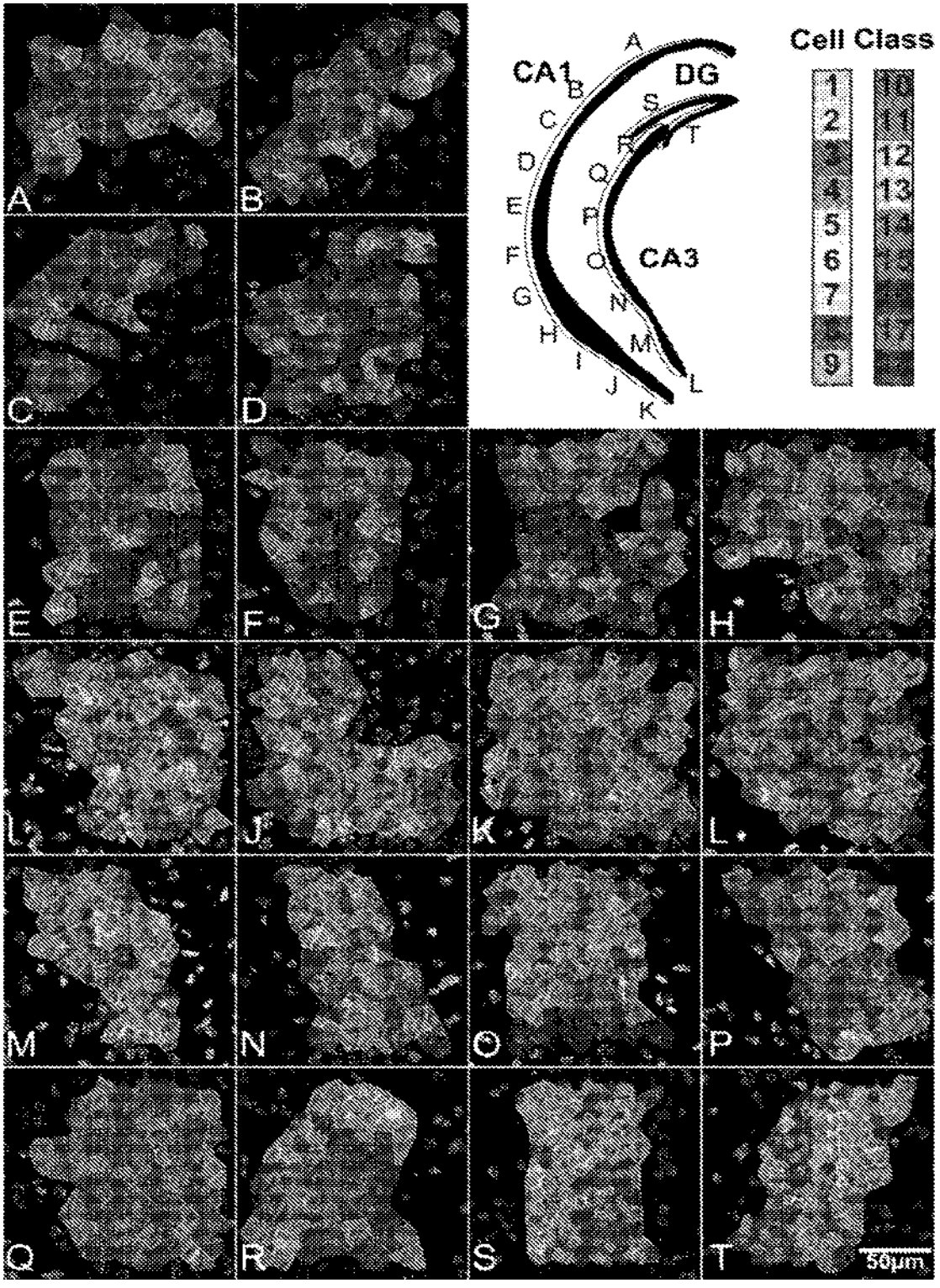

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
CA1
DG
CA3
Cell Class
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
50µm

Fig. 38

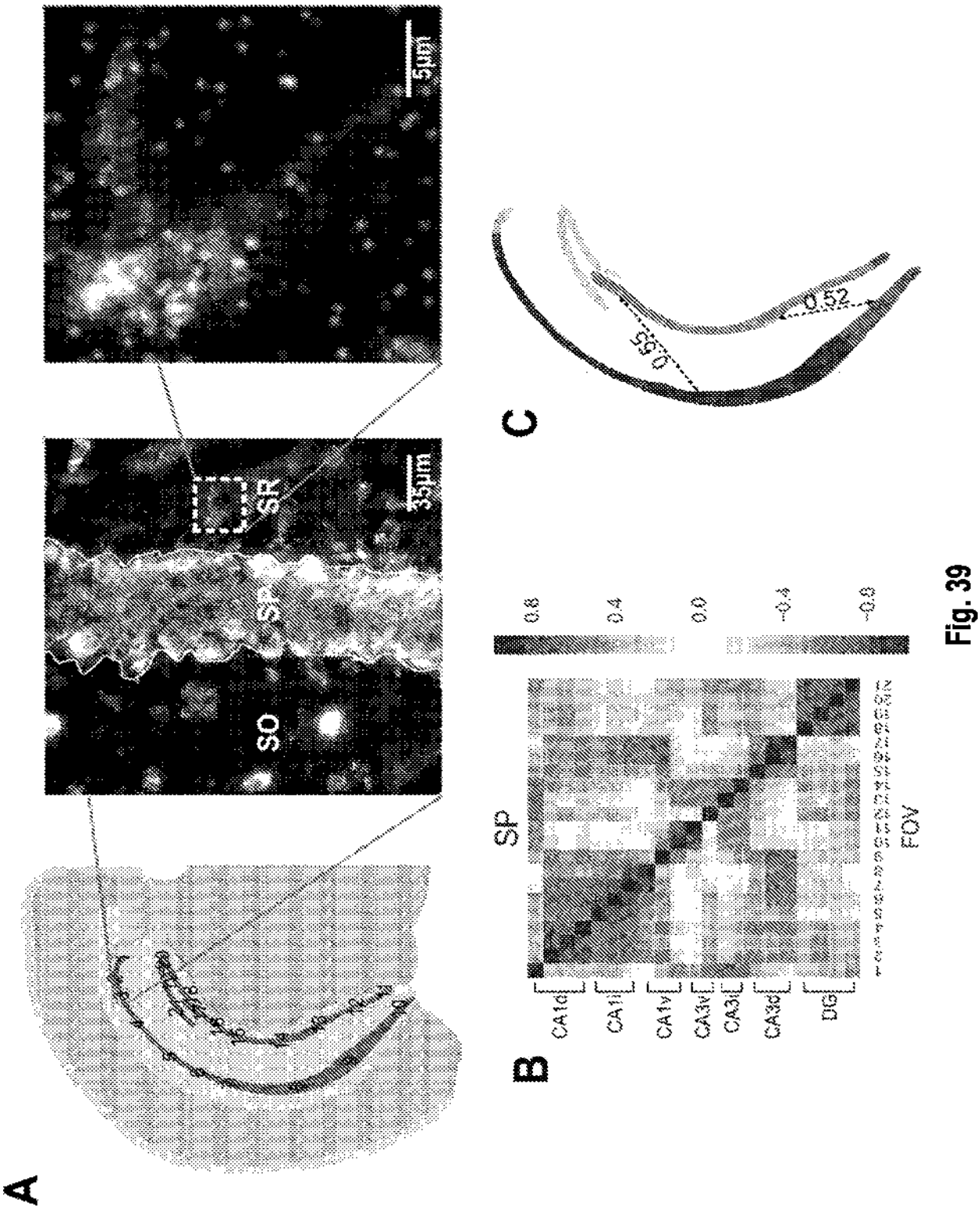

A
SO
SR
SR
35μm
5μm
B
SP
FOV
CA1d
CA1i
CA1v
CA3v
CA3i
CA3d
DG
0.8
0.4
0.0
-0.4
-0.8
C
0.52
0.55

Fig. 39

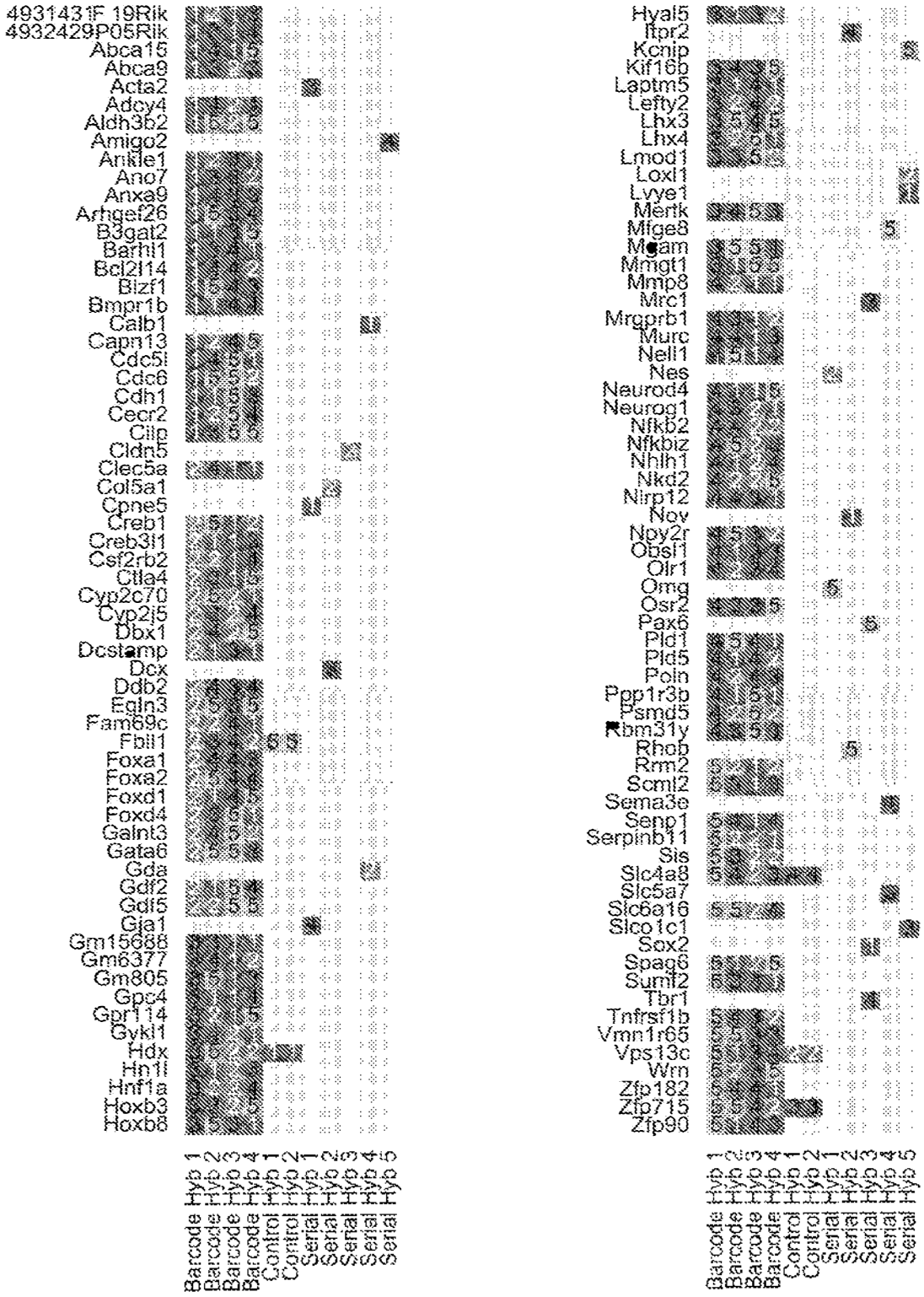

4931431F 19Rik
4932429P05Rik
Abca15
Abca9
Acta2
Adcy4
Aldh3b2
Amigo2
Ankle1
Ano7
Anxa9
Arhgef26
B3gat2
Barhl1
Bcl2l14
Bizf1
Bmpr1b
Calb1
Capn13
Cdc5l
Cdc6
Cdh1
Cecr2
Cilp
Cldn5
Clec5a
Col5a1
Cpne5
Creb1
Creb3l1
Csf2rb2
Ctla4
Cyp2c70
Cyp2j5
Dbx1
Dcstamp
Dcx
Ddb2
Egln3
Fam69c
Fbll1
Foxa1
Foxa2
Foxd1
Foxd4
Galnt3
Gata6
Gda
Gdf2
Gdf5
Gja1
Gm15685
Gm6377
Gm805
Gpc4
Gpr114
Gykl1
Hdx
Hn1l
Hnf1a
Hoxb3
Hoxb8
Hyal5
Itpr2
Kcnip
Kif16b
Laptm5
Lefty2
Lhx3
Lhx4
Lmod1
Loxl1
Lyve1
Mertk
Mfge8
Mgam
Mmgt1
Mmp8
Mrc1
Mrgprb1
Murc
Nell1
Nes
Neurod4
Neurog1
Nfkb2
Nfkbiz
Nhlh1
Nkd2
Nlrp12
Nov
Npy2r
Obsl1
Olr1
Omg
Osr2
Pax6
Pld1
Pld5
Poln
Ppp1r3b
Psmd5
Rbm31y
Rhob
Rrm2
Scml2
Sema3e
Senp1
Serpinb11
Sis
Slc4a8
Slc5a7
Slc6a16
Slco1c1
Sox2
Spag6
Sumf2
Tbr1
Tnfrsf1b
Vmn1r65
Vps13c
Wrn
Zfp182
Zfp715
Zfp90
Barcode Hyb 1
Barcode Hyb 2
Barcode Hyb 3
Barcode Hyb 4
Control Hyb 1
Control Hyb 2
Serial Hyb 1
Serial Hyb 2
Serial Hyb 3
Serial Hyb 4
Serial Hyb 5

Fig. 40

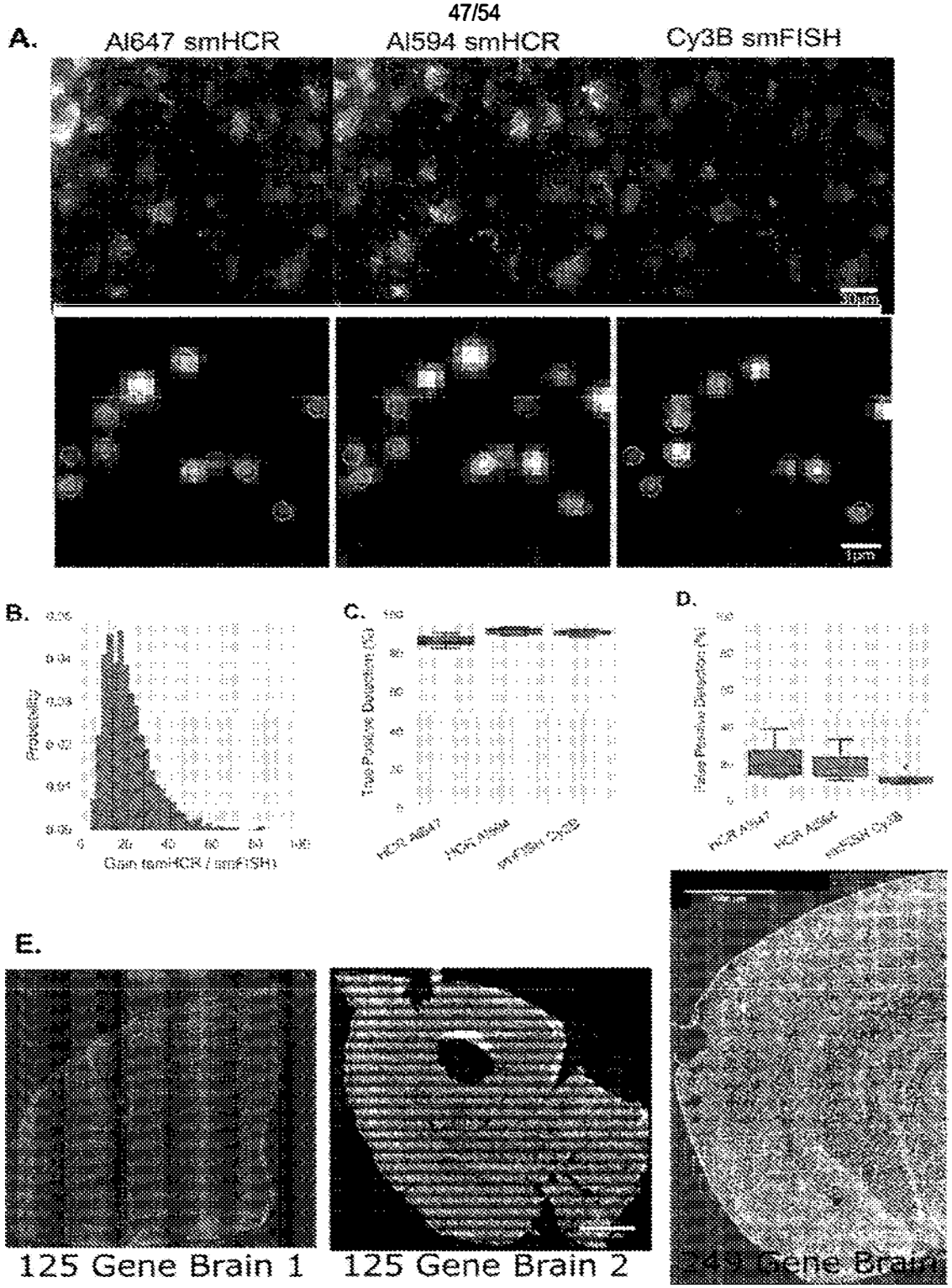

A.
Al647 smHCR
Al594 smHCR
Cy3B smFISH
50μm
1μm
B.
Probability
Gain tsmHCR / smFISH
C.
True Positive Detection (%)
HCR Al647
HCR Al594
smFISH Cy3B
D.
False Positive Detection (%)
HCR Al647
HCR Al594
smFISH Cy3B
E.
125 Gene Brain 1
125 Gene Brain 2
249 Gene Brain

Fig. 41

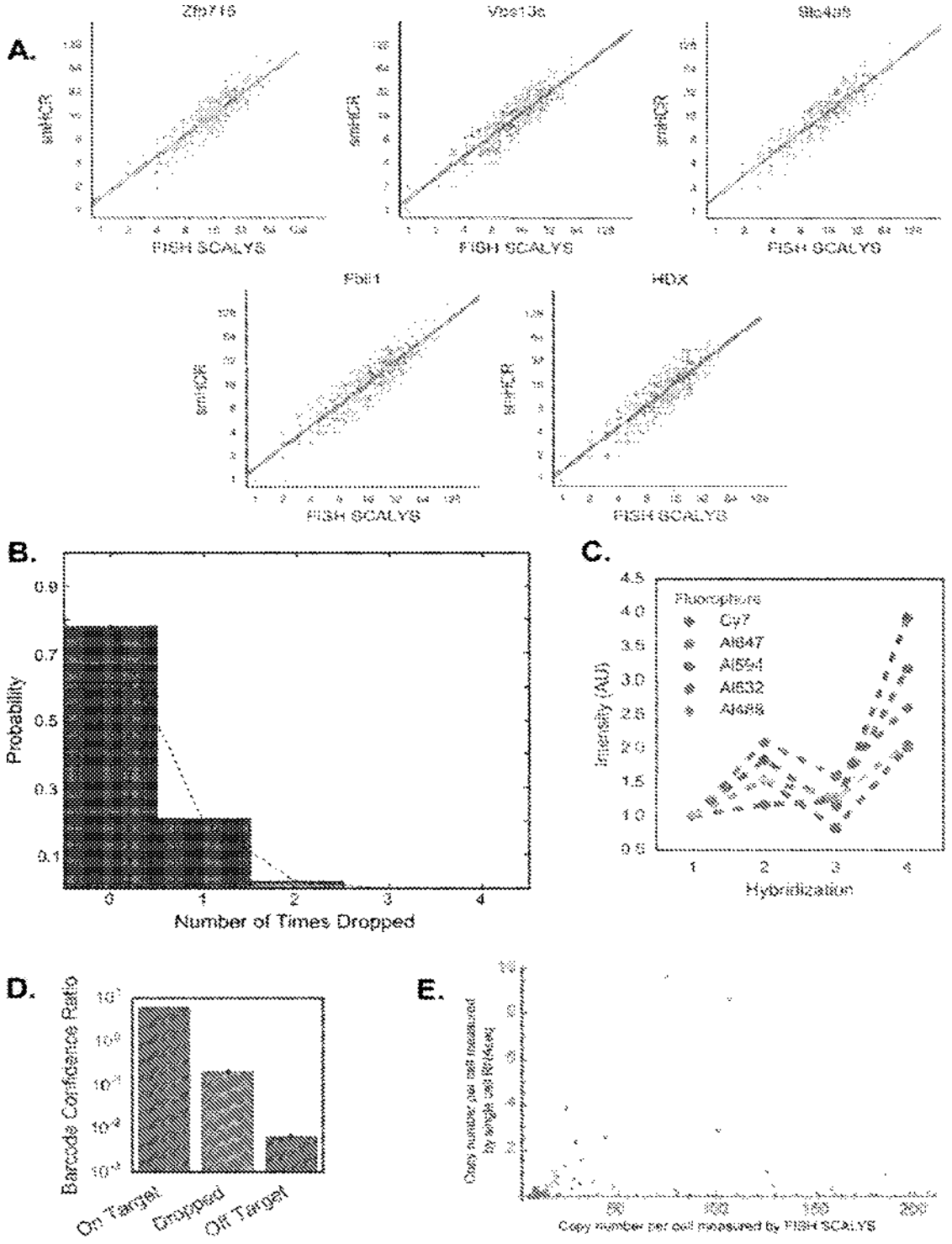

A.
Zfp716
Vps13a
Slc4a8
Pdk1
HDX
nCounter
FISH SCALYS
B.
Probability
Number of Times Dropped
C.
Fluorophore
Cy7
Alexa647
Alexa594
Alexa532
Alexa488
Intensity (AU)
Hybridization
D.
Barcode Confidence Ratio
On Target
Dropped
Off Target
E.
Copy number per cell measured by single cell RNAseq
Copy number per cell measured by FISH SCALYS

Fig. 42

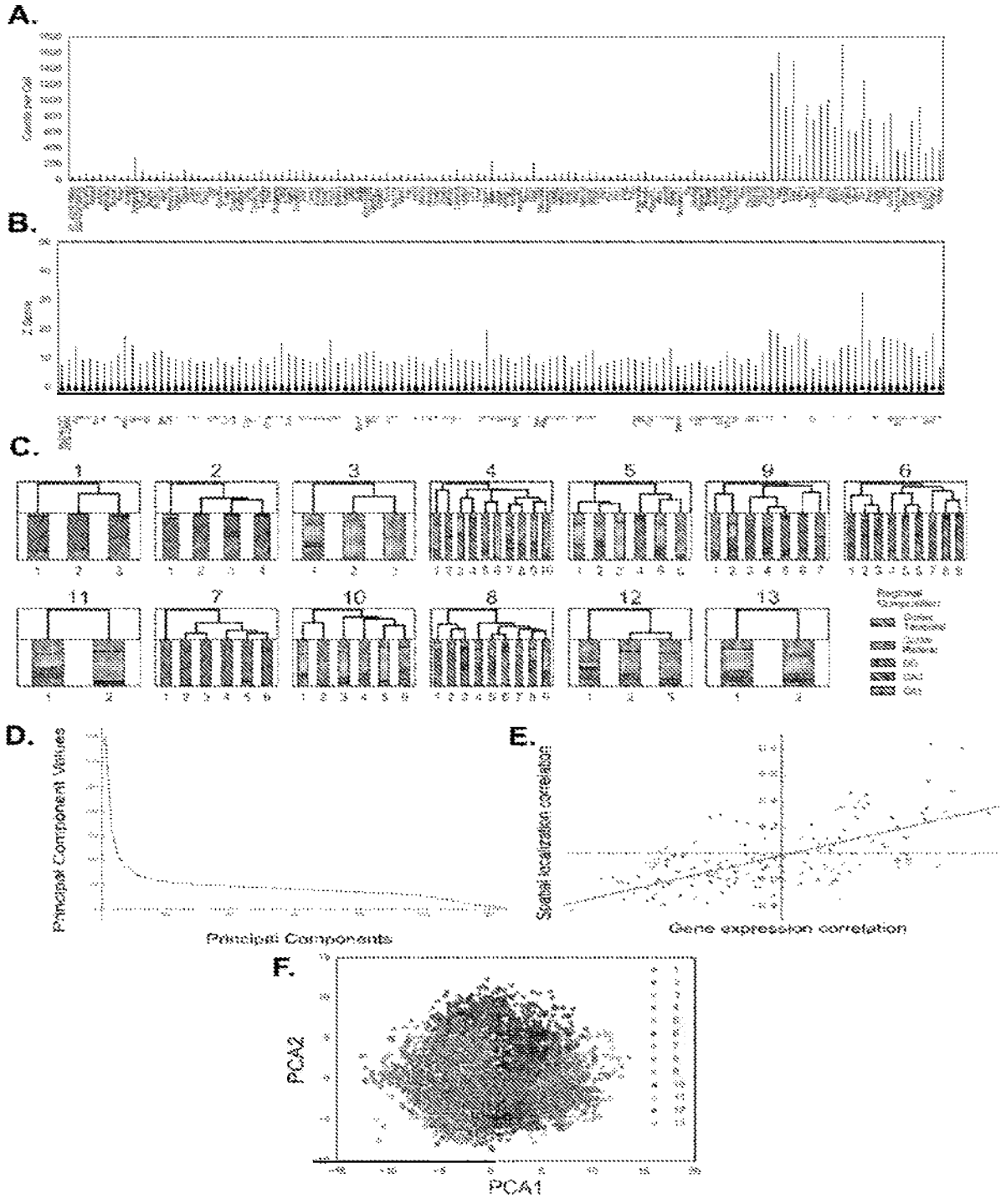

A.
B.
C.
1
2
3
4
5
9
6
11
7
10
8
12
13
D.
Principal Component Values
Principal Components
E.
Spatial localization correlation
Gene expression correlation
F.
PCA2
PCA1

Fig. 43

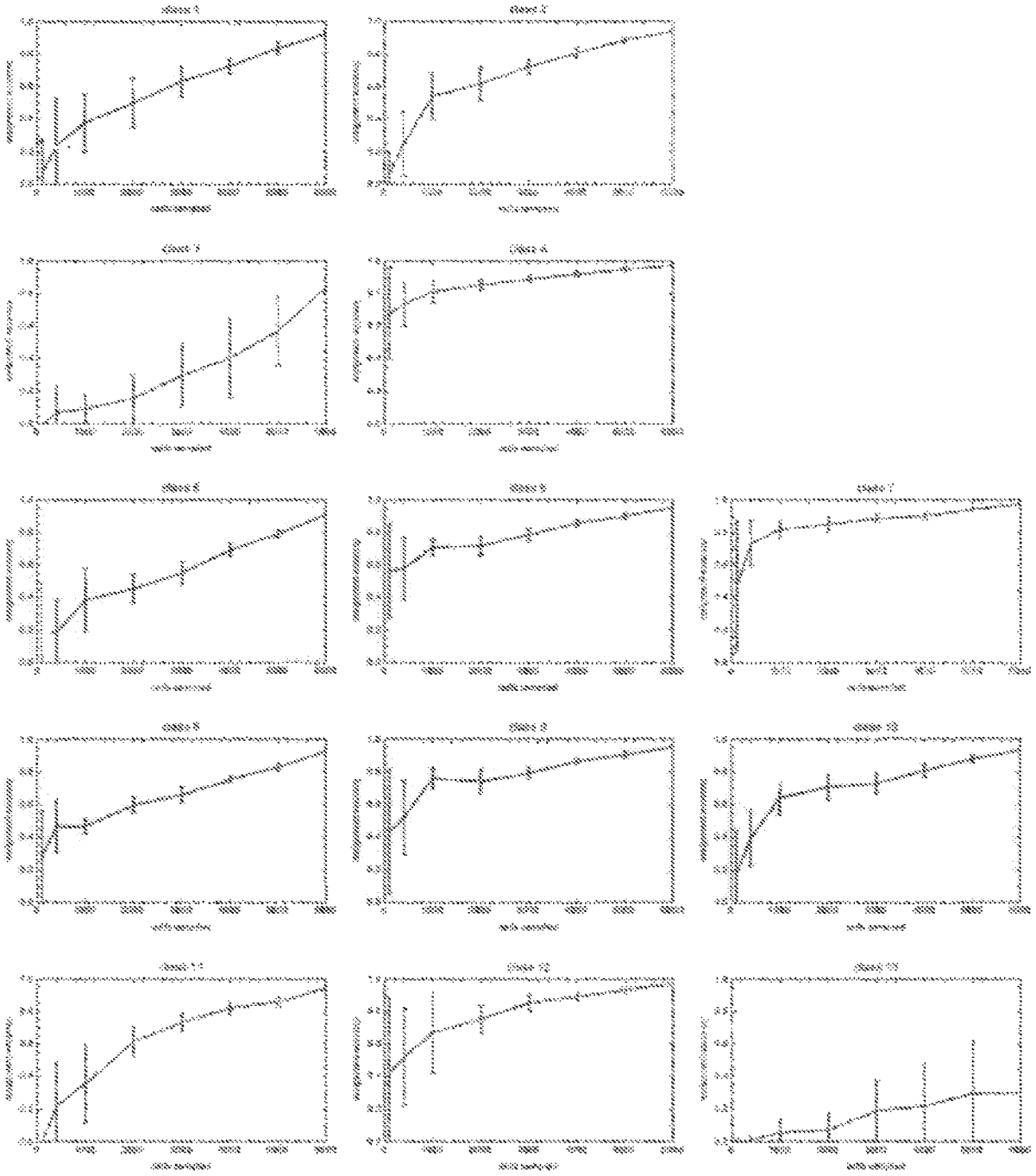


Fig. 44

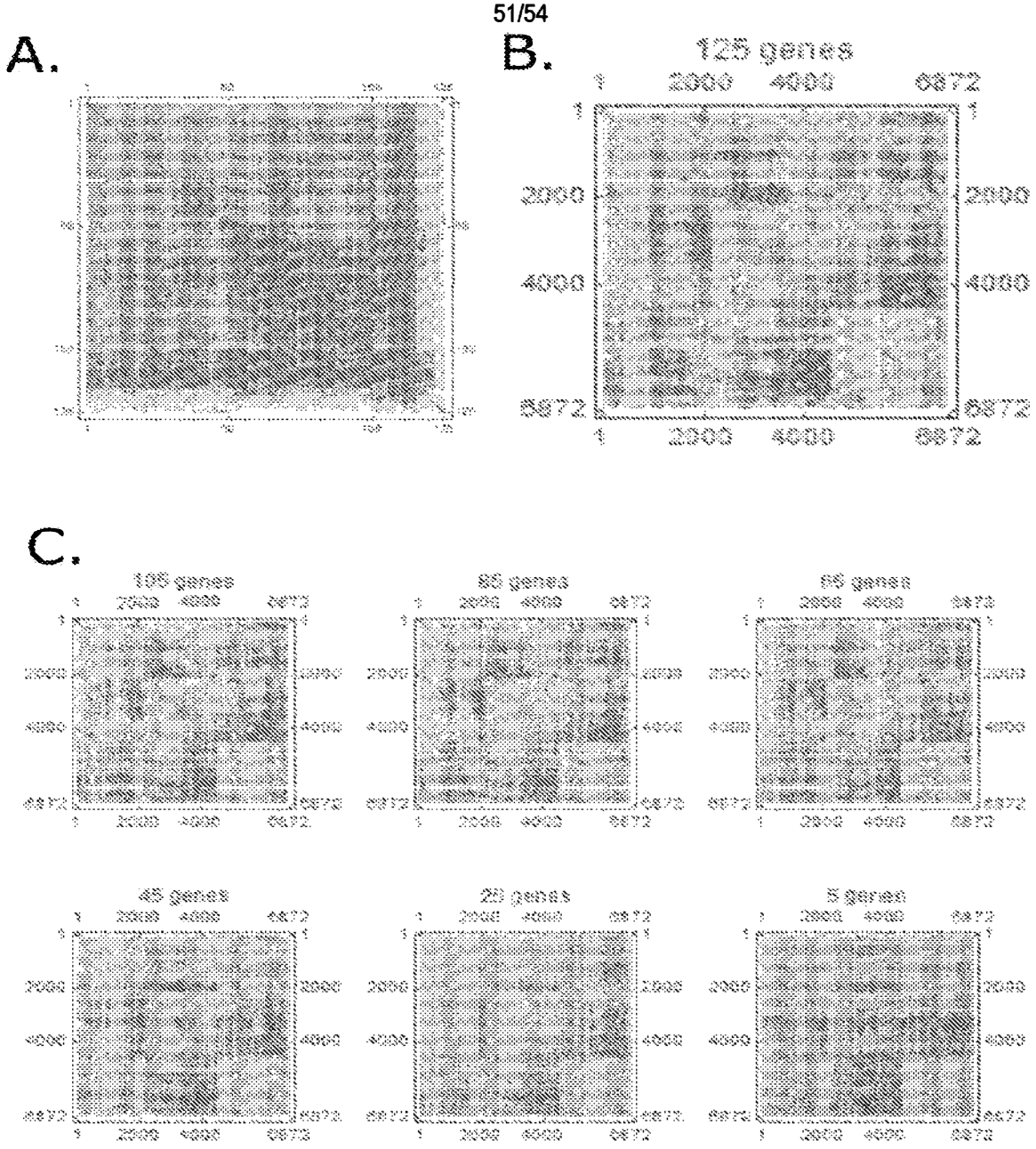

A.
B.
125 genes
1
2000
4000
6872
C.
105 genes
85 genes
65 genes
45 genes
25 genes
5 genes

Fig. 45

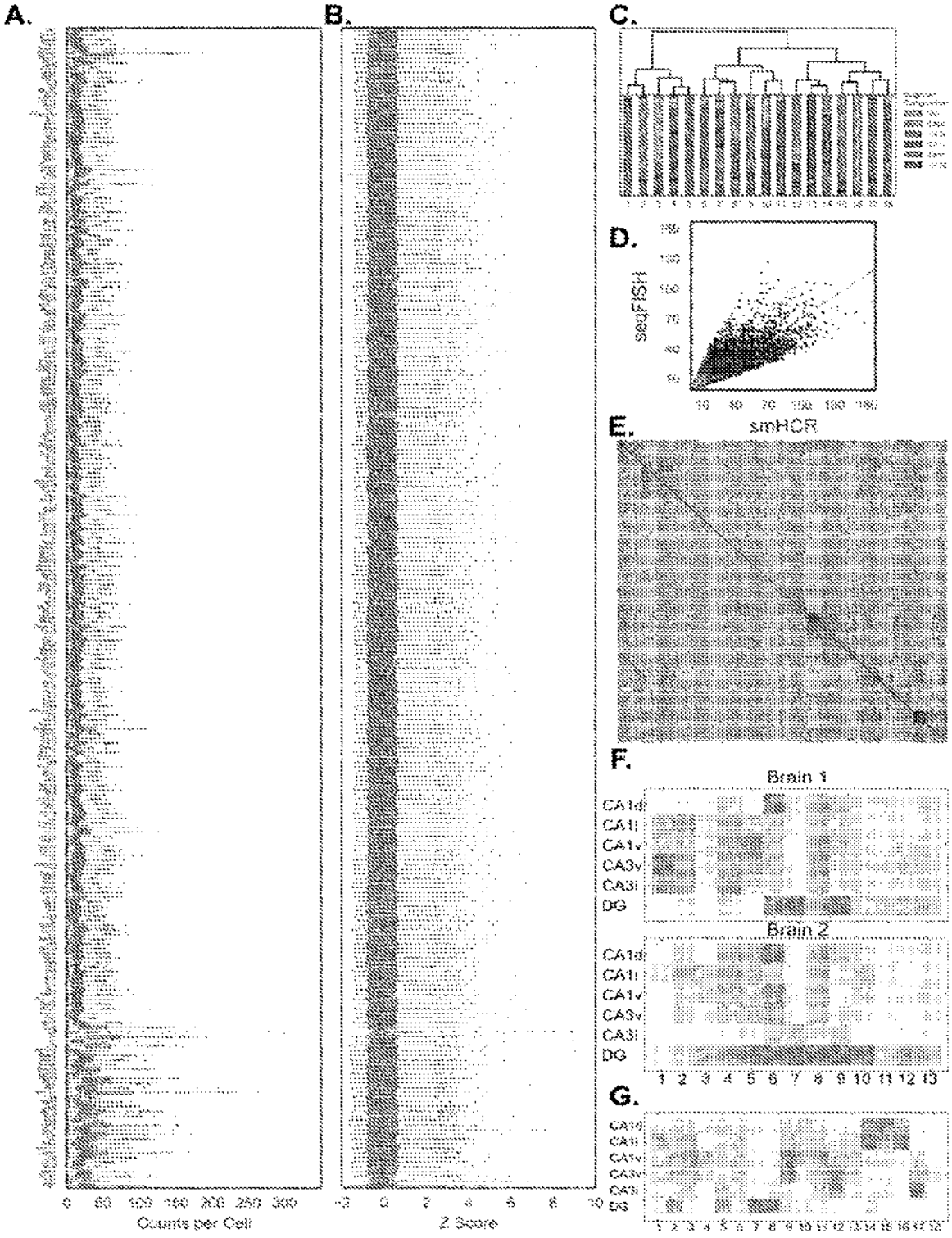

A.
B.
C.
D.
seqFISH
smHCR
E.
F.
Brain 1
Brain 2
CA1d
CA1l
CA1v
CA3v
CA3l
DG
1 2 3 4 5 6 7 8 9 10 11 12 13
G.
0 50 100 150 200 250 300
Counts per Cell
-2 0 2 4 6 8 10
Z Score

Fig. 46

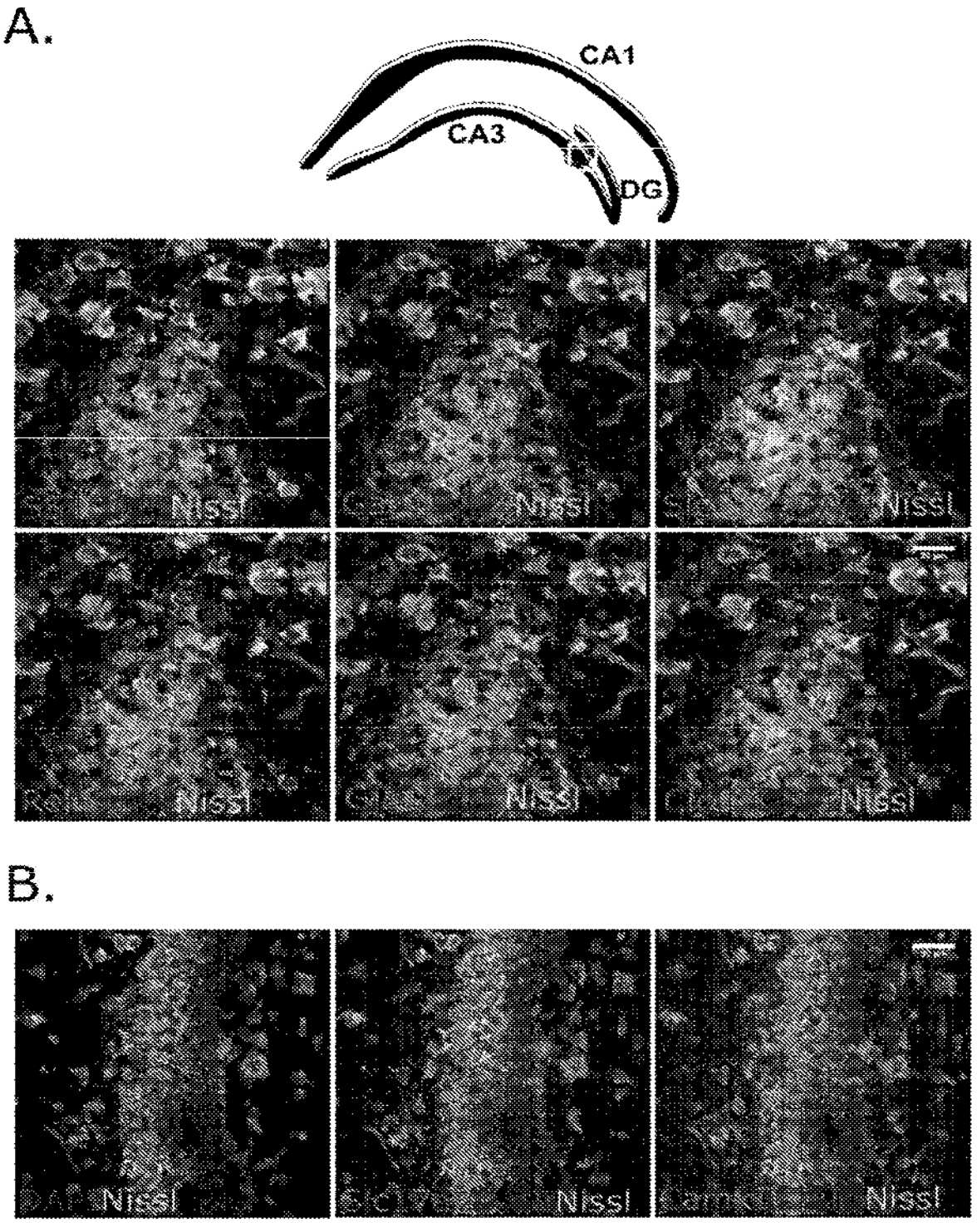

A.
CA1
CA3
DG
Nissl
Nissl
Nissl
Nissl
Nissl
Nissl
B.
Nissl
Nissl
Nissl

Fig. 47

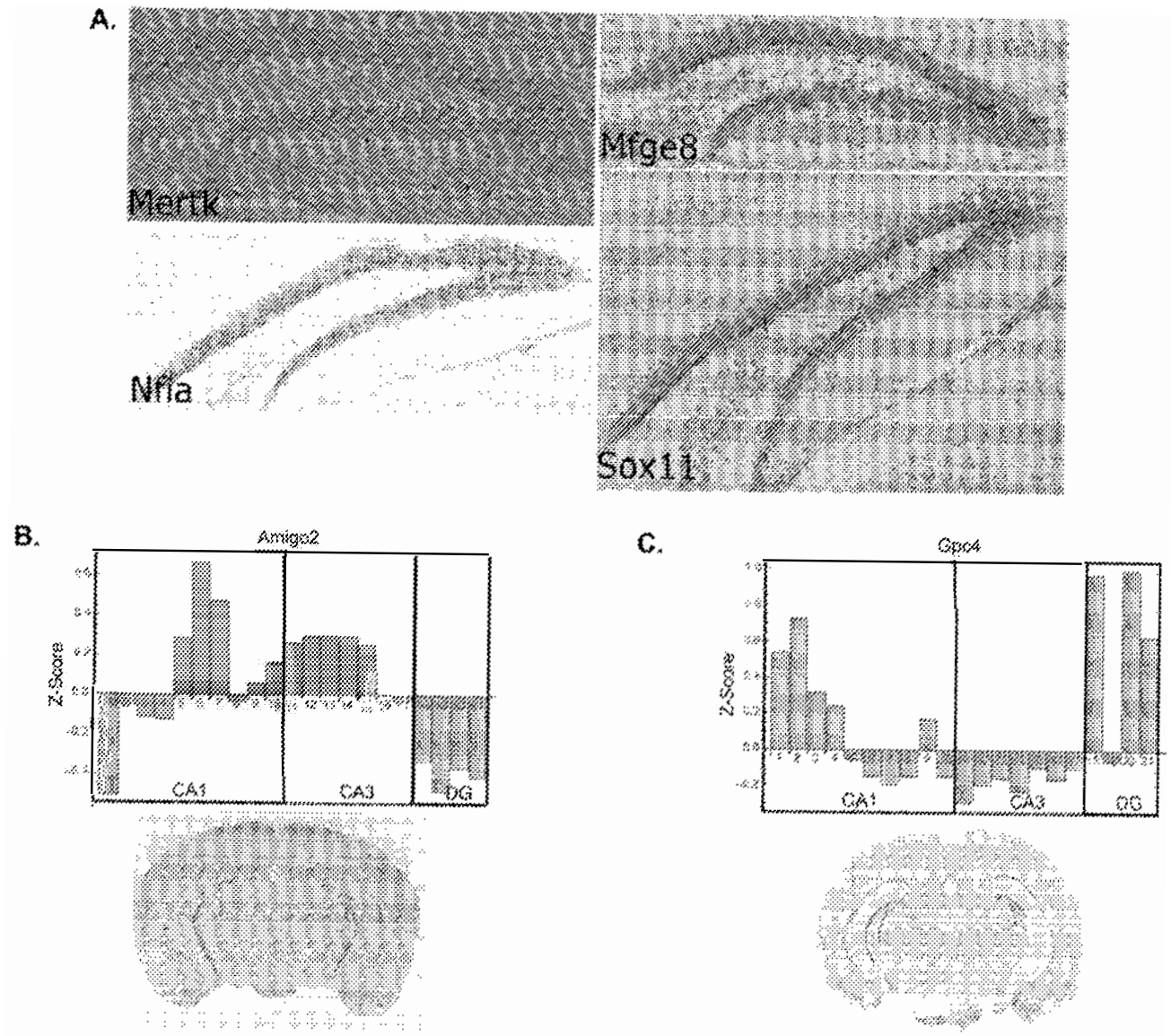


Fig. 48

NanoString EX2011.461

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/44994

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C12Q 1/68; G01N 21/64 (2017.01)
CPC - G01N 33/5308; G06F 19/24, 19/26; C12Q 1/6813, 1/6841, 1/6804

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2014/182528 A2 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 13 November 2014; paragraphs abstract, title, paragraphs [0007], [0012], [0031], [0039-40], [0065], [0067], [0069-71], [0087], [0093], [00121], [00161-164], [00168], [00175], [00183-184]; Figures 1-3; Claim 20	1, 2, 3/1, 3/2, 24, 25, 26/24, 26/25, 31 ---------- 30, 32/30, 32/31
Y	WO 2016/018960 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 4 February 2016; page 5, lines 5-9, page 13, lines 8-12, page 25, lines 5-7 and 17-22; page 81, lines 16-19	30, 32/30, 32/31
A	US 2016/0019334 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 21 January 2016; entire document	1, 2, 3/1, 3/2, 24, 25, 26/24, 26/25, 30, 31, 32/30, 32/31
A	US 2010/0323348 A1 (Hamady, ML et al.) 23 December 2010; entire document	1, 2, 3/1, 3/2, 24, 25, 26/24, 26/25, 30, 31, 32/30, 32/31
P, X	(Moffitt, JR, et al.) High-throughput single-cell gene-expression profiling with multiplexed error-robust fluorescence in situ hybridization, PNAS 27 September 2016, volume 113, no. 39, pages 11046–11051 doi: 10.1073/pnas.1612826113; entire document	1, 2, 3/1, 3/2, 24, 25, 26/24, 26/25, 30, 31, 32/30, 32/31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" 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 September 2017 (29.09.2017)	20 OCT 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/44994

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-23, 27-29, 33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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Electronic Patent Application Fee Transmittal

Application Number:	17476707
Filing Date:	16-Sep-2021
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Filer:	Robert E. Powers/Sylvana Householder
Attorney Docket Number:	NATE-037C01US 321329-2719

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:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	260	260
		Total in USD ($)		**260**

Electronic Acknowledgement Receipt

EFS ID:	45502686
Application Number:	17476707
International Application Number:	
Confirmation Number:	5473
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Customer Number:	141429
Filer:	Robert E. Powers/Francesca Vandermaelen
Filer Authorized By:	Robert E. Powers
Attorney Docket Number:	NATE-037C01US 321329-2719
Receipt Date:	19-APR-2022
Filing Date:	16-SEP-2021
Time Stamp:	13:37:59
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$260
RAM confirmation Number	E20224ID38140445
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

NanoString EX2011.466

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		NATE-037_C01US_Response.pdf	203030 110ce34b0e8ddd6b8c2fba522b0d54ed9509b0e9	yes	13

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Request for Reconsideration-After Non-Final Rejection	1	1
Claims	2	5
Drawings-other than black and white line drawings	6	6
Applicant Arguments/Remarks Made in an Amendment	7	13

Warnings:

Information:

2	Drawings-other than black and white line drawings	NATE-037_C01US_Replacement_Drawings.pdf	5113964 a1f9b44e29c46e44f68eba60e75ef970223a0521	no	4

Warnings:

Information:

3	Transmittal Letter	NATE-037-C01US_IDSTransmittal.pdf	121233 d4c6ca6c4277d0a7d6e6bf2dcc4c714a4585937f	no	3

Warnings:

Information:

4	Information Disclosure Statement (IDS) Form (SB08)	NATE-037-C01US_sb08.pdf	234815 e5dd6c317dce4d13940a26930facd9e9fe25ec46	no	1

Warnings:

Information:

This is not an USPTO supplied IDS fillable form

5	Foreign Reference	WO2018026873A1.pdf	14590958 b1212c01a714e06fc3a40b129117cd884cbbbe90	no	190
Warnings:					
Information:					
6	Fee Worksheet (SB06)	fee-info.pdf	38132 cd33fcc646c6d104f4dcd3c7cddd757df4e98f39	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	20302132		

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.

Docket No.: NATE-037/C01US 321329-2719
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Joseph M. BEECHEM	Confirmation No.:	5473
Application No.:	17/476,707	Group Art Unit:	1634
Filed:	September 16, 2021	Examiner:	F. W. Lu

For: CHEMICAL COMPOSITIONS AND USES THEREOF

VIA EFS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT/RESPONSE TO OFFICE ACTION

This paper is submitted as a Supplemental Response to the Response to Non-Final Office Action submitted April 19, 2022, which was in response to the Non-final Office Action mailed on January 19, 2022 in the above-identified application. Applicant hereby petitions for a one-month extension of time and submits the appropriate fee herewith. With the one-month extension of time, this paper is timely submitted on or before May 19, 2022. Applicant submits the following Amendments and/or Remarks, and respectfully requests reconsideration of the application in view thereof.

Any extensions of time necessary to prevent abandonment of this application are hereby petitioned for under 37 C.F.R. §1.136(a), and any additional fees required (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 50-1283.

Amendments to the Claims begin on page 2 of this paper. The Amendments to the Claims show the amendments filed by the Applicant in the Response to Non-Final Office Action submitted April 19, 2022.

Remarks/Arguments begin on page 7 of this paper.

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AMENDMENTS TO THE CLAIMS

Set forth below in ascending order, with status identifiers, is a complete listing of all claims currently under examination. Changes to any amended claims are indicated by [[double brackets]], ~~strikethrough~~ and/or underlining. This listing also reflects any cancellation and/or addition of claims. This listing includes the amendments filed by the Applicant in the Response to Non-Final Office Action submitted April 19, 2022.

1. (Currently Amended) A method for spatially detecting at least one target analyte in a tissue sample comprising:

a) contacting the tissue sample with a plurality of probes, wherein each of the probes ~~comprising~~comprise a target binding ~~domain~~domain that ~~hybridizes~~binds to the at least one target analyte, wherein the tissue sample has been treated to facilitate binding of the probes to the target analyte;

b) collecting the probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the probes, or portions thereof, from the first location of the tissue sample;

c) collecting the probes, or portions thereof, bound to the at least one target analyte in ~~an at least~~a second location of the tissue sample under conditions that release the probes, or portions thereof, from the second location of the tissue sample;

d) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the probes, or portions thereof, collected in step (b), thereby ~~to form~~forming a first plurality of extension products that ~~comprising~~comprise the probes, or portions thereof, collected in step (b) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;

e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the probes, or portions thereof, collected in step (c), thereby ~~to form~~forming a second plurality extension products that ~~comprising~~comprise the probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and

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f) identifying the first plurality of extension products and the second plurality of extension products ~~produced in step (d) and step (e)~~ by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location and the ~~at least~~ second location of the tissue sample.

2. – 3. (Canceled)

4. (Original) The method of claim 1, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.

5. (Canceled)

6. (Original) The method of claim 1, wherein the tissue sample is immobilized onto a microscope slide.

7. (Original) The method of claim 6, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.

8. (Currently Amended) The method of claim 7, wherein ~~the primers in~~ the plurality of primers [[are]]is immobilized on the microscope slide at their 5' ends.

9. (Currently Amended) The method of claim 8, wherein ~~performing the extension reaction~~each of step (d) and step (e) ~~comprises~~comprise performing a solid-phase amplification reaction, wherein the solid-phase amplification reaction is carried out ~~in association with~~on the microscope slide using the plurality of primers immobilized on the microscope slide.

10. (Currently Amended) The method of claim 1, wherein the sequencing step is next generation sequencing.

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11. (Currently Amended) The method of claim 1, ~~wherein the sequencing comprises~~further comprising amplifying a library using the ~~extension products~~first plurality of extension products and the second plurality of extension products as templates.

12. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (d) further comprise~~at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier.

13. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (e) further comprise~~ at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier.

14. (Canceled)

15. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (d) further comprise~~at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site.

16. (Currently Amended) The method of claim 1, wherein the ~~extension products of step (e) further comprise~~ at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one amplification primer binding site.

17. (Original) The method of claim 1, wherein the target binding domains comprise a single-stranded nucleic acid molecule.

18. (Original) The method of claim 1, wherein steps (b) and (c) are performed simultaneously.

19. (Original) The method of claim 1, wherein steps (d) and (e) are performed simultaneously.

20. (New) A method for spatially detecting at least one target analyte in a tissue sample comprising:

a) contacting the tissue sample with a first plurality of probes and a second plurality of probes,

wherein each of the probes in the first plurality of probes comprise a target binding domain that binds to the at least one target analyte,

wherein each of the probes in the second plurality of probes comprise a target binding domain that binds to the at least one target analyte,

wherein the tissue sample has been treated to facilitate binding of the probes to the at least one target analyte;

b) ligating together probes from the first plurality of probes and probes from the second plurality of probes that are bound to the same at least one target analyte;

c) collecting the ligated probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the ligated probes, or portions thereof, from the first location of the tissue sample;

d) collecting the ligated probes, or portions thereof, bound to the at least one target analyte in a second location of the tissue sample under conditions that release the probes, or portions thereof, from the second location of the tissue sample;

e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (c), thereby forming a first plurality of extension products that comprise the ligated probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;

f) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (d), thereby forming a second plurality extension products that

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comprise the ligated probes, or portions thereof, collected in step (d) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and

g) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location and the second location of the tissue sample.

21. (New) The method of claim 20, wherein the tissue sample is a formalin-fixed paraffin-embedded (FFPE) tissue sample.

22. (New) The method of claim 20, wherein the tissue sample is immobilized onto a microscope slide.

23. (New) The method of claim 22, wherein the microscope slide comprises a plurality of primers immobilized on the microscope slide.

24. (New) The method of claim 23, wherein the plurality of primers is immobilized on the microscope slide at their 5' ends.

25. (New) The method of claim 24, wherein each of step (e) and step (f) comprise performing a solid-phase amplification reaction, wherein the solid-phase amplification reaction is carried out on the microscope slide using the plurality of primers immobilized on the microscope slide.

26. (New) The method of claim 20, wherein the sequencing step is next generation sequencing.

27. (New) The method of claim 20, further comprising amplifying a library using the first plurality of extension products and the second plurality of extension products as templates.

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28. (New) The method of claim 20, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one unique molecular identifier.

29. (New) The method of claim 20, wherein the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one unique molecular identifier.

30. (New) The method of claim 20, wherein the probes in the first plurality of probes comprise an amplification primer binding site, the probes in the second plurality of probes comprise an amplification primer binding site, or both probes in the first plurality of probes comprise an amplification primer binding site and the probes in the second plurality of probes comprise an amplification primer binding site.

31. (New) The method of claim 20, wherein the at least one nucleic acid sequence that identifies the first location of the tissue sample comprises at least one amplification primer binding site, the at least one nucleic acid sequence that identifies the second location of the tissue sample comprises at least one amplification primer binding site, or both the at least one nucleic acid sequence that identifies the first location of the tissue sample and the at least one nucleic acid sequence that identifies the second location of the tissue sample comprise at least one amplification primer binding site.

32. (New) The method of claim 20, wherein the target binding domains comprise a single-stranded nucleic acid molecule.

33. (New) The method of claim 20, wherein steps (c) and (d) are performed simultaneously.

34. (New) The method of claim 20, wherein steps (e) and (f) are performed simultaneously.

REMARKS

Upon entry to these amendments, claims 1, 4 and 6-13 and 15-34 are pending in the present application. Claims 2, 3, 5 and 14 are canceled herein without prejudice or disclaimer. Applicant reserves the right to pursue the subject matter of the canceled claims in one or more continuation applications. Claims 1, 8, 9, 10-13, 15 and 16 are amended herein and claims 20-34 are added herein. Support for the claim amendments and new claims can be found throughout the application as originally filed. Amendment and cancelation of certain claims is not to be construed as a dedication to the public of any of the subject matter of the claims as previously presented. No new matter is added.

Applicant-Initiated Interview Summary

Applicant's representative, the undersigned, thanks Examiner Frank Wei Min Lu for the courtesy of the telephonic interview regarding the above-identified application conducted on April 28, 2022. Present during the telephonic interview were Examiner Frank Wei Min Lu for the USPTO and Robert E. Powers on behalf of the Applicant. The claim amendments submitted by the Applicant in the Response to Non-Final Office Action of April 19, 2022 and the rejections of record were discussed. Per this discussion, Applicant herein submits this supplemental response, which further cancels dependent claims 2 and 3, and incorporates the subject matter of these dependent claims into a new independent claim. To aid the Office in their review, this supplemental response includes all of the amendments presented by the Applicant in the Response to Non-Final Office Action of April 19, 2022.

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CONCLUSION

In view of the foregoing, and the remarks made in the Response to Non-Final Office Action of April 19, 2022, Applicant respectfully submits that no further impediments exist to the allowance of this application and, therefore, requests an indication of allowability. However, the Examiner is requested to call the undersigned if any questions or comments arise. The Director is hereby authorized to charge any appropriate fees under 37 C.F.R. §§1.16, 1.17, and 1.21 that may be required by this paper, and to credit any overpayment, to Deposit Account No. 50-1283 **referencing Docket No.** NATE-037/C01US 321329-2719**.**

Dated: May 3, 2022

Respectfully submitted,
COOLEY LLP

By: /Robert E. Powers/
Robert E. Powers
Reg. No. 77,802
Matthew Pavao
Reg. No. 50,572

USPTO CUSTOMER NO. 141429

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Electronic Patent Application Fee Transmittal

Application Number:	17476707
Filing Date:	16-Sep-2021
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Filer:	Matthew Pavao/Francesca Vandermaelen
Attorney Docket Number:	NATE-037C01US 321329-2719

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:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	220	220
Miscellaneous:				
			Total in USD ($)	**220**

Electronic Acknowledgement Receipt

EFS ID:	45622178
Application Number:	17476707
International Application Number:	
Confirmation Number:	5473
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Customer Number:	141429
Filer:	Matthew Pavao/Francesca Vandermaelen
Filer Authorized By:	Matthew Pavao
Attorney Docket Number:	NATE-037C01US 321329-2719
Receipt Date:	03-MAY-2022
Filing Date:	16-SEP-2021
Time Stamp:	14:30:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$220
RAM confirmation Number	E202253E30330491
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

NanoString EX2011.480

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		NATE-037_C01US_Response.pdf	145332 274e4d330fe3876f4fe300886911b392d71fe19a	yes	9
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Supplemental Response or Supplemental Amendment		1	1	
	Claims		2	7	
	Applicant Arguments/Remarks Made in an Amendment		8	9	
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38282 40b26191dd42ec0334fc08bfd7617df7097e434b	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	183614		

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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.

PTO/SB/06 (09-11)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 17/476,707	Filing Date 09/16/2021	☑ 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	05/03/2022	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 30	Minus	** 20	= 10		x $100 =	1000
	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	1000

		(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".	/ANGELA D JOHNSON/
*** 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.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037C01US 321329-2719	5473

141429 7590 05/04/2022

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

EXAMINER	
LU, FRANK WEI MIN	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
05/04/2022	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):

zIPPatentDocketingMailboxUS@cooley.com

NanoString EX2011.484

Applicant-Initiated Interview Summary	**Application No.** 17/476,707	**Applicant(s)** BEECHEM et al.		
	Examiner FRANK W LU	**Art Unit** 1634	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
FRANK W LU	Primary Examiner	Telephonic
Dr. Robert Powers (Reg. No. 77,802)	Attorney of Record	

Date of Interview: 28 April 2022

Issues Discussed:

Proposed Amendment(s)

Dr. Powers and the examiner discussed the amendment filed on April 19, 2020. Dr. Powers will consider to file a supplemental response to cancel claims 2 and 3 and add a new independent claim which includes all limitation recited in claims 1-3.

/FRANK W LU/ Primary Examiner, Art Unit 1634	April 28, 2022

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

Doc Code: DIST.E.FILE **Document Description: Electronic Terminal Disclaimer - Filed**	PTO/SB/25 U.S. Patent and Trademark Office Department of Commerce

Electronic Petition Request	**TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION**
Application Number	17476707
Filing Date	16-Sep-2021
First Named Inventor	Joseph BEECHEM
Attorney Docket Number	NATE-037C01US 321329-2719
Title of Invention	CHEMICAL COMPOSITIONS AND USES THEREOF

☒ 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
NanoString Technologies, Inc.	100%

The owner(s) of 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 any patent granted on pending reference Application Number(s)

17476712 filed on 09/16/2021

as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. 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 any patent granted on the reference application 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 any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: 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 shortened by any terminal disclaimer filed prior to its grant.

◉ 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 77802 ○ 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	/Robert Powers/
Name	Robert Powers

*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:	17476707
Filing Date:	16-Sep-2021
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Filer:	Robert E. Powers/Francesca Vandermaelen
Attorney Docket Number:	NATE-037C01US 321329-2719

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.: 17476707

Filing Date: 16-Sep-2021

Applicant/Patent under Reexamination: BEECHEM

Electronic Terminal Disclaimer filed on May 16, 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:	45720574
Application Number:	17476707
International Application Number:	
Confirmation Number:	5473
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Customer Number:	141429
Filer:	Robert E. Powers/Francesca Vandermaelen
Filer Authorized By:	Robert E. Powers
Attorney Docket Number:	NATE-037C01US 321329-2719
Receipt Date:	16-MAY-2022
Filing Date:	16-SEP-2021
Time Stamp:	16:42:27
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	E20225FG42232445
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

NanoString EX2011.491

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	43615 6a62bdc669a0fea11aa10b384a295fa9abce1c83	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38155 ddf496f8f0f03f69dc8553ee9cf6e61f5f495770	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	81770		

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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

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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.

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NOTICE OF ALLOWANCE AND FEE(S) DUE

141429 7590 05/24/2022

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

EXAMINER	
LU, FRANK WEI MIN	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 05/24/2022

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037C01US 321329-2719	5473

TITLE OF INVENTION: CHEMICAL COMPOSITIONS AND USES THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	08/24/2022

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

NanoString EX2011.493

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

141429 7590 05/24/2022

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037C01US 321329-2719	5473

TITLE OF INVENTION: CHEMICAL COMPOSITIONS AND USES THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1200	$0.00	$0.00	$1200	08/24/2022

EXAMINER	ART UNIT	CLASS-SUBCLASS
LU, FRANK WEI MIN	1634	435-006110

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑ Issue Fee ❑ Publication Fee (if required) ❑ Advance Order - # of Copies _____

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

❑ Electronic Payment via EFS-Web ❑ Enclosed check ❑ Non-electronic payment by credit card (Attach form PTO-2038)

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

NanoString EX2011.494

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.
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037C01US 321329-2719	5473

141429 7590 05/24/2022

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

EXAMINER	
LU, FRANK WEI MIN	

ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 05/24/2022

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

NanoString EX2011.495

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. 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 30 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, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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 disclosure of these records is required by the Freedom of Information Act.
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 inspection 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.

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Notice of Allowability	Application No. 17/476,707	Applicant(s) BEECHEM et al.	
	Examiner FRANK W LU	Art Unit 1634	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to amendments filed on 4/19/2022 and 5/3/2022.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ 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.

3. ☑ The allowed claim(s) is/are 1,4,6-13 and 15-34 . As a result of the allowed claim(s), 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.**

4. ☐ 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)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 4/19/2022.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☑ Interview Summary (PTO-413), Paper No./Mail Date. 5/17/2022.
5. ☑ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/FRANK W LU/ Primary Examiner, Art Unit 1634	May 18, 2022

NanoString EX2011.497

DETAILED ACTION

Reasons for Allowance

1. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Dr. Robert Powers (Reg. No.77,802) on May 17, 2022.

2. The application has been amended as follows:

1. (Currently Amended) A method for spatially detecting at least one target analyte in a first location and a second location of a tissue sample comprising:

a) contacting the tissue sample with a plurality of nucleic acid probes, wherein each of the nucleic acid probes comprise a target binding domain that binds to the at least one target analyte, wherein the tissue sample [has been] is treated to facilitate binding of the nucleic acid probes to the target analyte;

b) collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a first location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the first location of the tissue sample;

c) collecting the nucleic acid probes, or portions thereof, bound to the at least one target analyte in a second location of the tissue sample under conditions that release the nucleic acid probes, or portions thereof, from the second location of the tissue sample;

d) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the nucleic acid probes, or portions

thereof, collected in step (b), thereby forming a first plurality of extension products that comprise the nucleic acid probes, or portions thereof, collected in step (b) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;

e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the nucleic acid probes, or portions thereof, collected in step (c), thereby forming a second plurality extension products that comprise the nucleic acid probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the [at least] second location of the tissue sample; and

f) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location of the tissue sample and the second location of the tissue sample.

10. (Currently Amended) The method of claim 1, wherein the sequencing step is performed using a next generation sequencing reaction.

20. (Currently Amended) A method for spatially detecting at least one target analyte in a first location and a second location of a tissue sample comprising:

a) contacting the tissue sample with a first plurality of nucleic acid probes and a second plurality of nucleic acid probes,

wherein each of the nucleic acid probes in the first plurality of nucleic acid probes comprise a target binding domain that binds to the at least one target analyte,

wherein each of the nucleic acid probes in the second plurality of nucleic acid probes comprise a target binding domain that binds to the at least one target analyte,

wherein the tissue sample [has been] is treated to facilitate binding of the first plurality of nucleic acid probes and the second plurality of nucleic acid probes to the at least one target analyte;

b) forming ligated probes by ligating [together] each of nucleic acid probes from the first plurality of nucleic acid probes and each of nucleic acid probes from the second plurality of nucleic acid probes that are bound to [the same] each of identical molecules from the at least one target analyte;

c) collecting the ligated probes, or portions thereof, bound to each of the identical molecules from the at least one target analyte in a first location of the tissue sample under conditions that release the ligated probes, or portions thereof, from the first location of the tissue sample;

d) collecting the ligated probes, or portions thereof, bound to each of the identical molecules from the at least one target analyte in a second location of the tissue sample under conditions that release the probes, or portions thereof, from the second location of the tissue sample;

e) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the first location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (c), thereby forming a first plurality of extension products that comprise the ligated probes, or portions thereof, collected in step (c) and the at least one nucleic acid sequence that identifies the first location of the tissue sample;

f) performing an extension reaction that incorporates at least one nucleic acid sequence that identifies the second location of the tissue sample into each of the ligated probes, or portions thereof, collected in step (d), thereby forming a second plurality extension products that comprise the ligated probes, or portions thereof, collected in step (d) and the at least one nucleic acid sequence that identifies the at least second location of the tissue sample; and

g) identifying the first plurality of extension products and the second plurality of extension products by sequencing the first plurality of extension products and the second plurality of extension products, thereby spatially detecting the at least one target analyte in the first location of the tissue sample and the second location of the tissue sample.

26. (Currently Amended) The method of claim 20, wherein the sequencing step is performed using a next generation sequencing reaction.

30. (Currently Amended) The method of claim 20, wherein the nucleic acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site, the nucleic acid probes in the second plurality of nucleic acid probes comprise an amplification primer binding site, or both nucleic acid probes in the first plurality of nucleic acid probes comprise an amplification primer binding site and the nucleic acid probes in the second plurality of nucleic acid probes comprise an amplification primer binding site.

32. (Currently Amended) The method of claim 20, wherein the target binding domain[s comprise] of each probe from the first plurality of nucleic acid probes and the second plurality of nucleic acid probes comprises a single-stranded nucleic acid molecule.

3. The following is an examiner's statement of reasons for allowance:

Claims 1, 4, 6-13, and 15-34 are allowable in light of applicant's amendments filed on April 19, 2022 and May 3, 2022, the terminal disclaimer filed on May 16, 2022, and the examiner's amendment. The rejections under 35 U.S.C 112 (a) and (b) have been withdrawn in view of applicant's amendments filed on April 19, 2022 and May 3, 2022, and the examiner's amendment. The closest prior art in the record is Beechem *et al.,* (US 2020/0040385 A1, priority date: July 17, 2015). This prior art does not teach a combination of steps a) to f) of claim 1 and a combination of steps a) to g) of claim 20. This prior art either alone or in combination with the

other arts in the record does/do not teach or reasonably suggest a method for spatially detecting at least one target analyte in a first location and a second location of a tissue sample which comprises all limitations recited in claims 1 and 20

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance".

4. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center. The faxing of such papers must conform with the notices published in the Official Gazette, 1096 OG 30 (November 15, 1988), 1156 OG 61 (November 16, 1993), and 1157 OG 94 (December 28, 1993)(See 37 CAR § 1.6(d)). The CM Fax Center number is (571)273-8300.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Frank Lu, Ph.D., whose telephone number is (571)272-0746. The examiner can normally be reached on Monday-Friday from 9 A.M. to 5 P.M.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen, can be reached on (571)272-0731.

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). If you would

like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/FRANK W LU/
Primary Examiner, Art Unit 1634
May 18, 2022

Examiner-Initiated Interview Summary	**Application No.** 17/476,707	**Applicant(s)** BEECHEM et al.		
	Examiner FRANK W LU	**Art Unit** 1634	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
FRANK W LU	Primary Examiner	Telephonic
Dr. Robert Powers (Reg. No.77,802)	Attorney of Record	

Date of Interview: 17 May 2022

Issues Discussed:

Proposed Amendment(s)

Dr. Powers agreed with the examiner's amendment.

/FRANK W LU/ Primary Examiner, Art Unit 1634	May 17, 2022

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

NanoString EX2011.504

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/476,707	BEECHEM et al.
	Examiner	**Art Unit**
	FRANK W LU	1634

CPC					
Symbol				**Type**	**Version**
C12Q	/	1	/ 6876	F	2013-01-01
C12Q	/	1	/ 6818	I	2013-01-01
C12Q	/	1	/ 6858	I	2013-01-01
C12Q	/	1	/ 6848	I	2013-01-01
C12Q	/	1	/ 6869	I	2013-01-01
C12Q	/	1	/ 686	I	2013-01-01
C12Q	/	1	/ 6851	I	2013-01-01
C12Q	/	1	/ 6855	I	2013-01-01
C12Q	/	1	/ 6841	I	2013-01-01
C12Q	/	1	/ 6804	I	2013-01-01
C12Q	/	2600	/ 16	A	2013-01-01

CPC Combination Sets							
Symbol				**Type**	**Set**	**Ranking**	**Version**
C12Q	/	1	/ 6841	I	1	1	2013-01-01
C12Q	/	2523	/ 319	I	1	2	2013-01-01
C12Q	/	2531	/ 113	I	1	3	2013-01-01
C12Q	/	2537	/ 143	I	1	4	2013-01-01
C12Q	/	2563	/ 179	I	1	5	2013-01-01
C12Q	/	1	/ 6869	I	2	1	2013-01-01
C12Q	/	2525	/ 191	I	2	2	2013-01-01
C12Q	/	2531	/ 113	I	2	3	2013-01-01
C12Q	/	2565	/ 514	I	2	4	2013-01-01
C12Q	/	1	/ 6806	I	3	1	2013-01-01
C12Q	/	1	/ 6806	I	3	2	2013-01-01

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/FRANK W LU/ Primary Examiner, Art Unit 1634	18 May 2022	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

U.S. Patent and Trademark Office

Part of Paper No.: 5/2022

NanoString EX2011.505

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/476,707	BEECHEM et al.
	Examiner	**Art Unit**
	FRANK W LU	1634

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C12P	/	19	/	34

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**
435	91.2

CROSS REFERENCES(S)							
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**						

NONE (Assistant Examiner) (Date)	**Total Claims Allowed:** 30	
/FRANK W LU/ Primary Examiner, Art Unit 1634 (Primary Examiner) 18 May 2022 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure 1

U.S. Patent and Trademark Office

Part of Paper No.: 5/2022

NanoString EX2011.506

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/476,707	BEECHEM et al.
	Examiner	**Art Unit**
	FRANK W LU	1634

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1	10	13	19	23	28	32								
2	4	11	15	20	24	29	33								
3	6	12	16	21	25	30	34								
4	7	13	17	22	26										
5	8	14	18	23	27										
6	9	15	19	24	28										
7	10	16	20	25	29										
8	11	17	21	26	30										
9	12	18	22	27	31										

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/FRANK W LU/ Primary Examiner, Art Unit 1634	18 May 2022	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

U.S. Patent and Trademark Office

Part of Paper No.: 5/2022

NanoString EX2011.507

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	17/476,707	BEECHEM et al.
	Examiner	Art Unit
	FRANK W LU	1634

CPC - Searched*		
Symbol	Date	Examiner
Update	05/18/2022	/FL/

CPC Combination Sets - Searched*		
Symbol	Date	Examiner
Update	05/18/2022	/FL/

US Classification - Searched*			
Class	Subclass	Date	Examiner
Update		05/18/2022	/FL/

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Update (see attached files)	05/18/2022	/FL/

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
CPC C12Q	1/686, 6804, 6818, 6841, 6848, 6851, 6855, 6858, 6869, 6876; 2600/16	05/18/2022	/FL/

/FRANK W LU/ Primary Examiner, Art Unit 1634	May 18, 2022

NanoString EX2011.508

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(FILE 'HOME' ENTERED AT 23:27:34 ON 18 MAY 2022)

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     18 MAY 2022
L1     14662701 S DNA OR RNA OR NUCLEIC ACID OR POLYNUCLEOTIDE OR OLIGONUCLEOTI
L2      2404707 S PROBE OR PROBES
L3      5276491 S CONTACT OR CONTACTING OR HYBRIDIZE OR HYBRIDIZE OR HYBRIDIZAT
L4      2683818 S COLLECT OR COLLECTING OR COLLECTION
L5      2716133 S EXTENSION OR EXTENSE OR EXTENSING OR AMPLIFY OR AMPLIFYING OR
L6      2280786 S SEQUENCING
L7      6168094 S LOCATION OR LOCATIONS
L8            6 S L1 AND L2 AND L3 AND L4 AND L5 AND L6 AND L7
L9     10189563 S LOCATION OR LOCATONS OR POSITION OR POSITIONS
L10          14 S L1 AND L2 AND L3 AND L4 AND L5 AND L6 AND L9
L11           6 DUP REM L8 (0 DUPLICATES REMOVED)
L12          10 DUP REM L10 (4 DUPLICATES REMOVED)
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BIB DATA SHEET

CONFIRMATION NO. 5473

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
17/476,707	09/16/2021	435	1634	NATE-037C01US 321329-2719

APPLICANTS

NanoString Technologies, Inc., Seattle, WA;

INVENTORS

Joseph M. BEECHEM, Eugene, OR;
Dae KIM, Bellevue, WA;
Margaret HOANG, Seattle, WA;
Mark GREGORY, Boise, ID;
Erin PIAZZA, Edmonds, WA;
Denise ZHOU, Seattle, WA;

**** CONTINUING DATA ***************************

This application is a CON of 16/272,487 02/11/2019 ABN
which claims benefit of 62/771,212 11/26/2018
and claims benefit of 62/629,180 02/12/2018

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

09/21/2021

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
❑ Yes ☑ No	❑ Yes ❑ No	/FRANK LU/ Examiner's Signature	❑ Initials	OR	40	19	1

ADDRESS

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004
UNITED STATES

TITLE

CHEMICAL COMPOSITIONS AND USES THEREOF

FILING FEE RECEIVED		
2240	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	❑ All Fees ❑ 1.16 Fees (Filing) ❑ 1.17 Fees (Processing Ext. of time) ❑ 1.18 Fees (Issue) ❑ Other _____ ❑ Credit

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L18	6	probe same (DNA or RNA or "nucleic acid" or polynucleotide or oligonucleotide) same (contact or contacting or hybridize or hybridize or hybridization) same (collect or collecting or collection) same (extension or extense or extensing or amplify or amplifying or amplification) same sequencing same first near3 (location or position) same second near3 (location or position)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/05/18 23:27
L19	108	probe same (DNA or RNA or "nucleic acid" or polynucleotide or oligonucleotide) same (contact or contacting or hybridize or hybridize or hybridization) same (collect or collecting or collection) same (extension or extense or extensing or amplify or amplifying or amplification) same sequencing same (location or position)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/05/18 23:29
L20	78	probe same (DNA or RNA or "nucleic acid" or polynucleotide or oligonucleotide) same (contact or contacting or hybridize or hybridize or hybridization) same (collect or collecting or collection) same (extension or extense or extensing or amplify or amplifying or amplification) same sequencing same (identify or identifying or identification) same (location or position)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/05/18 23:30
L21	77	20 AND ((C12Q2537/143 OR C12Q1/686 OR C12Q1/6827 OR C12Q1/6806 OR C12Q1/6869 OR C12Q2600/156 OR C12Q2537/161 OR C12Q2537/165 OR C12Q1/6883 OR C12Q1/6874 OR C12Q2600/16 OR C12Q1/6848 OR C12Q2535/122 OR C12Q2563/179 OR C12Q2525/179 OR C12Q1/6862 OR C12Q2537/159 OR C12Q2545/114 OR C12Q2527/113 OR C12Q2527/143 OR C12Q2537/149 OR C12Q2525/191 OR C12Q2527/107 OR C12Q2531/113 OR C12Q2549/119 OR C12Q2565/514 OR C12Q1/6841 OR C12Q2533/107 OR C12Q1/68 OR C12Q1/6851 OR C12Q1/6886 OR C12Q2525/161 OR C12Q2525/301 OR C12Q2531/101 OR	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2022/05/18 23:30

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C12Q2523/319 OR C12Q1/6876 OR
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C12Q2537/16 OR C12Q2565/101 OR
C12Q1/6813 OR C12Q1/6816 OR
C12Q1/6888 OR C12Q2521/507 OR
C12Q2525/204 OR C12Q2531/137 OR
C12Q2561/125 OR C12Q2563/149 OR
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C12Q2525/173 OR C12Q2533/101 OR
C12Q2535/00 OR C12Q2537/1373 OR
C12Q2537/157 OR C12Q2561/109 OR
C12Q2563/131 OR C12Q2565/102 OR
C12Q2565/50 OR C12Q2565/601 OR
C12Q2600/118 OR C12Q2600/154 OR
C12Q2600/172 OR G16B20/00 OR
G16B20/10 OR G16B40/00 OR G16B20/20
OR G16B20/40 OR G16B30/00 OR
G16B30/10 OR G16B40/10 OR G16B5/00
OR G16B5/20 OR G16B25/20 OR
G01N2800/06 OR G01N33/57446 OR
G01N2458/10 OR G01N33/5308 OR
G01N2500/04 OR G01N33/54386 OR
G01N33/58 OR G01N33/6872 OR
C12N15/1003 OR C12N15/1065 OR
C12N15/1089 OR C12N15/1093 OR
C12N15/1017 OR C12N15/09 OR
C12N15/1068 OR C12N15/63 OR
C12N9/0083 OR B01J2219/00605 OR
B01J2219/0061 OR B01J2219/00612 OR
B01J2219/00617 OR B01J2219/00621 OR
B01J2219/00626 OR B01J2219/0063 OR
B01J2219/00637 OR B01J2219/00644 OR
B01J2219/00659 OR B01J2219/00662 OR
B01J19/0046 OR B01J2219/00722 OR
G06F19/18 OR G06F19/28 OR
G06F19/345 OR A23D9/00 OR
A61K2039/55522 OR A61K2039/55527 OR
A61K38/00 OR A61K38/193 OR
A61K39/39 OR A61P29/00 OR C07K14/53
OR C07K14/54 OR C07K2319/00 OR
C12P19/34 OR C12Y114/99001).CPC.)

5/18/2022 11:31:48 PM
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Complete if Known	
Application Number	17/476,707
Filing Date	09-16-2021
First Named Inventor	Joseph M. BEECHEM
Art Unit	1634
Examiner Name	F. W. Lu
Attorney Docket Number	NATE-037/C01US 321329-2719

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	9,376,677	06-28-2016	Mir	
	2.	9,644,204	05-09-2017	Hindson et al.	
	3.	9,975,122	05-22-2018	Masquelier et al.	
	4.	10,053,723	08-21-2018	Hindson et al.	
	5.	10,071,377	09-11-2018	Bharadwaj et al.	
	6.	2021/0403999	12-30-2021	Beechem et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	7.	WO 2018/026873 A1	02-08-2018	California Institute of Techology		

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /F.W.L/

Examiner Signature:	/FRANK W LU/	Date Considered	05/18/2022

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

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. 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 2 hours 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, 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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267356353 v1

PART B–FEE(S) TRANSMITTAL

Complete and send this form, together with the applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
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Alexandria, VA 22313-1450

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INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

COOLEY LLP/NanoString Technologies, Inc.
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO	CONFIRMATION NO
17/476,707	09/16/2021	Joseph M. BEECHEM	NATE-037/C01US 321329-2719	5473

TITLE OF INVENTION: CHEMICAL COMPOSITIONS AND USES THEREOF

APPLN: TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1,200.00	$0.00	$0.00	$1,200.00	08/24/2022

EXAMINER	ART UNIT	CLASS-SUBCLASS
F. W. Lu	1634	435-006110

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363)

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1. COOLEY LLP
2. Ivor R. Elrifi
3. Matthew Pavao

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: NanoString Technologies, Inc.

(B) RESIDENCE: (CITY and STATE or COUNTRY): Seattle, Washington

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment (*Please first reapply any previously paid fee shown above*):

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☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 50-1283

5. Change of Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29. NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27. NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status. NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Robert E. Powers/ Date May 24, 2022

Typed or printed name Robert E. Powers Registration No. 77,802

Electronic Patent Application Fee Transmittal

Application Number:	17476707
Filing Date:	16-Sep-2021
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Filer:	Robert E. Powers/Francesca Vandermaelen
Attorney Docket Number:	NATE-037C01US 321329-2719

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:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1200	1200

NanoString EX2011.516

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1200**

Electronic Acknowledgement Receipt

EFS ID:	45792419
Application Number:	17476707
International Application Number:	
Confirmation Number:	5473
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Customer Number:	141429
Filer:	Robert E. Powers/Francesca Vandermaelen
Filer Authorized By:	Robert E. Powers
Attorney Docket Number:	NATE-037C01US 321329-2719
Receipt Date:	24-MAY-2022
Filing Date:	16-SEP-2021
Time Stamp:	16:10:31
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1200
RAM confirmation Number	E20225NG10451407
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

NanoString EX2011.518

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	NATE-037_C01US_Issue_Fee.pdf	144085 ede979cdf6211f460c9389b5ae720f8958941154	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	38121 2447c5665f09484e9bc1cc6a35d1302589448a0e	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	182206		

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

Office of the Chief Financial Officer

Document Code:WFEE

User :71557

Sale Accounting Date:05/31/2022

Sale Item Reference Number
17476707

Effective Date
05/03/2022

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I20225SE23334399	1202	CLAIMS IN EXCESS OF 20	$1,000.00	Deposit Account

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.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/476,707	07/05/2022	11377689	NATE-037C01US 321329-2719	5473

141429 7590 06/15/2022

Cooley LLP/NanoString Technologies, Inc
1299 Pennsylvania Avenue NW
Suite 700
Washington, DC 20004

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PAIR WEB site http://pair.uspto.gov for additional inventors):

Joseph M. BEECHEM, Eugene, OR;
Dae KIM, Bellevue, WA;
Margaret HOANG, Seattle, WA;
Mark GREGORY, Boise, ID;
Erin PIAZZA, Edmonds, WA;

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

NanoString Technologies, Inc., Seattle, WA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

NanoString EX2011.521

DocCode - PATENT.GRANT

SCORE Placeholder Sheet for IFW Content

Application Number: 17476707 Document Date: 07/05/2022

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- Patent Grant with Color/Grayscale Drawings

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Form Revision Date: March 1, 2019

Docket No.: NATE-037/C01US 321329-2719
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Inventor:	Joseph M. BEECHEM	Confirmation No.:	5473
Application No.:	17/476,707	Filed:	September 16, 2021
Patent No.:	11,377,689	Issued:	July 5, 2022
Group Art Unit:	1634	Examiner:	F. W. Lu

For: CHEMICAL COMPOSITIONS AND USES THEREOF

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION
PURSUANT TO 37 C.F.R. § 1.322

Upon reviewing the above-identified patent, Patentee noted a typographical error which should be corrected.

In the Claims:

At column 145, claim number 1, line number 57-58,

"wherein the tissue sample [has been] is treated to facilitate binding of the nucleic acid"

should read

--wherein the tissue sample is treated to facilitate binding of the nucleic acid--

The error was not in the application as filed by applicant; accordingly no fee is required.

Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

NanoString EX2011.523

The Director is hereby authorized to charge any additional fees which may be required with respect to this communication, or credit any overpayment, to Deposit Account No. 50-1283, under Order No. NATE-037/C01US 321329-2719.

Dated: July 13, 2022

Respectfully submitted,
COOLEY LLP

USPTO CUSTOMER NO. 141429

COOLEY LLP
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Washington, DC 20004

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Electronic signature: / Matthew Pavao /
Robert Powers, Ph.D.
Reg. No. 77,802
Matthew Pavao, Ph.D.
Reg. No. 50,572

PTO/SB/44 (09-07)
Approved for use through 03/31/2023. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.	:	11,377,689
APPLICATION NO.	:	17/476,707
ISSUE DATE	:	July 5, 2022
INVENTOR(S)	:	Joseph M. BEECHEM et al.

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims:

At column 145, claim number 1, line number 57-58,

"wherein the tissue sample [has been] is treated to facilitate binding of the nucleic acid"

should read

--wherein the tissue sample is treated to facilitate binding of the nucleic acid--

MAILING ADDRESS OF SENDER (Please do not use Customer Number below):
Matthew Pavao, Ph.D.
COOLEY LLP
14th Floor
500 Boylston Street
Boston, MA 02116

NanoString EX2011.525

Electronic Acknowledgement Receipt

EFS ID:	46179168
Application Number:	17476707
International Application Number:	
Confirmation Number:	5473
Title of Invention:	CHEMICAL COMPOSITIONS AND USES THEREOF
First Named Inventor/Applicant Name:	Joseph M. BEECHEM
Customer Number:	141429
Filer:	Matthew Pavao/Francesca Vandermaelen
Filer Authorized By:	Matthew Pavao
Attorney Docket Number:	NATE-037C01US 321329-2719
Receipt Date:	13-JUL-2022
Filing Date:	16-SEP-2021
Time Stamp:	13:46:11
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	Request for Certificate of Correction	NATE-037_C01US_Request.pdf	218588 2fe7636c711ffb9b9ca3eb231a0614d4e495adf6	no	3

Warnings:

NanoString EX2011.526

Information:	
Total Files Size (in bytes):	218588

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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.

AO 120 (Rev. 08/10)

TO:	REPORT ON THE FILING OR DETERMINATION OF AN ACTION REGARDING A PATENT OR TRADEMARK
Mail Stop 8 Director of the U.S. Patent and Trademark Office P.O. Box 1450 Alexandria, VA 22313-1450	

In Compliance with 35 U.S.C. § 290 and/or 15 U.S.C. § 1116 you are hereby advised that a court action has been filed in the U.S. District Court District of Delaware on the following

☐ Trademarks or ☑ Patents. (☐ the patent action involves 35 U.S.C. § 292.):

DOCKET NO.	DATE FILED	U.S. DISTRICT COURT
22-cv-261-MFK	2/28/2022	District of Delaware

PLAINTIFF	DEFENDANT
10X GENOMICS, INC. and PRESIDENT AND FELLOWS OF HARVARD COLLEGE	NANOSTRING TECHNOLOGIES, INC.

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 10,227,639 B2	3/12/2019	PRESIDENT AND FELLOWS OF HARVARD COLLEGE
2	US 11,021,737 B2	6/1/2021	PRESIDENT AND FELLOWS OF HARVARD COLLEGE
3			
4			
5			

In the above—entitled case, the following patent(s)/ trademark(s) have been included:

DATE INCLUDED 5/12/2022 and 8/16/2022

INCLUDED BY ☒ Amendment ☒ Answer ☐ Cross Bill ☐ Other Pleading

	PATENT OR TRADEMARK NO.	DATE OF PATENT OR TRADEMARK	HOLDER OF PATENT OR TRADEMARK
1	US 11,293,051 B2	4/5/2022	PRESIDENT AND FELLOWS OF HARVARD COLLEGE
2	US 11,293,052 B2	4/5/2022	PRESIDENT AND FELLOWS OF HARVARD COLLEGE
3	US 11,293,054 B2	4/5/2022	PRESIDENT AND FELLOWS OF HARVARD COLLEGE
4	US 11,377,689 B2	7/5/2022	NanoString Technologies, Inc.
5			

In the above—entitled case, the following decision has been rendered or judgement issued:

DECISION/JUDGEMENT

CLERK	(BY) DEPUTY CLERK	DATE

Copy 1—Upon initiation of action, mail this copy to Director Copy 3—Upon termination of action, mail this copy to Director
Copy 2—Upon filing document adding patent(s), mail this copy to Director Copy 4—Case file copy

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 11,377,689 B2
APPLICATION NO. : 17/476707
DATED : July 5, 2022
INVENTOR(S) : Joseph M. Beechem et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

At Column 145, Claim number 1, Line number 57-58:
"wherein the tissue sample [has been] is treated to facilitate binding of the nucleic acid"
Should read:
--wherein the tissue sample is treated to facilitate binding of the nucleic acid--

Signed and Sealed this
Twenty-third Day of August, 2022

Katherine Kelly Vidal

Katherine Kelly Vidal
Director of the United States Patent and Trademark Office