EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	133	((array$4 or microarray$4 or support$4 or surface) near5 (dna or (nucleic adj acid) or protein or antibod$4)) same (probe near5 pool$6) same (probe near5 sequenc$6)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:54
L2	7450	(((high adj density) or (high adj resolution)) near5 (array$4 or microarray$4))near10 (dna or (nucleic adj acid) or protein or antibod$4)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:56
L3	7548	l1 or l2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:56
L4	3326193	@rlad<"20110405"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:57
L5	5137	l3 and l4	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:57
L6	1686	l5 and @pd>"20101231"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:58
L7	5	L5 and @pd<"20000101"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:59
L8	1479	L5 and @pd<"20050101"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 15:59
L9	3451	l5 not l6	US-PGPUB; USPAT;	OR	ON	2019/04/14 16:00

			USOCR; FPRS; EPO; JPO; DERWENT			
L10	1972	l9 not l8	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/04/14 16:00

4/14/2019 4:05:21 PM
C:\Users\jmartinell\Documents\EAST\Workspaces\16-276235-a.wsp

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 NEWS  1  DEC 20  2019 MeSH Version Now Available in MEDLINE on STN
 NEWS  2  JAN  4  DWPI Manual Code revision in 2019
 NEWS  3  JAN 17  First 2019 Emtree(R) update now available in Embase(TM)
 NEWS  4  JAN 29  The 2019 Version of MEDLINE Now Available on STN(TM)
 NEWS  5  FEB  4  INPADOC/INPAFAM: Major enhancements for PCT
 NEWS  6  FEB 26  FRFULL and GBFULL reloaded
 NEWS  7  MAR  7  Search Limits Significantly Expanded Exclusively in STNext(R)
 NEWS  8  MAR  7  New Key Terms field supports efficient full-text searching
                  and results evaluation in Patent Full-text Databases
 NEWS  9  MAR  8  CPC Combination Sets Now Fully Available in Derwent
                  World Patents Index
 NEWS 10  MAR 25  Derwent World Patent Index Coverage Expanded to Include
                  Eight Former Soviet Republics and the Eurasian Patent
                  Organization (EAPO)
 NEWS 11  APR  3  Derwent World Patents Index and Derwent Markush
                  Resource (DWPIM) Now Updated Twice Weekly
 NEWS 12  APR 11  Updates in database World Surface Coatings Abstracts
                  (file WSCA) have resumed

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FILE 'HOME' ENTERED AT 13:24:01 ON 12 APR 2019

=> file biosis caplus medline
COST IN U.S. DOLLARS                                SINCE FILE       TOTAL
                                                      ENTRY     SESSION
FULL ESTIMATED COST                                    0.40        0.40

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FILE 'MEDLINE' ENTERED AT 13:24:25 ON 12 APR 2019

=> s ((locat? or address or spac?)(20a)(bead? or microbead? or sphere? or microsphere?) and target? and (cod? or barcod? or
tag?))/bi,ab
L1            85 ((LOCAT? OR ADDRESS OR SPAC?)(20A)(BEAD? OR MICROBEAD? OR SPHERE
                 ? OR MICROSPHERE?) AND TARGET? AND (COD? OR BARCOD? OR TAG?))/BI
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                ,AB

=> s l1 not 2019/py
L2            81 L1 NOT 2019/PY

=> s l2 not 2018/py
L3            78 L2 NOT 2018/PY

=> s l3 not 2017/py
L4            76 L3 NOT 2017/PY

=> s l4 not 2016/py
L5            70 L4 NOT 2016/PY

=> s l5 not 2015/py
L6            62 L5 NOT 2015/PY

=> s l6 not 2014/py
L7            38 L6 NOT 2014/PY

=> s l7 not 2013/py
L8            27 L7 NOT 2013/PY

=> s l8 not 2012/py
L9            24 L8 NOT 2012/PY

=> dup rem l9
PROCESSING COMPLETED FOR L9
L10           20 DUP REM L9 (4 DUPLICATES REMOVED)

=> d his

     (FILE 'HOME' ENTERED AT 13:24:01 ON 12 APR 2019)

     FILE 'BIOSIS, CAPLUS, MEDLINE' ENTERED AT 13:24:25 ON 12 APR 2019
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L2            81 S L1 NOT 2019/PY
L3            78 S L2 NOT 2018/PY
L4            76 S L3 NOT 2017/PY
L5            70 S L4 NOT 2016/PY
L6            62 S L5 NOT 2015/PY
L7            38 S L6 NOT 2014/PY
L8            27 S L7 NOT 2013/PY
L9            24 S L8 NOT 2012/PY
L10           20 DUP REM L9 (4 DUPLICATES REMOVED)

=> d l10 1-20 bib ab
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L10 ANSWER 1 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2011:246257 CAPLUS Full-text
DN 155:164894
TI SearchFill: A stochastic optimization code for detecting atomic vacancies in crystalline and non-crystalline systems
AU Davis, Sergio M.; Belonoshko, Anatoly B.; Johansson, Boerje
CS Applied Materials Physics, Department of Materials Science and Engineering, KTH, Stockholm, SE-100 44, Swed.
SO Computer Physics Communications (2011), 182(5), 1105-1110
CODEN: CPHCBZ; ISSN: 0010-4655
DOI 10.1016/j.cpc.2010.12.009
PB Elsevier B.V.
DT Journal
LA English
AB We present an implementation of a stochastic optimization algorithm applied to location of atomic vacancies. Our method labels an empty point in space as a vacancy site, if the total spatial overlap of a "virtual sphere", centered around the point, with the surrounding atoms (and other vacancies) falls below a tolerance parameter. A Metropolis-like algorithm displaces the vacancies randomly, using an "overlap temperature" parameter to allow for acceptance of moves into regions with higher overlap, thus avoiding local min. Once the algorithm has targeted a point with low overlap, the overlap temperature is decreased, and the method works as a steepest descent optimization. Our method, with only two free parameters, is able to detect the correct number and coordinates of vacancies in a wide spectrum of condensed-matter systems, from crystals to amorphous solids, in fact in any given set of atomic coordinates, without any need of comparison with a reference initial structure.
OSC.G 3 THERE ARE 3 CAPLUS RECORDS THAT CITE THIS RECORD (3 CITINGS)
RE.CNT 11 THERE ARE 11 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 2 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2011:1346525 CAPLUS Full-text
DN 156:526034
TI Development of DNA point mutation detection methods using probe DNA-immobilized microbeads
AU Hashimoto, Masahiko; Tamai, Yusuke; Tsukagoshi, Kazuhiko
CS Department of Chemical Engineering and Materials Science, Doshisha University, Kyoto, Japan
SO Science and Engineering Review of Doshisha University (2011), 52(2), 93-98
CODEN: DDRKAZ; ISSN: 0036-8172
PB Doshisha University, Science and Engineering Research Institute
DT Journal
LA Japanese
AB One technique that can distinguish low-abundant mutant DNA from wild-type DNA is the ligase detection reaction (LDR) coupled to a primary polymerase chain reaction (PCR). The LDR products as a target obtained by the sequential PCR/LDR reactions can be analyzed in a variety of fashions such as microarray and slab gel electrophoresis. In the present work, we employed probe DNA-immobilized magnetic microbeads to selectively capture the fluorescently labeled-LDR products via the target hybridization with the probe. After the hybridization, the microbeads were rinsed and introduced into a capillary tube and packed in one space in the tube with a magnet. Photoluminescence from the beads aggregates in the tube was successfully obtained with fluorescence or chemiluminescence detection system, showing that the present method can be amenable to mutation detection.

L10 ANSWER 3 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN DUPLICATE 1
AN 2011:1147644 CAPLUS Full-text
DN 156:142398
TI Profiling and quantitative evaluation of three nickel-coated magnetic matrices for purification of recombinant proteins: helpful hints for the optimized nanomagnetisable matrix preparation
AU Nejadmoghaddam, Mohammad Reza; Chamankhah, Mahmood; Zarei, Saeed; Zarnani, Amir Hassan
CS Nanobiotechnology Research Center (NBRC), Avicenna Research Institute, ACECR, Tehran, Iran
SO Journal of Nanobiotechnology (2011), 9, 31
CODEN: JNOAAO; ISSN: 1477-3155
URL: http://www.jnanobiotechnology.com/content/pdf/1477-3155-9-31.pdf
DOI 10.1186/1477-3155-9-31
PB BioMed Central Ltd.
DT Journal; (online computer file)
LA English
AB Background: Several materials are available in the market that work on the principle of protein magnetic fishing by their histidine (His) tags. Little information is available on their performance and it is often quoted that greatly improved purification of histidine-tagged proteins from crude exts. could be achieved. While some com. magnetic matrixes could be used successfully for purification of several His-tagged proteins, there are some which have been proved to operate just for a few extent of His-tagged proteins. Here, we address quant. evaluation of three com. available Nickel nanomagnetic beads for purification of two His-tagged proteins expressed in Escherichia coli and present helpful hints for optimized purification of such proteins and preparation of nanomagnetisable matrixes. Results: Marked differences in the performance of nanomagnetic matrixes, principally on the basis of their specific binding capacity, recovery profile, the amount of imidazole needed for protein elution and the extent of target protein loss and purity were obtained. Based on the aforesaid criteria, one of these materials featured the best purification results (SiMAG/N-NTA/Nickel) for both proteins at the concentration of 4 mg/mL, while the other two (SiMAC-Nickel and SiMAG/CS-NTA/Nickel) did not work well with respect to specific binding capacity and recovery profile. Conclusions: Taken together, functionality of different types of nanomagnetic matrixes vary considerably. This variability may not only be dependent upon the structure and surface chemical of the matrix which in turn determine the affinity of interaction, but, is also influenced to a lesser extent by the phys. properties of the protein itself. Although the results of the present study may not be fully applied for all nanomagnetic matrixes, but provide a framework which could be used to profiling and quant. evaluation of other magnetisable matrixes and also provide helpful hints for those researchers facing same challenge.
OSC.G 5 THERE ARE 5 CAPLUS RECORDS THAT CITE THIS RECORD (5 CITINGS)
RE.CNT 24 THERE ARE 24 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 4 OF 20 BIOSIS COPYRIGHT (c) 2019 Clarivate Analytics on STN
AN 2011:624137 BIOSIS Full-text
DN PREV201100624137
TI Profiling and quantitative evaluation of three Nickel-Coated magnetic matrices for purification of recombinant proteins: Ielpful hints for the optimized nanomagnetisable matrix preparation.
AU Nejadmoghaddam, Mohammad Reza; Chamankhah, Mahmood; Zarei, Saeed; Zarnani, Amir Hassan [Reprint Author]
CS ACECR, Avicenna Res Inst, Nanobiotechnol Res Ctr NBRC, Tehran, Iran
zarnani25@yahoo.com
SO Journal of Nanobiotechnology, (AUG 8 2011) Vol. 9, pp. Article No.: 31.
http://www.jnanobiotechnology.com/.
ISSN: 1477-3155.
DOI 10.1186/1477-3155-9-31
DT Article
LA English
ED Entered STN: 28 Sep 2011
Last Updated on STN: 13 Jan 2016
AB Background: Several materials are available in the market that work on the principle of protein magnetic fishing by their histidine (His) tags. Little information is available on their performance and it is often quoted that greatly improved purification of histidine-tagged proteins from crude extracts could be achieved. While some commercial magnetic matrices could be used successfully for purification of several His-tagged proteins, there are some which have been proved to operate just for a few extent of His-tagged proteins. Here, we address quantitative evaluation of three commercially available Nickel nanomagnetic beads for purification of two His-tagged proteins expressed in Escherichia coli and present helpful hints for optimized purification of such proteins and preparation of nanomagnetisable matrices.Results: Marked differences in the performance of nanomagnetic matrices, principally on the basis of their specific binding capacity, recovery profile, the amount of imidazole needed for protein elution and the extent of target protein loss and purity were obtained. Based on the aforesaid criteria, one of these materials featured the best purification results (SiMAG/N-NTA/Nickel) for both proteins at the concentration of 4 mg/ml, while the other two (SiMAC-Nickel and SiMAG/CS-NTA/Nickel) did not work well with respect to specific binding capacity and recovery profile.Conclusions: Taken together, functionality of different types of nanomagnetic matrices vary considerably. This variability may not only be dependent upon the structure and surface chemistry of the matrix which in turn determine the affinity of interaction, but, is also influenced to a lesser extent by the physical properties of the protein itself. Although the results of the present study may not be fully applied for all nanomagnetic matrices, but provide a framework which could be used to profiling and quantitative evaluation of other magnetisable matrices and also provide helpful hints for those researchers facing same challenge.

L10 ANSWER 5 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2009:901702 CAPLUS Full-text
DN 151:261208
TI Liquid phase chip for detecting CYP2C9 and CYP2C19 gene mutation
IN Xu, Jiasen; He, Jiaying; Guo, Yuanjie
PA Guangzhou Surexam Bio-Tech Co., Ltd., Peop. Rep. China
SO Faming Zhuanli Shenqing, 30pp.
CODEN: CNXXEV
DT Patent
LA Chinese
FAN.CNT 1
PI

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
CN 101487052	A	20090722	CN 2009-10037358	20090224
CN 101487052	B	20111228		

PRAI CN 2009-10037358 20090224

AB A liquid-phase chip comprises: (1) microspheres coated with specific anti-tag sequences and having different colors, (2) wild-type and mutant ASPE primers for each mutation, wherein the primers are composed of 3'-terminal specific sequences and 5'-terminal tag sequences complementary with the anti-tag sequences, and (3) primers for amplifying the target sequences having CYP2C9 and/or CYP2C19 gene mutations. Spacing arms are arranged between the anti-tag sequences and the microspheres. The above 3'-terminal specific sequences of wild-type and mutant ASPE primers are disclosed. The detection with the liquid-phase chip has largely increased fluorescent signal value, sensitivity, signal-to-noise ratio, accuracy and reliability.

L10 ANSWER 6 OF 20 BIOSIS COPYRIGHT (c) 2019 Clarivate Analytics on STN
AN 2010:443399 BIOSIS Full-text
DN PREV201000443399
TI Transcolonic NOTES Peritoneoscopy for the Detection of Peritoneal Metastases.
AU Voermans, Rogier P.; Faigel, Douglas O.; Henegouwen, Mark I. van Berge; Conlon, Sean; Spivey, James T.; Rowe, Renee L.; Sheppard, Brett C.; Fockens, Paul
SO Gastroenterology, (MAY 2009) Vol. 136, No. 5, Suppl. 1, pp. A648.
Meeting Info.: Digestive Disease Week/110th Annual Meeting of the American-Gastroenterological-Association. Chicago, IL, USA. May 30 -June 04, 2009. Amer Gastroenterol Assoc.
CODEN: GASTAB. ISSN: 0016-5085.
DT Conference; (Meeting)
Conference; Abstract; (Meeting Abstract)
LA English
ED Entered STN: 4 Aug 2010
Last Updated on STN: 4 Aug 2010
AB Background: Natural Orifice Transluminal Endoscopic Surgery (NOTES) peritoneoscopy could replace laparoscopic peritoneoscopy (LAP) for staging of gastrointestinal malignancies. In previously performed porcine experiments we successfully created a model for peritoneal metastases and established a benchmark for LAP detection. In this model the NOTES transgastric peritoneoscopy (TGP) appeared to be inferior to LAP, mainly due to limited visualization of the liver. Visualisation of the liver could be improved by approaching through the colon. Aim: To compare the transcolonic peritoneoscopy (TCP) with previously set LAP standard in detecting peritoneal metastases. Methods: This investigator-initiated protocol was performed in a live acute porcine model. 2.5 mm color-coded beads were stapled via LAP in the peritoneum to simulate metastases. In the same model we previously established the benchmark for LAP detection: yield 95% of beads (90% CI: 91-100%). Using a non-inferiority design a sample size of 31 beads was determined. Three to 7 beads were placed in each of 6 animals. Randomization was performed for number and location of beads. Locations were based on the most likely targets for metastases and included: abdominal peritoneum (4 beads), diaphragm (8), surface of liver and hepatoduodenal ligament (18), and miscellaneous sites (1). TCP was then performed with a 2 channel therapeutic endoscope using either standard accessories (forceps, cap; TCP-s) or with a specially designed toolkit (bendable overtube, articulating retractors and graspers; TCP-t) in randomized order by one of 2 endoscopists, blinded to bead placement. Results: A total of 31 beads were placed into 6 pigs. TCP-s found 23 beads (yield 74%, 90% CI: 67-88%, non-inferior to LAP minus the margin of equivalence). TCP-t found 17 beads (yield 55%; 90% CI: 39-70%, inferior to LAP minus the margin of equivalence). TCP-s was superior for detecting beads in comparison with TCP-t (p=0.034). TCP-s was, using a transparent cap, superior in detecting beads on the inferior liver surface. Exclusion of these liver beads resulted in non significant difference between TCP-s and TCP-t (p=0.317). Conclusions: In this prospective, blinded, comparative trial, TCP was non inferior in comparison with the predetermined standard. The use of a transparent cap improved visualization of the inferior liver surface and should be integrated in the bendable overtube to achieve optimal results. TCP seems to result in improved visualization of the liver in comparison with TGP.

L10 ANSWER 7 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2008:1051430 CAPLUS Full-text
DN 149:371618
TI Method and liquid-phase chip for detecting and typing human papilloma virus
IN Xu, Jiasen; Lin, Yiqun; Yang, Huiyi
PA Guangzhou Surexam Biotech Co., Ltd., Peop. Rep. China
SO Faming Zhuanli Shenqing, 29pp.
CODEN: CNXXEV
DT Patent
LA Chinese
FAN.CNT 1
PI

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
CN 101250593	A	20080827	CN 2008-10026275	20080203
CN 101250593	B	20110126		

PRAI CN 2008-10026275 20080203

AB The title liquid-phase chip comprises: microspheres coated with specific anti-tag sequences, specific ASPE primers designed according to each human papilloma virus (HPV) subtypes, and primers for amplifying the target sequence containing mutation site. The anti-tag sequences are immobilized on the microspheres via spacing arm sequence of 7-10 T bases. The anti-tag sequences are selected from two or more of SEQ ID Number 25-SEQ ID Number 47. The ASPE primers are selected from two or more of SEQ ID Number 1-SEQ ID Number 23. Each ASPE primer comprises mutation-specific sequence at 3'-end, and a tag sequence at 5'-end. The tag sequence is complementary with the anti-tag sequence. The liquid-phase chip has the advantages of wide application range, high sensitivity, high signal/noise ratio, and accurate results.

L10 ANSWER 8 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2008:918964 CAPLUS Full-text
DN 150:162515
TI Use of stagnation point flows for DNA trapping, manipulation, and target sequence detection
AU Dylla-Spears, Rebecca; Sohn, Lydia L.; Muller, Susan J.
CS Department of Chemical Engineering, University of California, Berkeley, CA, 94720, USA
SO AIP Conference Proceedings (2008), 1027(Pt. 2, 15th International Congress on Rheology, 2008), 959-961
CODEN: APCPCS; ISSN: 0094-243X
PB American Institute of Physics
DT Journal
LA English
AB We use a stagnation point flow to manipulate single mols. of double-stranded genomic DNA for applications requiring detection of specific sequences along the DNA backbone. Fluorescent polystyrene beads are surface-functionalized with site-specific probes and incubated with fluorescently labeled double-stranded γ-DNA. The solution is introduced into a microfluidic cross slot where the tagged DNA mols. are trapped and elongated at the stagnation point of the planar extensional flow. Beads bound along the stretched DNA may be directly observed, and their locations along the backbone determined, using fluorescence microscopy. We have demonstrated the feasibility of this method for detection of specific sequences. (c) 2008 American Institute of Physics.
OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)
RE.CNT 8 THERE ARE 8 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 9 OF 20 MEDLINE ® on STN
AN 2008717598 MEDLINE Full-text
DN PubMed ID: 18982660
TI Fast marker based C-Arm pose estimation.
AU Kainz Bernhard
CS Institute for Computer Graphics and Vision, Graz University of Technology, Austria. kainz@icg.tugraz.at
AU Grabner Markus; Ruther Matthias
SO Medical image computing and computer-assisted intervention : MICCAI ... International Conference on Medical Image Computing and Computer-Assisted Intervention, (2008) Vol. 11, No. Pt 2, pp. 652-9.
Journal code: 101249582.
CY Germany: Germany, Federal Republic of
DT (EVALUATION STUDIES)
Journal; Article; (JOURNAL ARTICLE)
(RESEARCH SUPPORT, NON-U.S. GOV'T)
LA English
FS MEDLINE; Priority Journals
FS Print
EM 200812
ED Entered STN: 6 Nov 2008
Last Updated on STN: 2 Jan 2009
Entered Medline: 30 Dec 2008
OSC.G 3 There are 3 MEDLINE records that cite this record
AB To estimate the pose of a C-Arm during interventions therapy we have developed a small sized X-Ray Target including a special set of beads with known locations in 3D space. Since the patient needs to remain in the X-Ray path for all feasible poses of the C-Arm during the intervention, we cannot construct a single marker which is entirely visible in all images. Therefore finding 2D-3D point correspondences is a non-trivial task. The marker pattern has to be chosen in a way such that its projection onto the image plane is unique in a minimal-sized window for all relevant poses of the C-Arm. We use a two dimensional adaption of a linear feedback shift register (LFSR) to generate a two-dimensional pattern with unique sub-patterns in a certain window range. Thereby uniqueness is not achieved by placing unique 2D sub patterns side by side but by the code property itself. The code is designed in a way that any sub window of a minimal size guarantees uniqueness and that even occlusions from medical instruments can be handled. Experiments showed that we were able to estimate the C-Arm's pose from a single image within one second with a precision below one millimeter and one degree.

L10 ANSWER 10 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2009:178700 CAPLUS Full-text
DN 150:253112
TI First application of PCR-Luminex system for molecular diagnosis of fungicide resistance and species identification of fungal pathogens
AU Ishii, Hideo; Tanoue, Junko; Oshima, Michiyo; Chung, Wen-Hsin; Nishimura, Kumiko; Yamaguchi, Junichiro; Nemoto, Fumihiro; So, Kazuhiro; Iwama, Toshitaka; Yoshimatsu, Hideaki; Shimizu, Motoshige; Kozawa, Toru
CS National Institute for Agro-Environmental Sciences, Kannondai 3-1-3, Tsukuba, Ibaraki, 305-8604, Japan
SO Journal of General Plant Pathology (2008), 74(6), 409-416
CODEN: JGPPBQ; ISSN: 1345-2630
DOI 10.1007/s10327-008-0119-z
PB Springer Japan
DT Journal
LA English
AB Fungicide resistance in plant pathogens is often caused by a single point mutation in a gene encoding fungicide target proteins. Such is the case for resistance to MBI-D (inhibitors of scytalone dehydratase in melanin biosynthesis) fungicides in rice blast fungus (Magnaporthe oryzae), which is caused by a mutation in the scytalone dehydratase gene that results in a replacement of valine with methionine at codon 75 of the fungicide target protein. PCR-Luminex, a novel system developed for high-throughput anal. of single nucleotide polymorphisms (SNPs) was successfully introduced to diagnose MBI-D resistance using specific oligonucleotide probes coupled with fluorescent beads. The PCR-Luminex system was further tested for its potential in identifying species causing Fusarium head blight on wheat. Four major pathogens, Fusarium graminearum (=F. asiaticum), F. culmorum, F. avenaceum, and Microdochium nivale, known to cause the disease, were tested, and the species were identified using the PCR-Luminex method. So far, this report is the first on the application of the DNA-based PCR-Luminex system in the area of crop protection and/or agricultural sciences.
OSC.G 9 THERE ARE 9 CAPLUS RECORDS THAT CITE THIS RECORD (9 CITINGS)
RE.CNT 29 THERE ARE 29 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 11 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2007:359116 CAPLUS Full-text
DN 146:352601
TI Purifying nucleic acids using oligonucleotide attached to solid support
IN Slepnev, Vladimir I.
PA Primera Biosystems, Inc., USA
SO PCT Int. Appl., 37 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1
PI

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2007035685	A2	20070329	WO 2006-US36382	20060918
WO 2007035685	A3	20080124		

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW

RW: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG, BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AP, EA, EP, OA

CA 2622712	A1	20070329	CA 2006-2622712	20060918
US 20070072223	A1	20070329	US 2006-523255	20060918
EP 1924591	A2	20080528	EP 2006-814900	20060918

R: AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LI, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR, AL, BA, HR, MK, RS

PRAI US 2005-60717593 P 20050916
WO 2006-US36382 W 20060918
ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT
AB Described herein are compns. and methods for enriching a nucleic acid sample for target sequence(s). The compns. comprise an oligonucleotide attached to a solid support, directly or indirectly, wherein the oligonucleotide does not serve as a primer for a polymerase enzyme. Also provided is a method of performing a polymerase reaction using the compns. provided herein. The composition of the present invention can further comprise a second oligonucleotide. The second oligonucleotide comprises a tag binding sequence is substantially complementary to and hybridizes to a tag sequence within the first oligonucleotide. In one embodiment, the second oligonucleotide further comprises a target binding sequence, which is covalently linked to the tag binding sequence. Two examples of one oligonucleotide composition are single oligonucleotide attached at its 3' end to a magnetic bead, and attached at an internal location to a silica bead; the 3' terminal nucleotide being a substituted dideoxy nucleotide which prevents the oligonucleotide from serving as a primer for a polymerase enzyme.
OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)

L10 ANSWER 12 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN DUPLICATE 2
AN 2008:615762 CAPLUS Full-text
DN 149:4179
TI Neutron spectrometry in a pet cyclotron with a Bonner sphere system
AU Fernandez, F.; Amgarou, K.; Domingo, C.; Garcia, M. J.; Quincoces, G.; Marti-Climent, J. M.; Mendez, R.; Barquero, R.
CS Group de Fisica de les Radiations, Departament de Fisica, Universitat Autonoma de Barcelona, Bellaterra, E-08193, Spain
SO Radiation Protection Dosimetry (2007), 126(1-4), 371-375
CODEN: RPDODE; ISSN: 0144-8420
DOI 10.1093/rpd/ncm077
PB Oxford University Press
DT Journal
LA English
AB Positron emission tomog. (PET) is a non-invasive medical imaging technique normally used for diagnostic purposes to determine the location and concentration of physiol. active compds. in a human body. An unshielded cyclotron is used for PET at the Clinica Universitaria de Navarra to produce short-lived positron emitting radionuclides (15O, 13N, 11C and 18F) by bombarding appropriate target material with proton or deuteron beams with energies up to 18 and 9 MeV, resp. Subsequent nuclear reactions may generate undesirable neutrons that should be evaluated and controlled. In this study, the neutron measurements performed with an active and a passive Bonner sphere systems at different locations outside and inside the cyclotron vault during operation have been presented. The neutron spectrum at each location was determined with an unfolding code developed by the authors.
OSC.G 7 THERE ARE 7 CAPLUS RECORDS THAT CITE THIS RECORD (7 CITINGS)
RE.CNT 10 THERE ARE 10 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 13 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2006:294061 CAPLUS Full-text
DN 145:391545
TI Injection and manipulation of silicon microbeads in a customized microfluidic platform
AU Hoffmann, Daniel; Brennan, Des; Loughran, Michael
CS Biophotonics and Microfluidics Research, Tyndall National Institute, Univ. College Cork, Cork, Ire.
SO Proceedings of SPIE-The International Society for Optical Engineering (2006), 6112(Microfluidics, BioMEMS, and Medical Microsystems IV), 61120E/1-61120E/8
CODEN: PSISDG; ISSN: 0277-786X
DOI 10.1117/12.660342
PB SPIE-The International Society for Optical Engineering
DT Journal
LA English
AB The first on-chip injection and manipulation of optically encoded, silicon microbeads in a microfluidic platform is reported. Encoded microbeads of different shapes and sizes were fabricated in silicon via standard microfabrication techniques. The optical signature consisted of a series of lithog. defined bar-codes, which can be identified by a laser detection system. In-situ identification of encoded microbeads was possible at microbead velocities ≤ 50 cm per s. The microbeads can also be transported within a channel network in accordance with the encoded optical signature of each bead. The microbead transport is controlled by the laminar flow of a liquid in pressure driven microchannels. Hydrodynamic pulsing facilitated single and multiple injection of microbeads from a reservoir into the laminar fluid stream of a branched microfluidic network. Careful control of the fluid velocity and alteration of the microchannel geometry also enabled manipulation of microbead velocity. The incorporation of five pillars to retain microbeads at a specific location within the microchannel network formed the basis of a reaction chamber for on chip functionalization of microbeads. The principle of hydrodynamic switching was utilized to re-direct the transport of microbeads at a branched microfluidic network. In the final part of this research the authors verify that this microbead technol. is suitable for detection of specific target DNA.
RE.CNT 10 THERE ARE 10 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 14 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2005:371399 CAPLUS Full-text
DN 142:405531
TI Mutation typing method by identification tag ligation and sequencing
IN Koyama, Nobuto; Mineno, Junichi; Yamashita, Shusaku; Yamanaka, Nao; Sato, Masahiro; Izu, Hiroyuki; Takayama, Masanori; Asada, Kiyozo; Kato, Ikunoshin
PA Takara Bio Inc., Japan
SO PCT Int. Appl., 42 pp.
CODEN: PIXXD2
DT Patent
LA Japanese
FAN.CNT 1
PI

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2005038026	A1	20050428	WO 2004-JP15214	20041015

W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW
RW: BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

PRAI JP 2003-356850 A 20031016
AB A method of typing a mutation characterized by comprising the step of preparing target DNA containing a mutation site to be typed, the step of attaching a sample identification code to the target DNA, and the step of determining the base sequence of a region containing the sample identification code and the mutation site in the target DNA having the sample identification code attached thereto. A variation of the method comprises (a) ligation of ID code and the mutation site-containing region to a tag vector to construct a tag library, (b) preparing tag-ligated DNA from the library, (c) ligating to microbeads, (d) ligating initiation adaptor to the end of labeled DNA on microbeads, (e) spreading microbeads two dimensionally on flow cell and recording the location of each beads, (f) cleaving DNA on microbeads with restriction enzyme to obtain sticky ends, (g) ligating complementary encoded adaptor to the target DNA on microbeads, (h) hybridizing microbeads with decoder probe to analyze encoded adaptor, (i) removing decoder probe from microbeads to use microbeads in steps after (f).
OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)
RE.CNT 13 THERE ARE 13 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 15 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2003:856135 CAPLUS Full-text
DN 139:347728
TI Method for detecting ligands and targets in a mixture
IN Hammond, David J.; Lathrop, Julia Tait
PA American National Red Cross, USA
SO PCT Int. Appl., 39 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 6
PI

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2003089922	A1	20031030	WO 2003-US11799	20030414
W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW				
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG				
CA 2482529	A1	20031030	CA 2003-2482529	20030414
AU 2003231731	A1	20031103	AU 2003-231731	20030414
US 20030212253	A1	20031113	US 2003-414524	20030414
US 7223734	B2	20070529		
EP 1495318	A1	20050112	EP 2003-747009	20030414
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO, MK, CY, AL, TR, BG, CZ, EE, HU, SK				
JP 2005522712	T	20050728	JP 2003-586604	20030414
JP 4585766	B2	20101124		
CN 1659432	A	20050824	CN 2003-813727	20030414
CN 100525829	C	20090812		
HK 1082040	A1	20100423	HK 2006-102446	20060223
US 20080160501	A1	20080703	US 2007-797318	20070502
US 20090258834	A1	20091015	US 2007-802730	20070524
PRAI US 2002-60372091	P	20020415		
US 2003-414524	A1	20030414		
WO 2003-US11799	W	20030414		

ASSIGNMENT HISTORY FOR US PATENT AVAILABLE IN LSUS DISPLAY FORMAT

AB The invention provides a method of characterizing a target that binds to a ligand. The method comprises providing ligands, optionally attached to a support, and contacting the ligands with targets to allow at least one target to bind to at least one ligand. The method further comprises immobilizing the resulting complexes in a first matrix, such that each complex has a different position within the first matrix, and transferring the target of the complex to a second matrix. The position of the target within the second matrix corresponds to the position of the ligand-support complex within the first matrix. The target on the second matrix is then detected. Ligand-support complexes displaying a library of peptide hexamers were incubated with diluted blood plasma. The beads were washed, suspended in buffer, combined with low m.p. agarose and spread across an agarose base. The resin-agarose mixture was allowed to harden to form a gel system before target protein was transfered onto a second matrix membrane and detected. Resin beads aligning with locations of target protein on the membrane were selected, washed, and reincubated with plasma. Transfer and detection of target protein on the membrane was repeated. Several peptides were identified that bound to both von Willebrand factor and to Factor VIII.

OSC.G 1 THERE ARE 1 CAPLUS RECORDS THAT CITE THIS RECORD (1 CITINGS)
RE.CNT 3 THERE ARE 3 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 16 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2002:664468 CAPLUS Full-text
DN 137:334742
TI Detection of DNA hybridization via fluorescent polymer superquenching
AU Kushon, Stuart A.; Ley, Kevin D.; Bradford, Kirsten; Jones, Robert M.; McBranch, Duncan; Whitten, David
CS QTL Biosystems LLC, Santa Fe, NM, 87507, USA
SO Langmuir (2002), 18(20), 7245-7249
CODEN: LANGD5; ISSN: 0743-7463
DOI 10.1021/la026211u
PB American Chemical Society
DT Journal
LA English
AB An assay for a target single strand 20-base sequence of DNA coding for the anthrax lethal factor, based on conjugated polymer fluorescence superquenching, is reported. The assay employs a platform in which the receptor (a biotinylated complementary sequence "capture strand") and polymer (two components: an anionic poly(phenylene ethynylene) (PPE) and a biotinylated -PPE) are co-located on streptavidin-derivatized polystyrene microspheres. A conjugate of the target strand with the energy transfer quencher QSY-7 (DNA-QTL) is used to construct competition assays for the target. A direct competition assay between the target-DNA and DNA-QTL for the microsphere-bound capture is only marginally successful due evidently to greater kinetic affinity of the polymer-capture ensemble for the conjugate. However a sequential addition of target, followed by DNA-QTL affords a quant. assay for the target by attenuation of PPE fluorescence quenching by the DNA-QTL. Likewise a direct competition in solution between the target and DNA-QTL for the biotinylated capture strand followed by addition of microspheres provides a sensitive and quant. assay for the target single strand DNA.
OSC.G 121 THERE ARE 121 CAPLUS RECORDS THAT CITE THIS RECORD (121 CITINGS)
RE.CNT 28 THERE ARE 28 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 17 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2000:191259 CAPLUS Full-text
DN 132:232708
TI Method for identification in Drosophila of dominant suppressor genes useful for gene therapy
IN Rorth, Pernille; Cohen, Stephen M.
PA European Molecular Biology Laboratory, Germany
SO PCT Int. Appl., 44 pp.
CODEN: PIXXD2
DT Patent
LA English
FAN.CNT 1
PI

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
WO 2000015843	A1	20000323	WO 1999-IB1548	19990907
W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW				
RW: GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
AU 9955283	A	20000403	AU 1999-55283	19990907
PRAI GB 1998-19914	A	19980911		
WO 1999-IB1548	W	19990907		

AB The invention relates to a method for the identification of a dominant suppressor gene useful in gene therapy and the use of the identified gene for diagnosis or treatment of a disease caused by a genetic mutation in a mammal. The genes are identified in Drosophila by a screening technique that utilizes a modular misexpression system that allows conditional expression of genes tagged by insertion of a P-element carrying a GAL4 regulatable Enhancer and a basal promoter oriented to direct expression of adjacent genes. A Drosophila strain carrying a mutant gene is crossed with a library of target strain to form a crossed strain, and the gene that alters the level or effects of expression of the mutant gene is identified. The gene therapy consists of introducing the dominant suppressor gene cloned in a vector under the control of regulatory sequences to block the abnormal mutant gene activity in a mammal. In particular, gene therapy for cancer, neurodegenerative disease, or T cell acute lymphocytic lymphoma, using LDB1 dominant suppressor gene is proposed. Two EP element insertions on the Drosophila X chromosome producing phenotypes suggesting a role in dorsal-ventral axis formation when expressed in the developing wing, were independently isolated. These elements, EP1306 and EP1394, were mapped to cytol. position 17C1-4 on polytene chromosome corresponding to Beadex gene location, and the sequence adjacent to the insertion sites were overexpressed in Beadex1 mutant wing disks, suggesting that the EP elements direct the expression of the Beadex gene. Genetic interactions between Beadex and apterous or chip genes were observed Studies suggested that the protein encoded by the Beadex gene, dLMO protein, competes with Apterous for binding to Chip and thus interferes with Apterous-Chip complex formation.

RE.CNT 5 THERE ARE 5 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

L10 ANSWER 18 OF 20 BIOSIS COPYRIGHT (c) 2019 Clarivate Analytics on STN
DUPLICATE 3
AN 1996:361748 BIOSIS Full-text
DN PREV199699084104
TI Selection of chymotrypsin inhibitors from a conformationally-constrained combinatorial peptide library.
AU McBride, Jeffrey D.; Freeman, Neil; Domingo, Gonzalo J.; Leatherbarrow, Robin J. [Reprint author]
CS Dep. Chemistry, Imperial Coll. Sci. Technol. Med., South Kensington, London SW7 2AY, UK
SO Journal of Molecular Biology, (1996) Vol. 259, No. 4, pp. 819-827.
CODEN: JMOBAK. ISSN: 0022-2836.
DT Article
LA English
ED Entered STN: 14 Aug 1996
Last Updated on STN: 14 Aug 1996
AB A synthetic library of cyclic peptides was constructed utilizing the anti-tryptic loop region of the Bowman-Birk inhibitor, D4 from Macrotyloma axillare, as a template. The loop region of this proteinase inhibitor was reproduced by an 11 residue sequence, conformationally constrained by the presence of a disulfide bridge, to act as a mimetic of the functional reactive site region of this protein. This sequence, plus a pentaglycine spacer arm, was used to create a "one bead, one peptide" combinatorial library after on-resin deprotection and cyclization. Randomization at three positions considered to be important for proteinase specificity (P-2, P-1 and P-2) with the genetically coded amino acids (minus cysteine) plus norleucine generated 8000 permutations. Screening this library with biotinylated alpha-chymotrypsin under appropriate conditions revealed a small number (lt 0.05%) of beads that selectively bound the labeled proteinase. The sequences present on these active beads were determined, and found to have a well-defined consensus. Analysis of chymotrypsin inhibition in solution using re-synthesized peptides reveals that the sequences identified are potent inhibitors with K-i values in the nanomolar range. These results show that directed randomization of the canonical loop is a powerful way of generating proteinase inhibitors with targeted specificities. Incorporation of selective random changes within a defined structural framework is found to be an effective means of generating variation in large synthetic systems. The functional basis for inhibition by the identified sequences is discussed.

L10 ANSWER 19 OF 20 BIOSIS COPYRIGHT (c) 2019 Clarivate Analytics on STN
AN 1993:79987 BIOSIS Full-text
DN PREV199395044487
TI Quantitative single photon emission tomography: Verification for sources in an elliptical water phantom.
AU Ljungberg, Michael H. [Reprint author]; King, Michael A.; Strand, Sven-Erik
CS Radiation Physics Dep, Univ. Lund, Lasarettet, S-22185 Lund, Sweden
SO European Journal of Nuclear Medicine, (1992) Vol. 19, No. 10, pp. 838-844. CODEN: EJNMD9. ISSN: 0340-6997.
DT Article
LA English
ED Entered STN: 26 Jan 1993
Last Updated on STN: 26 Jan 1993
AB Accurate absorbed dose calculations are important for a proper dose planning in internal radionuclide therapy. The activity distribution must be measured and the target volume defined. This can be done with single photon emission tomography (SPET) if proper attenuation and scatter correction are employed. This study investigated the calculation of the activity and the volume of different spherical sources. These two parameters are essential for a proper dose calculation. The scatter and attenuation correction method is based on spatially variant scatter functions and density maps. The volume calculation method is based on obtaining a threshold from a grey-level histogram. Both point sources and spheres of different diameters containing technetium-99m were placed in different locations in an elliptical water phantom and imaged by SPET. The activity and the volume of the spheres were calculated from the SPET images and compared with known activities. Results show a quantification of activity within 10% for most of the sources. Important influences on the quantification are (a) the presence of artefacts due to improper reconstruction and (b) the finite spatial resolution which affects the total number of counts within the determined volume.

L10 ANSWER 20 OF 20 CAPLUS COPYRIGHT 2019 ACS on STN
AN 1976:23528 CAPLUS Full-text
DN 84:23528
OREF 84:3831a,3834a
TI Neutron spectrum from an oralloy sphere
AU Ragan, C. E.; Auchampaugh, G. F.; Hemmendinger, A.; Silbert, M. G.
CS Los Alamos Sci. Lab., Los Alamos, NM, USA
SO Report (1975), LA-5949-MS, 15 pp. Avail.: NTIS
From: Nucl. Sci. Abstr. 1975, 32(7), Abstr. No. 18502
DT Report
LA English
AB The neutron [12586-31-1] spectrum from a 38-kg fully enriched U (orally) sphere, pulsed by a centrally located 14-MeV n source, was measured by using time-of-flight (TOF) techniques. The nearly isotropic source of 14-MeV n was produced by bombarding a T gas target with low-energy d. Neutrons of 0.180-15.0 MeV were detected at the end of a partially evacuated, 39-m flight path by a NE-213 liquid scintillator that used pulse-shape discrimination to distinguish n events from γ-ray events. Detector thresholds of 0.15 and 0.85 MeV were used to cover the energy range, and the detector efficiency was measured over this range by using monoenergetic n from T(p,n), T(d,n), and D(d,n) reactions. Several measurements of the TOF spectrum were used to determine the n flux at the detector, and a comparison of this flux with the results of Monte-Carlo calcns. is presented. These calcns. were performed with the code MCN using 2 cross-section sets, those from Lawrence Livermore Laboratory and those from ENDF/B-IV library. The results calculated by using ENDF/B-IV cross sections are in excellent agreement with measurements, especially at 1-6 MeV, where the uncertainties in both the calculated and exptl. results are smallest.

=> d his

(FILE 'HOME' ENTERED AT 13:24:01 ON 12 APR 2019)

FILE 'BIOSIS, CAPLUS, MEDLINE' ENTERED AT 13:24:25 ON 12 APR 2019
L1 85 S ((LOCAT? OR ADDRESS OR SPAC?)(20A)(BEAD? OR MICROBEAD? OR SPH
L2 81 S L1 NOT 2019/PY
L3 78 S L2 NOT 2018/PY
L4 76 S L3 NOT 2017/PY
L5 70 S L4 NOT 2016/PY
L6 62 S L5 NOT 2015/PY
L7 38 S L6 NOT 2014/PY
L8 27 S L7 NOT 2013/PY
L9 24 S L8 NOT 2012/PY
L10 20 DUP REM L9 (4 DUPLICATES REMOVED)

=> log y

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	261.79	262.19

STN INTERNATIONAL LOGOFF AT 13:27:36 ON 12 APR 2019

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	Unknown
Patent No.	:	9,371,598	Examiner	:	Unknown
Issue Date	:	June 21, 2016	Conf. No.	:	2142
Application No.	:	13/080,616			
Filed	:	April 5, 2011			
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

REVOCATION AND NEW POWER OF ATTORNEY

The undersigned for **10X Genomics, Inc.** ("10X") and **Prognosys Biosciences, Inc.**, ("Prognosys") acting on behalf of **10X** and **Prognosys**, hereby revokes all powers of attorney previously granted in the application.

By virtue of an agreement effective as of November 18, 2018 between **10X** and **Prognosys**, **10X** is authorized to direct prosecution of the above-identified patent application.

The undersigned for **10X** and **Prognosys**, acting on behalf of **10X** and **Prognosys**, hereby appoints **10X**'s attorney(s) and agent(s) at Fish & Richardson P.C. (the "Firm"), which are associated with the Customer Number provided below, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, said appointment to be to the exclusion of the inventor(s) and his/their attorney(s) and agent(s) in accordance with the provisions of 37 CFR §3.71, et seq. of the Patent Office Rules of Practice.

159367

The undersigned also direct all correspondence be addressed to that Customer Number.

PTO Customer Number: 159367

By executing this Power of Attorney, **Prognosys** is allowing the Firm to prosecute the above-referenced patent application and to prosecute any matters that may arise before the U.S. Patent & Trademark Office ("PTO") in the enforceable life of the above-referenced patent. Although this Power of Attorney states that **Prognosys** is appointing the Firm with "power of attorney" for the sole purpose of prosecuting the matters arising before the PTO in connection with the patent and patent application, that appointment is understood solely in light of

Prognosys's ownership interest in the application. So, despite the use of the phrase "power of attorney," the executed Power of Attorney does not create an attorney-client relationship between **Prognosys** and the Firm, or, for that matter, between the Firm and any other person. Moreover, communications between **Prognosys** and the Firm will not be withheld from **10X**.

10X will remain the Firm's sole client with respect to the subject application and patent. Accordingly, the Firm understands, and your signature on the Power of Attorney confirms, that the Firm has no attorney-client relationship with **Prognosys**, despite your execution of this Power of Attorney.

Each undersigned, whose title is supplied below, is empowered to act on behalf of the indicated party.

Respectfully submitted,

Date: April 12, 2019

Printed Name: Eric S. Whitaker
10X Genomics, Inc.

Title: General Counsel

Date: May 3, 2019

Printed Name: MARK CHEE
Prognosys Biosciences, Inc.

Title: CEO

Curio EX1010-p. 557
Curio v Prognosys

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61850684.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	Unknown
Application No.	:	15/187,661	Examiner	:	Unknown
Filed	:	June 20, 2016	Conf. No.	:	1038
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

REVOCATION AND NEW POWER OF ATTORNEY

The undersigned for **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby revokes all powers of attorney previously granted in the application.

By virtue of an agreement effective as of November 18, 2018 between **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, **10X Genomics, Inc.** is authorized to direct prosecution of the above-identified patent application.

The undersigned for **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby appoints **10X Genomics, Inc.**'s attorney(s) and agent(s) at Fish & Richardson P.C. (the "Firm"), which are associated with the Customer Number provided below, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, said appointment to be to the exclusion of the inventor(s) and his/their attorney(s) and agent(s) in accordance with the provisions of 37 CFR §3.71, et seq. of the Patent Office Rules of Practice.

159367

The undersigned also direct all correspondence be addressed to that Customer Number.

PTO Customer Number: 159367

By executing this Power of Attorney, **Prognosys Biosciences, Inc.** is allowing the Firm to prosecute the above-referenced patent application and to prosecute any matters that may arise before the U.S. Patent & Trademark Office ("PTO") in the enforceable life of the above-referenced patent. Although this Power of Attorney states that **Prognosys Biosciences, Inc.** is appointing the Firm with "power of attorney" for the sole purpose of prosecuting the matters

arising before the PTO in connection with the patent and patent application, that appointment is understood solely in light of **Prognosys Biosciences, Inc.**'s ownership interest in the application. So, despite the use of the phrase "power of attorney," the executed Power of Attorney does not create an attorney-client relationship between **Prognosys Biosciences, Inc.** and the Firm, or, for that matter, between the Firm and any other person. Moreover, communications between **Prognosys Biosciences, Inc.** and the Firm will not be withheld from **10X Genomics, Inc.**.

10X Genomics, Inc. will remain the Firm's sole client with respect to the subject application and patent. Accordingly, the Firm understands, and your signature on the Power of Attorney confirms, that the Firm has no attorney-client relationship with **Prognosys Biosciences, Inc.**, despite your execution of this Power of Attorney.

Each undersigned, whose title is supplied below, is empowered to act on behalf of the indicated party.

Respectfully submitted,

Date: April 12, 2019

Printed Name: Eric S. Whitaker
10X Genomics, Inc.

Title: General Counsel

Date: May 3, 2019

Printed Name: MARK CHEE
Prognosys Biosciences, Inc.

Title: CEO

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61886512.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1636
Application No.	:	16/276,235	Examiner	:	Unknown
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

REVOCATION AND NEW POWER OF ATTORNEY

The undersigned for **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby revokes all powers of attorney previously granted in the application.

By virtue of an agreement effective as of November 18, 2018 between **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, **10X Genomics, Inc.** is authorized to direct prosecution of the above-identified patent application.

The undersigned for **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10X Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby appoints **10X Genomics, Inc.**'s attorney(s) and agent(s) at Fish & Richardson P.C. (the "Firm"), which are associated with the Customer Number provided below, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, said appointment to be to the exclusion of the inventor(s) and his/their attorney(s) and agent(s) in accordance with the provisions of 37 CFR §3.71, et seq. of the Patent Office Rules of Practice.

159367

The undersigned also direct all correspondence be addressed to that Customer Number.

PTO Customer Number: 159367

By executing this Power of Attorney, **Prognosys Biosciences, Inc.** is allowing the Firm to prosecute the above-referenced patent application and to prosecute any matters that may arise before the U.S. Patent & Trademark Office ("PTO") in the enforceable life of the above-referenced patent. Although this Power of Attorney states that **Prognosys Biosciences, Inc.** is appointing the Firm with "power of attorney" for the sole purpose of prosecuting the matters

arising before the PTO in connection with the patent and patent application, that appointment is understood solely in light of **Prognosys Biosciences, Inc.**'s ownership interest in the application. So, despite the use of the phrase "power of attorney," the executed Power of Attorney does not create an attorney-client relationship between **Prognosys Biosciences, Inc.** and the Firm, or, for that matter, between the Firm and any other person. Moreover, communications between **Prognosys Biosciences, Inc.** and the Firm will not be withheld from **10X Genomics, Inc.**.

10X Genomics, Inc. will remain the Firm's sole client with respect to the subject application and patent. Accordingly, the Firm understands, and your signature on the Power of Attorney confirms, that the Firm has no attorney-client relationship with **Prognosys Biosciences, Inc.**, despite your execution of this Power of Attorney.

Each undersigned, whose title is supplied below, is empowered to act on behalf of the indicated party.

Curio EX1010-p. 563
Curio v Prognosys

Respectfully submitted,

Date: April 12, 2019

Printed Name: Eric S. Whitaker
10X Genomics, Inc.

Title: General Counsel

Date: May 3, 2019

Printed Name: MARK CHEE
Prognosys Biosciences, Inc.

Title: CEO

CVL/jxw

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61886515.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1636
Application No.	:	16/276,260	Examiner	:	Unknown
Filed	:	February 14, 2019	Conf. No.	:	8647
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

REVOCATION AND NEW POWER OF ATTORNEY

The undersigned for **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby revokes all powers of attorney previously granted in the application.

By virtue of an agreement effective as of November 18, 2018 between **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, **10x Genomics, Inc.** is authorized to direct prosecution of the above-identified patent application.

The undersigned for **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby appoints **10x Genomics, Inc.**'s attorney(s) and agent(s) at Fish & Richardson P.C. (the "Firm"), which are associated with the Customer Number provided below, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, said appointment to be to the exclusion of the inventor(s) and his/their attorney(s) and agent(s) in accordance with the provisions of 37 CFR §3.71, et seq. of the Patent Office Rules of Practice.

159367

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PTO Customer Number: 159367

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Each undersigned, whose title is supplied below, is empowered to act on behalf of the indicated party.

Curio EX1010-p. 566
Curio v Prognosys

Respectfully submitted,

Date: April 12, 2019

Printed Name: Eric S. Whitaker
10x Genomics, Inc.

Title: General Counsel

Date: May 3, 2019

Printed Name: MARK CHEE
Prognosys Biosciences, Inc.

Title: CEO

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61886516.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark Chee	Art Unit	:	Unknown
Patent No.	:	9,868,979	Examiner	:	Unknown
Issue Date	:	January 16, 2018	Conf. No.	:	9096
Application No.	:	14/900,602			
Filed	:	December 21, 2015			
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS USING A MICROFLUIDIC DEVICE			

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

REVOCATION AND NEW POWER OF ATTORNEY

The undersigned for **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby revokes all powers of attorney previously granted in the application.

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The undersigned also direct all correspondence be addressed to that Customer Number.

PTO Customer Number: 159367

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Respectfully submitted,

Date: April 12, 2019

Printed Name: Eric S. Whitaker
10x Genomics, Inc.

Title: General Counsel

Date: May 3, 2019

Printed Name: MARK CHEE
Prognosys Biosciences, Inc.

Title: CEO

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61886518.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	Unknown
Patent No.	:	9,879,313	Examiner	:	Unknown
Issue Date	:	January 30, 2018	Conf. No.	:	5726
Application No.	:	14/900,604			
Filed	:	December 21, 2015			
Title	:	METHODS AND SYSTEMS FOR DETERMINING SPATIAL PATTERNS OF BIOLOGICAL TARGETS IN A SAMPLE			

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

REVOCATION AND NEW POWER OF ATTORNEY

The undersigned for **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, acting on behalf of **10x Genomics, Inc.** and **Prognosys Biosciences, Inc.**, hereby revokes all powers of attorney previously granted in the application.

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PTO Customer Number: 159367

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Respectfully submitted,

Date: April 12, 2019

Printed Name: Eric S. Whitaker
10x Genomics, Inc.

Title: General Counsel

Date: May 3, 2019

Printed Name: MARK CHEE
Prognosys Biosciences, Inc.

Title: CEO

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61886532.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	Unknown
Application No.	:	15/831,158	Examiner	:	Unknown
Filed	:	December 4, 2017	Conf. No.	:	5500
Title	:	METHODS AND SYSTEMS FOR DETERMINING SPATIAL PATTERNS OF BIOLOGICAL TARGETS IN A SAMPLE			

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

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Respectfully submitted,

Date: April 12, 2019

Printed Name: Eric S. Whitaker
10x Genomics, Inc.

Title: General Counsel

Date: May 3, 2019

Printed Name: MARK CHEE
Prognosys Biosciences, Inc.

Title: CEO

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61886523.doc

Electronic Acknowledgement Receipt

EFS ID:	35965577
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Judith Witzel
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	09-MAY-2019
Filing Date:	14-FEB-2019
Time Stamp:	13:31:47
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	Power of Attorney	PrognosysnonclientPOAs.pdf	2336907 4a16d7b258c5ce8211aab57536e85442414 28c14	no	21

Warnings:

Information:	
Total Files Size (in bytes):	2336907

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

REMARKS

Claims 2, 13, 14, 21, 28, and 30-31 have been amended in accordance with Applicant's discussion with Examiner Martinell on May 8, 2019. Claim 29 has been canceled without prejudice. New claim 32 has been added. Support for new claim 32 is found throughout Applicant's specification and in currently pending claim 22.

No new matter has been added. Applicant reserves the right to pursue any subject matter canceled as a result of the present amendments in future prosecution, either in this application or in one or more continuing applications.

In light the present amendments, arguments, and submissions, Applicant respectfully requests reconsideration and allowance of claims 2-28 and 30-31, and consideration and allowance of newly added claim 32.

Examiner Interview

Applicant thanks Examiner Martinell for the courtesy of having the telephonic interview conducted on May 8, 2019 with the undersigned and Teresa A. Lavoie, Ph.D., J.D., USPTO Registration Number 42,782. The various claim amendments, cancellations, and additions that were discussed are reflected in the above listing of the claims.

Information Disclosure Statement

Applicant thanks Examiner Martinell for pointing out certain errors in the listing of references in the IDS filed March 15, 2019.

Regarding the list of US patent applications provided in the March 15, 2019 IDS transmittal, each of these was cited by a corresponding patent or publication number in that IDS.

Reference Number 165, originally cited by application number, was cited by its publication patent number in the March 15, 2019 IDS. Reference Numbers 345 and 346 are provisional applications that matured into applications that issued as US Patent Numbers 9,879,313 and 9,868,979, respectively, each of which was cited in the March 15, 2019 IDS. Reference Number 347 is a provisional application with a priority date much later than the instant application. Reference Number 644 is not in English and will not be cited.

Objections to the Drawings

The drawings were objected to for including reference characters not mentioned in the description. The specification has been amended to include reference characters 150, 160, 200, 306, and 550. As such, the specification now complies with the requirements of 37 CFR 1.121(b), rendering this objection moot.

Non-Statutory Double Patenting Rejection

Claims 2-31 were provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12, 20, 24-28, 34, 36-39, and 58-77 of copending Application No. 15/187,661 in view of any one of Fu et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), Rothberg et al (U.S. Patent Application Publication 2009/0026082 Al, particularly paragraph 0339), Chee et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), or Leamon et al (U.S. Patent Application Publication 2005/0130173 Al, particularly paragraph 0083). Without conceding the merits of this rejection and solely to advance prosecution of the present application, Applicant will submit a terminal disclaimer over the '661 application. As such, Applicant respectfully requests withdrawal of this rejection upon acceptance of the terminal disclaimer.

Claims 2-31 were provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 2-23 of copending Application No. 16/276,260 in view of any one of Fu et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), Rothberg et al (U.S. Patent Application Publication 2009/0026082 Al, particularly paragraph 0339), Chee et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), or Leamon et al (U.S. Patent Application Publication 2005/0130173 Al, particularly paragraph 0083). Without conceding the merits of this rejection and solely to advance prosecution of the present application, Applicant will submit a terminal disclaimer over the '260 application. As such, Applicant respectfully requests withdrawal of this rejection upon acceptance of the terminal disclaimer.

Claims 2-31 were rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-46 of U.S. Patent No. 9,371,598 in view of any one of Fu et al (U.S.

Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), Rothberg et al (U.S. Patent Application Publication 2009/0026082 Al, particularly paragraph 0339), Chee et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), or Leamon et al (U.S. Patent Application Publication 2005/0130173 Al, particularly paragraph 0083). Without conceding the merits of this rejection and solely to advance prosecution of the present application, Applicant will submit a terminal disclaimer over the '598 patent. As such, Applicant respectfully requests withdrawal of this rejection upon acceptance of the terminal disclaimer.

CONCLUSION

Applicant submits that claims 2-28 and 29-32 are in condition for allowance, which action is requested. The Examiner is invited to call the undersigned at the telephone number below if such will advance prosecution of this application.

Please apply any necessary charges or credit any overpayments to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: May 15, 2019

/Cameron Luitjens/

Cameron M. Luitjens, Ph.D., J.D.
Reg. No. 58,674

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61910709.doc

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 NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003

CONFIRMATION NO. 7944

IMPROPER CPOA LETTER

159367
FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

OC000000108167888

Date Mailed: 05/15/2019

NOTICE REGARDING POWER OF ATTORNEY

This is in response to the power of attorney filed 05/09/2019. The power of attorney in this application is not accepted for the reason(s) listed below:

- The power of attorney has not been accepted because the party who is giving power has not been identified. Power of attorney may only be signed by the applicant for patent (37 CFR 1.42) or the patent owner. A party who is not the applicant must become the applicant in accordance with 37 CFR 1.46(c) and appoint any power of attorney in compliance with 37 CFR 3.71 and 3.73. For a reissue application, reexamination proceeding, or supplemental examination proceeding, a patent owner who was not the applicant under 37 CFR 1.46 must appoint any power of attorney in compliance with 37 CFR 3.71 and 3.73. See 37 CFR 1.32(b)(4).

/atesfai/

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

159367
FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022
UNITED STATES

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 NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003

CONFIRMATION NO. 7944

IMPROPER CFR REQUEST



159367
FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

Date Mailed: 05/15/2019

RESPONSE TO REQUEST FOR CORRECTED FILING RECEIPT

Power of Attorney, Claims, Fees, System Limitations, and Miscellaneous

In response to your request for a corrected Filing Receipt, the Office is unable to comply with your request because:

- Any request to correct or update the name of the applicant must include an application data sheet (ADS) in compliance with 37 CFR 1.76 specifying the correct or updated name of the applicant in the applicant information section. Any request to change the applicant after an original applicant has been specified under 37 CFR 1.46(b) must include a new ADS in compliance with 37 CFR 1.76 specifying the applicant in the applicant information section and comply with 37 CFR 3.71 and 3.73. See 37 CFR 1.46(c).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/atesfai/

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/276,235	Filing Date 02/14/2019	☐ 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 $50 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $230 =	
☐ 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	05/15/2019	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 $50 =	500
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0		x $230 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	500

		(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".	/CAROLYN E THOMAS/
*** 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.

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 05/23/2019

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United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

159367 7590 05/15/2019

FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
MARTINELL, JAMES	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
05/15/2019	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):

PATDOCTC@fr.com
ipadmin@10xgenomics.com

Applicant-Initiated Interview Summary	**Application No.** 16/276,235	**Applicant(s)** CHEE, Mark S.	
	Examiner JAMES MARTINELL	**Art Unit** 1634	**AIA (FITF) Status** No

All participants (applicant, applicants representative, PTO personnel):

(1) JAMES MARTINELL.

(2) Ms. Lavoie.

(3) Mr. Luitjens.

(4) _____.

Date of Interview: 08 May 2019.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☑Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 2-31.

Identification of prior art discussed: None.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Applicant intends to address the Nonstatutory Double Patenting issues. Concept of mapping locations of biological nucleic acids back to locations in tissue samples was discussed ("spatial trancriptomics"). Applicant intends to respond with amendments to claims to reflect mapping concept..

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.

☐ Attachment

/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiners responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicants correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,-
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
(The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicants record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiners version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, Interview Record OK on the paper recording the substance of the interview along with the date and the examiners initials.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

REPLY TO ACTION OF APRIL 18, 2019

Please consider the following reply.

Electronic Acknowledgement Receipt

EFS ID:	36023681
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Judith Witzel
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	15-MAY-2019
Filing Date:	14-FEB-2019
Time Stamp:	16:18:41
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		RESPONSE.pdf	131395 e49b6c7714aed3f0c7120aa3509868b513f96202	yes	12

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Applicant Arguments/Remarks Made in an Amendment	9	12
	Claims	5	8
	Specification	2	4
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Warnings:			
Information:			
	Total Files Size (in bytes):	131395	

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

REMARKS

Claims 2, 13, 14, 21, 28, and 30-31 have been amended in accordance with Applicant's discussion with Examiner Martinell on May 8, 2019. Claim 29 has been canceled without prejudice. New claim 32 has been added. Support for new claim 32 is found throughout Applicant's specification and in currently pending claim 22.

No new matter has been added. Applicant reserves the right to pursue any subject matter canceled as a result of the present amendments in future prosecution, either in this application or in one or more continuing applications.

In light the present amendments, arguments, and submissions, Applicant respectfully requests reconsideration and allowance of claims 2-28 and 30-31, and consideration and allowance of newly added claim 32.

Examiner Interview

Applicant thanks Examiner Martinell for the courtesy of having the telephonic interview conducted on May 8, 2019 with the undersigned and Teresa A. Lavoie, Ph.D., J.D., USPTO Registration Number 42,782. The various claim amendments, cancellations, and additions that were discussed are reflected in the above listing of the claims.

Information Disclosure Statement

Applicant thanks Examiner Martinell for pointing out certain errors in the listing of references in the IDS filed March 15, 2019.

Regarding the list of US patent applications provided in the March 15, 2019 IDS transmittal, each of these was cited by a corresponding patent or publication number in that IDS.

Reference Number 165, originally cited by application number, was cited by its publication patent number in the March 15, 2019 IDS. Reference Numbers 345 and 346 are provisional applications that matured into applications that issued as US Patent Numbers 9,879,313 and 9,868,979, respectively, each of which was cited in the March 15, 2019 IDS. Reference Number 347 is a provisional application with a priority date much later than the instant application. Reference Number 644 is not in English and will not be cited.

Objections to the Drawings

The drawings were objected to for including reference characters not mentioned in the description. The specification has been amended to include reference characters 150, 160, 200, 306, and 550. As such, the specification now complies with the requirements of 37 CFR 1.121(b), rendering this objection moot.

Non-Statutory Double Patenting Rejection

Claims 2-31 were provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-12, 20, 24-28, 34, 36-39, and 58-77 of copending Application No. 15/187,661 in view of any one of Fu et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), Rothberg et al (U.S. Patent Application Publication 2009/0026082 Al, particularly paragraph 0339), Chee et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), or Leamon et al (U.S. Patent Application Publication 2005/0130173 Al, particularly paragraph 0083). Without conceding the merits of this rejection and solely to advance prosecution of the present application, Applicant will submit a terminal disclaimer over the '661 application. As such, Applicant respectfully requests withdrawal of this rejection upon acceptance of the terminal disclaimer.

Claims 2-31 were provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 2-23 of copending Application No. 16/276,260 in view of any one of Fu et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), Rothberg et al (U.S. Patent Application Publication 2009/0026082 Al, particularly paragraph 0339), Chee et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), or Leamon et al (U.S. Patent Application Publication 2005/0130173 Al, particularly paragraph 0083). Without conceding the merits of this rejection and solely to advance prosecution of the present application, Applicant will submit a terminal disclaimer over the '260 application. As such, Applicant respectfully requests withdrawal of this rejection upon acceptance of the terminal disclaimer.

Claims 2-31 were rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-46 of U.S. Patent No. 9,371,598 in view of any one of Fu et al (U.S.

Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), Rothberg et al (U.S. Patent Application Publication 2009/0026082 Al, particularly paragraph 0339), Chee et al (U.S. Patent Application Publication 2009/0117573 Al, particularly paragraph 0116), or Leamon et al (U.S. Patent Application Publication 2005/0130173 Al, particularly paragraph 0083). Without conceding the merits of this rejection and solely to advance prosecution of the present application, Applicant will submit a terminal disclaimer over the '598 patent. As such, Applicant respectfully requests withdrawal of this rejection upon acceptance of the terminal disclaimer.

CONCLUSION

Applicant submits that claims 2-28 and 29-32 are in condition for allowance, which action is requested. The Examiner is invited to call the undersigned at the telephone number below if such will advance prosecution of this application.

Please apply any necessary charges or credit any overpayments to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: May 15, 2019

/Cameron Luitjens/

Cameron M. Luitjens, Ph.D., J.D.
Reg. No. 58,674

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61910709.doc

Amendments to Claims:

1. **(Canceled)**

2. **(Currently Amended)** A method for determining a spatial location of a biological molecule of a tissue sample comprising

(a) providing a plurality of beads ~~in a spatial pattern~~, wherein the plurality of beads comprise a plurality of binding agents, wherein a binding agent of the plurality of binding agents (i) comprises a coding identifier having a nucleic acid sequence and (ii) is configured to interact with a biological molecule of a tissue sample, wherein the coding identifier corresponds to a location ~~in the spatial pattern~~ at which the binding agent interacts with a biological molecule of a tissue sample;

(b) contacting the plurality of beads with the tissue sample such that the binding agent interacts with the biological molecule; and

(c) using the coding identifier to identify the location i~~n the spatial pattern~~ where the binding agent interacted with the biological molecule, thereby determining a spatial location of the biological molecule of the tissue sample.

3. **(Previously Presented)** The method of claim 2, wherein the plurality of beads is provided on a substrate surface.

4. **(Previously Presented)** The method of claim 3, wherein the plurality of beads is provided in wells on the substrate surface.

5. **(Previously Presented)** The method of claim 2, wherein the biological molecule is a nucleic acid.

6. **(Previously Presented)** The method of claim 5, wherein the binding agent of the plurality of binding agents comprises a nucleic acid.

7. (**Previously Presented**) The method of claim 6, wherein the binding agent interacts with the biological molecule via hybridization.

8. (**Previously Presented**) The method of claim 2, wherein the binding agent and coding identifier are provided as a single molecule.

9. (**Previously Presented**) The method of claim 8, wherein the binding agent and coding identifier are provided as a single molecule through nucleic acid synthesis.

10. (**Previously Presented**) The method of claim 8, wherein the binding agent and coding identifier are provided as a ligation product.

11. (**Previously Presented**) The method of claim 8, wherein the binding agent and coding identifier are provided as a single molecule through chemical coupling.

12. (**Previously Presented**) The method of claim 2, wherein the plurality of binding agents comprises an additional binding agent, which (i) comprises an additional coding identifier and (ii) is configured to interact with another biological molecule of the tissue sample, wherein the additional coding identifier is different from the coding identifier of step (a)(i).

13. (**Currently Amended**) The method of claim 12, wherein the additional coding identifier corresponds to a location ~~in the spatial pattern~~ at which the additional binding agent of the plurality of binding agents interacts with said another biological molecule of the tissue sample.

14. (**Currently Amended**) The method of claim 13, wherein the location ~~in the spatial pattern~~ at which the binding agent interacts with the biological molecule and the additional binding agent interacts with said another biological molecule is different.

15. (**Previously Presented**) The method of claim 14, wherein the spatial location of the biological molecule and said another biological molecule of the tissue sample is different.

16. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining the spatial locations of a plurality of biological molecules of the tissue sample simultaneously.

17. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining presence or absence of the biological molecule at the location at which it is identified.

18. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining the relative amount of the biological molecule at the location at which it is identified.

19. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining all or a portion of the nucleic acid sequence of the coding identifier.

20. (**Previously Presented**) The method of claim 2, wherein step (c) comprises sequencing all or a portion of the nucleic acid sequence of the coding identifier.

21. (**Currently Amended**) The method of claim 2, wherein prior to step (c), spatial organization of cells in the tissue sample is preserved.

22. (**Previously Presented**) The method of claim 2, wherein the biological molecule of the biological sample is selected from a protein, a nucleic acid, a lipid, a carbohydrate, and an ion.

23. (**Previously Presented**) The method of claim 22, wherein the biological molecule is part of a multicomponent complex.

24. (**Previously Presented**) The method of claim 2, wherein the tissue sample is a fresh tissue sample.

25. (**Previously Presented**) The method of claim 2, wherein the tissue sample is a preserved tissue sample.

26. (**Previously Presented**) The method of claim 25, wherein the preserved tissue sample is a frozen tissue sample.

27. (**Previously Presented**) The method of claim 26, wherein the preserved tissue sample is a paraformalin-fixed paraffin-embedded (FFPE) tissue sample.

28. (**Currently Amended**) The method of ~~claim 3~~ claim 2, further comprising analyzing gene expression in the tissue sample.

29. (**Canceled**

30. (**Currently Amended**) The method of ~~claim 3~~ claim 2, wherein the location of the binding agent is known prior to step (b).

31. (**Currently Amended**) The method of ~~claim 3~~ claim 2, wherein the binding agent is immobilized directly or indirectly onto a bead of the plurality of beads.

32. (**New**) The method of claim 2, wherein the biological molecule of the biological sample is a protein.

Amendments to the Specification:

Replace paragraph [0004] on page 7, under the Description of the Figures with the following amended paragraph:

[0004] Figure 4 illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention. Figure 4A shows two target-specific/encoding oligonucleotide constructs specifically bound to a target nucleic acid of interest in a sample. Figure 4B shows a scheme for delivering twenty different coding tags, a1 through a10 and b1 through b10, to a sample to form a 10x10 coding tag grid. Figure 4C shows a tissue section sample to which the coding tags are delivered, forming the coding tag grid in the sample.

On page 15, please replace the paragraph [00029] with the following amended paragraph:

[00029] Once the reacted (transformed) or interacted encoded probes are separated from the unreacted or un-interacted encoded probes, the sequence of the reacted and/or interacted encoded probes is determined at step 150 by, preferably, sequencing. The sequence of the encoded probes allows the mapping of the assay results at step 160 back to locations in the biological sample.

Please replace paragraph [00030] bridging pages 15 and 16 with the following amended paragraph:

Figure 2 provides a simplified overview of an assay system 200 of the present invention embodying an efficient implementation of a combinatorial coding scheme for the encoding of spatial information. For purposes of this overview, the probes are oligonucleotides, but as explained elsewhere, other types of probes can also be used. In step 210, a biological sample affixed to a support, e.g., a tissue sample or other biological structure, is provided. In step 220, one or more oligonucleotide probes are delivered to the biological sample, where the oligonucleotide probes are capable of hybridizing with biological targets in the biological

sample. In step 230, the oligonucleotide probes are allowed to interact with (hybridize to) the nucleic acid targets; that is, appropriate conditions are provided where oligonucleotide probes can hybridize to the target nucleic acids.

On page 19, please replace paragraph [00038] with the following amended paragraph:

Two encoding agents, both comprising a coding tag (seen at 315 and 317), a ligation region (seen at 311 and 313), and a primer region (seen at 319 and 321) are combined with and ligated to the extended probe at step 304 to form an encoded target-specific oligonucleotide. Again, in contrast with Figure 1, the probes and encoding agents are delivered at separate steps. Doing so allows use of the combinatorial embodiments described infra. In preferred embodiments, the encoding oligonucleotides within a pair of encoding oligonucleotides ligate specifically to one side of the target sequence or the other (i.e., 5' or 3' of the target sequence) in step 306. Also, typically, the ligation and primer regions of the encoding oligonucleotides and probes are universal; that is, the set of ligation and primer regions used in constructing the probes and encoding oligonucleotides are constant, and only the target-specific regions of the probes and the coding tags of the encoding oligonucleotides differ. However, again in alternative embodiments, the ligation and primer regions are not universal and differ between probes and encoding agents.

Please replace paragraph [00050] bridging pages 22 and 23 with the following amended paragraph:

The pool of resulting sequences is shown at 540. A sequence readout was obtained for target specific oligonucleotide 1 only at a4bl, a4b2, alb3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at a1b1 (position shown with vertical lines). A sequence readout was obtained for both target specific oligonucleotides 1 and 2 at positions a2bl, a3bl, alb2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at alb4, a2b4 or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a

large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample, and neither target nucleic acid was detected in the upper right portion of the biological sample. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample, as shown in 550.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

INFORMATION DISCLOSURE STATEMENT

Please consider the documents listed on the disclosure form submitted with this statement. Copies of the documents are being submitted with this statement to the extent required under 37 C.F.R. § 1.98(a)(2).

This statement is being filed after a first action on the merits, but before receipt of a final action or a notice of allowance.

Apply any necessary charges or credits applicable under 37 C.F.R. § 1.17(p) to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: May 16, 2019

/Cameron Luitjens/
Cameron M. Luitjens, Ph.D., J.D.
Reg. No. 58,674

Customer Number 159367
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Telephone: (612) 335-5070
Facsimile: (877) 769-7945

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Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		47706-0004003	16/276,235
		Applicant: Prognosys Biosciences, Inc.	
(37 CFR §1.98(b))		Filing Date: February 14, 2019	Group Art Unit: 1634

cU.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	1.	KR 10-20090081260	7/28/2009	KR				
	2.	WO 2001/090415	11/29/2001	WO				
	3.	WO 2002/059364	8/1/2002	WO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document

Examiner Signature	Date Considered

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ASSAY METHOD OF MICROARRAY HYBRIDIZATION

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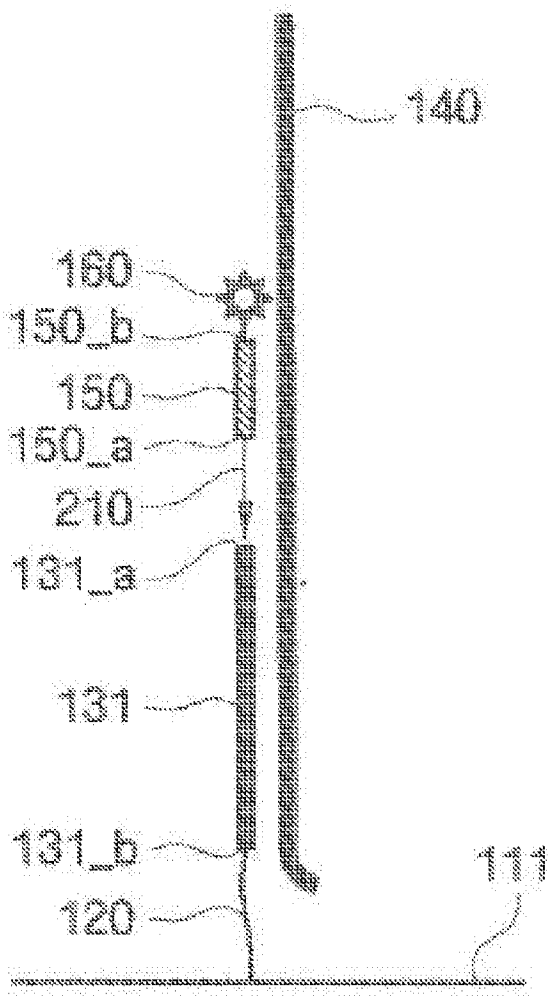
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A method for detecting hybridization in a microarray is provided to observe the hybridization with probe on the microarray and varify the presence of sequence of a target sample. A method for detecting the hybridization in a microarray comprises: a step of providing the microarray in which plural probes(131) are formed; a step of hybridizing random primer(150) with target sample(140); a step of connecting each end of the probes in random primer and free end; a step of removing the target sample; a step of measuring the intensity of random primer.

FIG. 4



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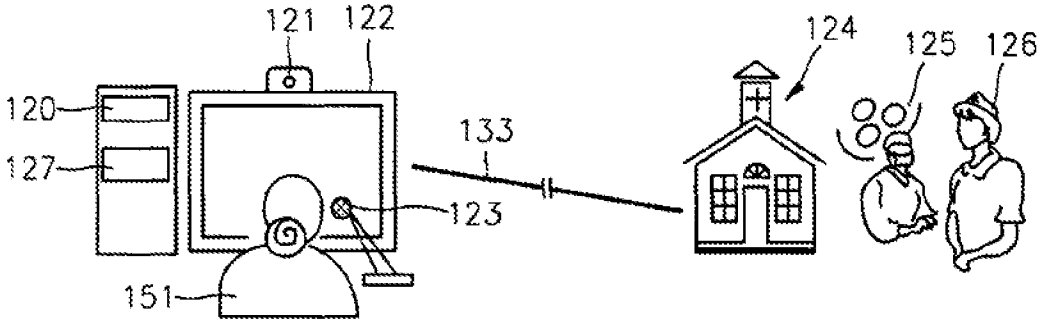
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(54) Title: NUCLEIC HYBRIDIZATION ASSAY METHOD AND DEVICE USING A CLEAVAGE TECHNIQUE RESPONSIVE TO THE COMPLEMENTARY DOUBLE STRAND OR THE SINGLE STRAND OF NUCLEIC ACIDS OR OLIGONUCLEOTIDES



(57) Abstract: A cleavable signal element applicable to quantitative and qualitative assay devices, using a cleavable technique specifically responsive to a complementary double strand or single strand of nucleic acids, and a nucleic acid hybridization assay method and device using the cleavable signal element are provided. Using the cleavable technique responsive to the complementary double strand or single strand of nucleic acids, detection sensitivity to a target nucleic acid can be increased, and diagnosis and detection reliability can be improved twice through in-situ determinations. Through simultaneous single nucleotide polymorphism (SNP) detection and expression profile determination, more accurate diagnosis for many diseases can be achieved. The assay device can be easily modified to be suitable for detection with general laser-based detection systems such as CD-ROM readers. Information read from the assay device is digitized as software and transmitted to and received by doctors and patients through a computer network or wirelessly, which enables construction of remote diagnosis systems.

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NUCLEIC HYBRIDIZATION ASSAY METHOD AND DEVICE USING A CLEAVAGE TECHNIQUE RESPONSIVE TO THE COMPLEMENTARY DOUBLE STRAND OR THE SINGLE STRAND OF NUCLEIC ACIDS OR OLIGONUCLEOTIDES

Technical Field

The present invention relates to cleavable signal elements using a cleavage technique specifically responsive to a complementary double strand or single strand of nucleic acids, which are applicable to quantitative and qualitative assay devices, and a nucleic acid hybridization assay method and device using the cleavable signal element.

Background Art

To date, most clinical diagnostic assays for the detection of small quantities of analytes in fluids have been conducted as individual tests; that is, as single tests conducted upon single samples to detect individual analytes. More recently, multiple-sample preparation and automated reagent addition devices and multiple-sample assay devices, either in parallel or in serial procession, have been designed to improve efficiency and economy. Such automated reagent preparation devices and automated multiplex analyzers are often integrated into a single device.

Large-scale clinical laboratory analyzers of this type can accurately perform hundreds of assays in one hour automatically or semi-automatically. However, these analyzers are expensive and only centralized laboratories and hospitals can afford them. Such centralization necessitates sample transport to the laboratory or hospital, and often precludes urgent or emergent analysis of time-critical samples.

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Thus, to address these problems, there is an increasing need for clinical analyzers which are cheap and easy-to-handle for everyone, such as clinical analyzers suitable for use at the patient bedside or in the patient's home without dedicated detectors. Blood glucose and pregnancy testers are well known examples.

Although useful tests of this sort have been offered for many years, a major breakthrough was the introduction of solid phase immunoassays and other strip tests since 1980. Most notable are Advance® test (Johnson & Johnson), TAM™ hCG assay (Monoclonal Antibodies, Inc.), Clear Blue Easy™ (Unipath Ltd.), and ICON (Hybritech). Commercially Available are Quantab™ (Environmental Test Systems), AccuLevel® (Syva), AccuMeter® (ChemTrak), Clinimeter™ (Crystal Diagnostics), and Q.E.D.™ (Enxymatics). One of the newest is a thermometer-type assay device (Ertinghausen G., U.S. Patent No. 5,087,556) that is not yet commercially available. These systems can be used to assay blood levels of therapeutic drugs and general chemical analytes such as cholesterols.

One disadvantage, however, of each of these formats is that only one, or a very limited number, of assays can conveniently be performed simultaneously.

To fill the gap between massive analyzers and strip testers, some small instruments have been developed. The most notable is Eclipse ICA™ (Biotope, Inc.). This device is an automated centrifugal immunoassay and chemistry system. Patient samples are pipetted into cassettes that are placed into a rotary device. Sixteen tests can be run in approximately 17 minutes. The results are measured by UV/Visible spectrometry or by fluorometry.

Despite these developments, there still exists a need for a simple device that can easily be used for multiple quantitative assays without a

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specialized detector.

< Spatially Addressable Probe Assays >

Recently, spatially addressable arrays of different biomaterials have been fabricated on solid supports. These probe arrays permit the simultaneous analysis of a large number of analytes. Examples are arrays of oligonucleotides or peptides that are fixed to a solid support and that capture complementary analytes. One such system is described by Fodor et al., *Nature*, Vol. 364, August 5, 1993. Short oligonucleotide probes attached to a solid support bind complementary sequences contained in longer strands of DNA in liquid sample; the sequence of the sample nucleic acids is then calculated by computer based on the hybridization data so collected.

There remains a need for an economical system to fabricate spatially addressable probe arrays in a simplified format that provides both for ready detection and the ability to assay for large numbers of test substances (i.e. analytes) in a fluid test sample in a single step, or a minimum number of steps, or assay for a single test substance or analyte in a large number of fluid test samples.

< Spatially Addressable Laser-Based Detection Systems >

Several devices permit spatially addressable detection of digital information. In particular, several formats have been developed based on differential reflectance and transmittance of recording information.

In conventional audio or CD-ROM compact disks, digital information or digitally encoded analog information is encoded on a circular plastic disk by means of indentations in the disk. Typically, such indentations are on the order of one-eighth to one-quarter of the wavelength of the incident beam of a laser that is used to read the information from the disk. The indentations on the disk cause

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destructive interference within the reflected beam, which corresponds to a bit having a "zero" value. The flat areas of the disk reflect the laser beam back to a detector and the detector gives a value of "one" to the corresponding bit.

In another convention, a change of intensity of a reflected light beam gets a value of one while a constant intensity corresponds to zero.

Since the indentations have been formed in the disk in a regular pattern from a master copy containing a predetermined distribution of bits of "zero" and bits of "one", the resultant signal received by the detector is able to be processed to reproduce the same information that was encoded in the master disk.

The standard compact disk is formed from a 12-cm polycarbonate substrate, a reflective metal layer, and a protective lacquer coating. The format of current CDs and CD-ROMs is described by the ISO 9660 industry standard.

The polycarbonate substrate is optical-quality clear polycarbonate. In a standard pressed, or mass-replicated CD, the data layer is part of the polycarbonate substrate, and the data are impressed in the form of a series of pits by a stamper during the injection molding process. The stamping master is typically glass.

Pits are continuously spirally impressed in the CD substrate. The reflective metal layer applied thereupon, typically aluminum, assumes the shape of the solid polycarbonate substrate, and differentially reflects the laser beam to the reading assembly depending on the presence or absence of "pits." An acrylic lacquer is spin-coated as a thin layer on top of the reflective metal layer to protect it from abrasion and corrosion.

Although similar in concept and compatible with CD readers, the information is recorded differently in a recordable compact disk (CD-R). In CD-R, the data layer is separate from the polycarbonate substrate.

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The polycarbonate substrate instead has impressed upon it a continuous spiral groove as an address for guiding the incident laser. An organic dye is used to form the data layer. Cyanine or a metal-stabilized cyanine compound is generally used to form the data layer. An alternative material is phthalocyanine. One such metallophthalocyanine compound is described in U.S. Patent No. 5,580,696.

In CD-R, the organic dye layer is sandwiched between the polycarbonate substrate and the metallized reflective layer, usually 24 carat gold, but alternatively silver, of the media. Information is recorded by a recording laser of appropriate preselected wavelength that selectively melts "pits" into the dye layer, causing the pits to become non-translucent. The reading sensor reads the presence or absence of pits from refractivity rather than differential reflectivity by physical pits in the standard CD. As in a standard CD, a lacquer coating protects the information layer.

Other physical formats for recording and storing information have been developed based on the same concept as the compact disk: creation of differential reflectance or transmittance on a substrate to be read by laser. One such format is termed digital versatile disk (DVD). A DVD looks like standard CD: it is a 120-mm (4.75 inch) disk with a hole in the center for engaging a rotatable drive mechanism. Like a CD, data is recorded on the disk in a spiral trail of tiny pits, and the disks are read using a laser beam. In contrast to a CD, which can store approximately 680 million bytes of digital data under the ISO 9660 standard, the DVD can store from 4.7 billion to 17 billion bytes of digital data. The DVD's larger capacity is achieved by making the pits smaller and the spiral tighter, that is, by reducing the pitch of the spiral, and by recording the data in as many as four layers, two on each side of the disk. The smaller pit size and tighter pitch require that the reading laser wavelength be smaller. While the smaller wavelength is compatible

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with standard pressed CDs, it is incompatible with current versions of the dye-based CD-R.

Thus, a single sided/single layer DVD can contain 4.7 GB of digital information. A single sided/dual layer DVD can contain 8.5 GB of information. A Dual sided/single layer disk can contain 9.4 GB of information, while a dual sided/dual layer DVD contains up to 17 GB of information.

Depending on the capacity, the disk may have one to four information layers. In the 8.5 GB and 17 GB options, a semi-reflector is used in order to access two information layers from one side of the disk. For the 8.5 GB DVD and 17 GB options, the second information layer per side may be molded into the second substrate or may be added as a photopolymer layer. In either case, a semi-reflector layer is required to allow both information layers to be read from one side of the disk. For the 17 GB DVD, it is necessary to produce two dual-layer substrates, and bond them together.

The DVD laser reader is designed to adjust its focus to either layer depth so that both of them can be quickly and automatically accessed.

All of the above-described formats require that the disk be spun. The nominal constant linear velocity of a DVD system is 3.5 to 4.0 meters per second (slightly faster for the larger pits in the dual layer versions), which is over 3 times the speed of a standard CD, which is 1.2 mps.

< Detection Method of DNA Chips>

DNA chips refer to chips having highly immobilized DNA probes of interest on solid substrates and are used for the analysis of a gene expression profile, genetic defects, etc., in samples. To investigate whether the sample contains a target nucleic acid that binds to the probe

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immobilized on the substrate, a detection system therefor is required.

Most currently-available genetic analysis DNA chips employ a method of fluorescently labeling a sample DNA, reacting it with the proves immobilized on the chip, and detecting the unreacted fluorescent material remaining on the chip surface using a confocal microscope or charge coupled device (CCD) imager (U.S. Patent No. 6,141,096). However, such optical detection method is disadvantageous in size reduction and cannot display digitized outputs. For these reasons, research on the development of a new detection method for electrical signal outputs is actively being conducted.

Many research institutes, including Clinical Micro Sensors, are researching the electrochemical detection of DNA hybridization using a metal compound that is liable to oxidation/reduction (U.S. Patent Nos., 6,096,273, 6,090,933). Separate compounds containing easily oxidizable/reducible metals form a complex upon DNA hybridization, and the complex is electrochemically detected (*Anal. Chem., Vol.*, 70, pp. 4670-4677, 1998; *J. Am. Chem. Soc.*, Vol. 119, pp. 9861-9870, 1997; *Analytica Chemica Acta*, Vol, Vo. 2886, pp. 216-224, 1994; *Bioconjugate Chem.*, Vol. 8, pp. 906-913, 1997). However, this electrochemical method still needs separate labeling.

Approaches to assay methods not using the fluorescent label or any other labels have been actively made. As a result, a method of measuring a difference in mass before and after binding using a quartz crystal microbalance (*Anal. Chem., Vol.* 70, pp, 1288-1296, 1998), an assay method using matrix assisted laser description ionization (MALDI) mass spectrometry (*Anal. Chem., Vol.* 69, pp. 4540-4546, 1997, U.S. Patent No. 6,043,031) were developed.

Even a single-base difference can be analyzed using a microfabricated cantilever, which is a mechanical sensor type for measuring molecular binding force before and after binding of DNA

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probe and target (*Science*, Vol., 288, pp. 316-318, 2000; *Proc. Natl., Acad. Sci.* USA, 98, 2560, 2001). However, this method needs additional equipment, such as a laser, for accurate measurement of cantilever beam deflection.

The present invention relates to the field of diagnosis and detection of small quantities of materials in fluids. It is an object of the present invention to provide cleavable signal elements using a cleavage technique specifically responsive to a double strand or single strand of nucleic acids or oligonucleotides having a complementary sequence, which are applicable to quantitative and qualitative assay devices, and a nucleic acid hybridization assay method and device using the cleavable signal element.

It is another object of the present invention to provide an accurate method and device of diagnosing a variety of diseases from both single nucleotide polymorphism (SNP) detection and gene expression profile obtained using the nucleic acid hybridization assay device.

An analytical apparatus based on the nucleic acid hybridization assay method and device using the cleavage technique can be modified to use the standard laser-based detection system, such as CD-ROM reader or DVD reader, and can be coupled to a detector including an optical device, an electrochemical device, or a capacitance and impedance measurement device. The analytical apparatus and method according to the present invention are useful in both detecting a number of individual analytes in a test sample and detecting a single analyte in a large number of separate samples.

It is still another object of the present invention is to provide an remote diagnostic system providing convenience to both patients and doctors by transmitting and receiving the information read from the analytical apparatus and digitalized as computer software, through an existing communication network, such as the Internet.

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Disclosure of the Invention

It is an object of the present invention to provide a nucleic acid hybridization assay method and device applicable to quantitative and qualitative assay devices, which use a cleavage technique specifically responsive to a complementary double strand of nucleic acids of a particular sequence.

In the present invention, cleavage is performed using a restriction enzyme specifically responsive to only a double strand of a particular sequence. Hereinafter, the particular sequence is referred to as a "restriction sequence", and each single strand of the restriction sequence is referred to as a "restriction probe". The restriction probe is additionally ligated to one end of a capture probe that has a complementary sequence to a target DNA and thus is hybridized to the target DNA, and then immobilized on a substrate. The restriction probe and capture probe can be designed collectively as a single probe. In forming a double strand from the capture probe and restriction probe, the restriction probe is cleaved by the restriction enzyme, and thus the restriction and capture probes are collectively referred to as "cleavable capture probes" or "cleavable signal elements".

Although use of the restriction enzyme that specifically responds to a restriction sequence of the designed restriction probe is well known in the field, it is preferable to design the restriction probe such that the sequence of the restriction probe ligated to one end of the capture probe does not overlap with the sequence of the capture probe.

The sequence of the capture probe is determined to be specific to an analyte of interest for diagnosis or analysis purpose. As the capture probe contacts a sample containing a target nucleic acid having a complementary sequence to the capture probe, the capture probe forms

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a double strand with the target nucleic acid through hybridization. At this time, the restriction probe attached to one end of the capture probe still remains as a single strand and does not form the double stand.

To form a double strand of the non-hybridized restriction probe, a solution mixture containing four dNTPs and a DNA polymerase required for DNA extension are added. Formation of a double-strand of the restriction probe is achieved through DNA extension using the target nucleic acid hybridized to the capture probe as a primer.

Once the cleavable capture probe forms a complete double strand through hybriziation of the capture probe to the target nucleic acid and formation of the restriction probe double strand, the double-stranded restriction probe is cleavable by the restriction enzyme. After cleavage of the double strand by the restriction enzyme, the cleaved signal element is removed from the substrate through washing.

In contrast, when the capture probe contacts a sample that does not have a complementary nucleic acid sequence to the capture probe, the cleavable capture probe cannot form the double strand, so it remains attached to the substrate after the addition of the restriction enzyme and washing. The cleavable capture probe remaining on the substrate is referred to as an "uncleaved probe". To improve the sensitivity of a detector, a "label-attached uncleaved probe" structure can be optionally formed on the substrate by contacting the uncleaved probe with fluorescent labels or other labeling elements such as metal microspheres.

After sample-to-probe contact, reaction using the DNA polymerization solution and the restriction enzyme, and washing, detection of the presence or absence of the uncleaved probe or "label-attached uncleaved probe" structure on the substrate by a detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement

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device, indicates the presence or absence of a particular analyte.

As described above, "uncleaved probes (cleavable capture probes not cleaved and adhering to the substrate)" or "label-attached uncleaved probe" structures act as signal elements for the presence of particular analytes. The presence of the cleavable signal element (uncleaved signal element) on the substrate means that sample does not contain a particular analyte, and the absence of the cleavable signal element (cleaved signal element) from the substrate means that sample contains a particular analyte.

The present invention provides a nucleic acid hybridization assay method using the cleavable capture probe as a cleavable signal element.

In general, the assay method using the assay device and a cleavage technique specifically responsive to a particular sequence involves: contacting the assay device with a liquid sample; reacting the cleavable signal probe with a DNA polymerization solution to form a double strand; contacting the cleavable capture probe with a restriction enzyme to cleave the double strand; removing the cleaved double strand through washing; and detecting the presence or absence of the cleavable signal element on a solid support substrate using the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, as described above.

After washing, a label may be optionally attached to the uncleaved probe remaining on the solid support substrate to form a "label-attached uncleaved probe" structure. This label attachment improves the sensitivity of the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device.

According to the present invention, the presence of a particular

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analyte can be double checked through two steps, thereby increasing assay reliability. As a first step, after contact with a sample, whether the capture probe is double-stranded or not with the target nucleic acid is determined. As a second step, after the reaction with the DNA polymerization solution, the restriction enzyme treatment, and the washing, whether the cleavable capture probe remains on the substrate or not is determined in situ. Therefore, the particular analyte can be detected with higher reliability.

In another aspect, the present invention provides an assay device comprising a solid support substrate on which a plurality of cleavable capture probes (cleavable signal elements) are deposited in a spatially-addressable pattern. Suitable materials for the solid support substrate of the assay device include gold, glass, and silicon, with polycarbonate being preferred. Alternative examples of the solid support substrate include disks of any shape compatible for detection using existing laser reflectance-based detectors, including audio compact disk (CD) readers, CD-ROM (compact disk read-only memory) readers, recordable CD readers, DVD (digital versatile disk) readers, and the like.

In a preferred embodiment of the present invention, an "uncleaved probe" or a "label-attached uncleaved probe" structure and the cleaved double strand differentially reflect or scatter incident light, in particular, incident laser light, which can be adapted for detection using existing laser-reflectance based detectors, including CD readers, CD-R readers, CD-ROM readers, or DVD readers. Furthermore, according to the present invention, a bioinformatics-related database for diagnosis and assay interpretation, as well as hospital telephone numbers and web link information for remote diagnosis, may be loaded to a spatial address. In addition, the present invention enables personal medical history management by writing the diagnosis result to a CD-R or a hard disk.

The deposition of cleavable signal elements (cleavable capture

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probes) on the assay device in a spatially-addressable pattern permits a single-sample assay for multiple analytes, a multi-sample assay for a single analyte, and a multi-sample assay for multiple analytes.

Another aspect of the present invention provides a nucleic acid assay device including cleavable signal elements responsive to a variety of nucleic acid sequences. In view of this, the present invention provides an assay method and device for assaying a nucleic acid sequence present in a sample from the spatial address of a signal generated upon contact with the nucleic acid containing sample.

The present invention simultaneously provides single nucleotide polymorphism (SNP) detection and expression profile determination, thereby enabling more accurate diagnosis of many kinds of diseases.

Another aspect of the present invention provides a remote diagnostic system providing convenience to both patients and doctors, which detects a plurality of cleavable signal elements attached to a solid support substrate using the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, digitizes the detected information as computer software, and transmits to both doctor and patient through an existing communication network, such as the Internet.

The assay device and method according to the present invention uses cleavable capture probes as cleavable signal elements for detection of analytes in fluid test samples. Binding of the analyte preselected for detection enables cleavage of the cleavable capture probe at its restriction probe portion and removal of the cleavable capture probe from the substrate surface.

When the sample does not contain the target nucleic acid, the cleavable capture probe remains attached to the substrate surface. Therefore, the presence or absence of the cleavable signal element (cleavable capture probe) can be used as digital (binary) information

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indicating whether a particular analyte exists or not in the sample. A differential signal between the uncleaved signal element and the cleaved signal element indicates whether the particular analyte exists or not in the sample.

In a preferred embodiment, the signal element according to the present invention reflects or scatters incident light and is light addressable. Binding of the analyte preselected for detection enables cleavage and removal of the signal element. Reflection or scattering of incident light, in particular, incident laser light, from the uncleaved signal element indicates the absence of a particular analyte in the sample, and reflection or scattering from the cleaved signal element indicates the presence of the analyte in the sample.

The cleavable signal elements of the present invention are particularly adapted for detection using existing laser reflectance-based detectors, including CD readers, CD-ROM readers, laser disk readers, DVD readers, and the like. The cleavable signal elements of the present invention thus permits the ready adaptation of existing assay chemistries and existing assay schemes to detection using existing laser reflectance-base detectors. This leads to substantial cost savings per assay over standard assays using dedicated detectors.

Applicable assay examples are immunoassays, cell counting, genetic detection assays based upon hybridization, genetic detection assays based upon nucleic acid sequencing, nucleic acid sequencing itself, and the like. The present invention thus allows distribution of assay devices to research laboratories, physician's offices, and individual homes that must currently be performed at centralized locations.

The spatially addressable capabilities of the laser reflectance-based detectors currently used to detect and interpret information encoded on CDs and the like confer particular advantages

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on assays adapted to use the cleavable reflective signal elements of the present invention.

Thus, patterned deposition of multiple signal elements on a single support or substrate, coupled with use of a detector capable of addressing the spatial location of these individual signal elements, permits the concurrent assay of a single sample for multiple different analytes, multiple samples for a single analyte, or multiple samples for multiple analytes. The present invention is thus further directed to assay devices, commonly referred to herein as disk, bio-compact disks, bio-DCs, or bio-DVDs, comprising spatially addressable combinations (diverse geometries) of cleavable signal elements of different analyte specificity. Among such useful combinations are those that increase the predictive value or specificity of each of the individual assays, combinations that inculpate or exculpate particular diagnoses in a differential diagnosis, combinations that provide broad general screening tools, and the like.

Patterned deposition of multiple signal elements with identical specificity further permits the detection, using a single assay device, of large concentration ranges of a single analyte. It is thus another aspect of the present invention to provide assay devices comprising spatially addressable cleavable signal elements of identical specificity, the physical location of which is capable of conveying concentration information.

The spatially addressable capabilities of the laser reflectance-based digital detectors further permits the combination of interpretive software and the assay elements themselves on a single assay device. Another aspect of the present invention, therefore, is an assay device upon which software is encoded in an area spatially distinct from the patterned deposition of cleavable signal elements. The software may include information important for correct tracking by

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the incident laser, assay interpretive algorithms, standard control values, bioinformatics information, self-diagnostics, and the like. The software may include device drivers and software capable of uploading the diagnostic information to remote locations. The software may include educational information for patients on clinical assays, and may be adapted for chosen audiences. The software may include a variety of web sites and links, for example, a web site enabling a patient to communicate with a doctor or hospital based on his/her diagnosis result.

To increase detection sensitivity to reflection variations in the nucleic acid hybridization assay according to the present invention, one end of the cleavable capture probe (cleavable signal element) is covalently bound to the substrate, and the other end of the cleavable capture probe is labeled with, for example, a conducting polymer (e.g., polyaniline), a fluorescent label, or a metal microsphere, to form a label-attached signal element ("label-attached cleavable capture probe"), thereby increasing a reflectivity variation relative to the cleaved signal element.

In another preferred embodiment, the cleavable signal element according to the present invention provides information (signal) on the presence or absence of analytes in the sample to a capacitance and impedance measurement device for measuring conductance variations. Binding of the analyte preselected for detection enables removal of the cleavable signal element through cleavage. A conductance difference between the uncleaved signal element and the cleaved signal element signals whether the analyte exists or not in the sample. After washing, a "label-attached uncleaved probe" structure may be optionally formed on the substrate by contacting the uncleaved signal element with a label, such as a metal microsphere, to increase detector sensitivity.

To increase detection sensitivity to conductance variations in the nucleic acid hybridization assay according to the present invention, one

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end of the cleavable capture probe is bound to the substrate, and the other end of the cleavable capture probe is labeled with, for example, a conducting polymer (e.g., polyaniline), a fluorescent label, or a metal microsphere, to form a label-attached signal element ("label-attached cleavable capture probe"), thereby increasing a conductance (capacitance and impedance) variation relative to the cleaved signal element.

Another object of the present invention is to provide a nucleic acid hybridization assay method and device using a cleavage enzyme specifically responsive to a complementary double strand of nucleic acids, which are applicable to quantitative and qualitative assay devices.

In the present invention, cleavage is achieved by a cleavage enzyme, such as a DNAse, specific to double stands. In this case, only a capture probe acts as a cleavable signal element without a restriction probe. After the capture probe forms a double stand, the DNAse cleaves the double strand at a capture probe portion. Therefore, the capture probe functions as a "cleavable signal element"

Once a double strand that is cleavable by the DNAse is formed through hybridization of the capture probe to a target nucleic acid, the double strand is cleaved by the DNAse and separated from the substrate through washing.

In contrast, when the capture probe contacts a sample not including a complementary sequence to the capture probe, the capture probe does not form the double strand and thus remains as an uncleaved capture probe, attached to the substrate even after the addition of the DNAse and washing. After washing, a "label-attached uncleaved probe" structure may be optionally formed on the substrate by contacting the uncleaved signal element with a label, such as a fluorescent label or a metal microsphere, to increase detector sensitivity.

After contacting the sample, reaction with the DNAse, and

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washing, detection of the presence of the uncleaved probe or "label-attached uncleaved probe" on the substrate by the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, indicates whether the analyte is present or not in the sample. Therefore, the "uncleaved probe" or "label-attached uncleaved probe" acts as an analyte presence/absence signal element. The presence of the cleavable signal element (uncleaved signal element) on the substrate indicates that sample does not contain a particular analyte, and the absence of the cleavable signal element (the cleaved signal element remain) indicates that sample contains a particular analyte.

Still another object of the present invention to provide a nucleic acid hybridization assay method and device using a cleavage enzyme specifically responsive to a single strand of nucleic acids, which are applicable to quantitative and qualitative assay devices.

In the present invention, cleavage is achieved by a cleavage enzyme, such as a nuclease, for example, derived from mung bean, specific to single strands. In this case, only a capture probe acts as a cleavable signal element without a restriction probe.

When the capture probe contacts a sample not including a complementary sequence to the capture probe, the capture probe does not form a double strand and remains as a single strand which is cleavable by the nuclease. The single strand is cleaved by the nuclease and separated from the substrate through washing.

In contrast, when the capture probe is double-stranded through hybridization to a target nucleic acid, the capture probe remains as an uncleaved probe, attached to the substrate even after the addition of the nuclease and washing.

After washing, a "label-attached uncleaved probe" structure may be optionally formed on the substrate by contacting the uncleaved signal

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element with a label, such as a fluorescent label or a metal microsphere, to increase detector sensitivity.

After contacting the sample, reaction with the nuclease, and washing, detection of the presence of the uncleaved probe or "label-attached uncleaved probe" on the substrate by the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, indicates whether the analyte is present or not in the sample. Therefore, the "uncleaved probe" or "label-attached uncleaved probe" acts as an analyte presence/absence signal element. The presence of the cleavable signal element (uncleaved signal element) on the substrate indicates that sample contains a particular analyte, and the absence of the cleavable signal element (the cleaved signal element remain) indicates that sample does not contain a particular analyte.

Hereinafter, the structure of the present invention provided to achieve the above objects will be described.

To achieve an object of the present invention, there is provided a cleavable signal element comprising: a restriction probe of a single strand having a particular sequence cleavable by a restriction enzyme specific to a double strand of a particular sequence; and a capture probe of a single strand having a complementary sequence to a target nucleic acid for diagnosis or assay to form a double strand through hybridization to the target nucleic acid, wherein one end of the restriction probe is attached to a solid support substrate, and the other end of the restriction probe is ligated to the capture probe, thus forming a single-stranded, cleavable capture probe.

In the present invention, when the capture probe contacts a sample including the target nucleic acid of the complementary sequence, the capture probe is double-stranded through hybridization to the target nucleic acid, the restriction probe is double-stranded through DNA

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extension using the target nucleic acid hybridized to the capture probe as a primer with the addition of a DNA polymerization solution, the double-stranded restriction probe is cleaved by the restriction enzyme, and the cleaved cleavable capture probe is removed from the solid support substrate through washing, thus resulting in a cleaved signal element. In contrast, when the capture probe contacts a sample not including the target nucleic acid of the complementary sequence, the single-stranded, cleavable capture probe remains attached to the solid support substrate even after additions of the DNA polymerization solution and the restriction enzyme and washing, thus resulting in an uncleaved signal element. Preferably, the DNA polymerization solution comprises a solution of four dNTPs and a DNA polymerase solution.

Another cleavable signal element according to the present invention comprises a capture probe of a single strand having a complementary sequence to a target nucleic acid for diagnosis or assay to form a double strand through hybridization to the target nucleic acid, wherein one end of the capture probe is attached to a solid support substrate, and the capture probe itself forms a single-stranded, cleavable capture probe which is cleavable by a cleavage enzyme specifically responsive to a double strand or single strand of nucleic acids.

In this case, when the capture probe contacts a sample including the target nucleic acid of the complementary sequence, the capture probe is double-stranded through hybridization to the target nucleic acid, the double-stranded capture probe is cleaved by the cleavage enzyme specifically responsive to the double strand of nucleic acids, and the cleaved cleavable capture probe is removed from the solid support substrate through washing, thus resulting in a cleaved signal element. In contrast, when the capture probe contacts a sample not including the target nucleic acid of the complementary sequence, the single-stranded,

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cleavable capture probe remains attached to the solid support substrate even after the addition of the cleavage enzyme and washing, thus resulting in an uncleaved signal element. Preferably, the cleavage enzyme is a DNAse.

When the capture probe contacts a sample not containing the target nucleic acid of the complementary sequence, the capture probe remains as a single strand without hybridization, the single-stranded capture probe is cleaved by the cleavage enzyme specifically responsive to the single strand of nucleic acids, and the cleaved cleavable capture probe is removed from the solid support substrate through washing, thus resulting in a cleaved signal element. In contrast, when the capture probe contacts a sample including the target nucleic acid of the complementary sequence, the capture probe is double-stranded through hybridization to the target nucleic acid, and the double-stranded, cleavable capture probe remains attached to the solid support substrate even after the addition of the cleavage enzyme and washing, thus resulting in an uncleaved signal element. Preferably, the cleavage enzyme is a nuclease, more preferably, derived from mung bean.

In the cleavable signal elements according to the present invention, the solid support substrate may be a plastic substrate, a glass substrate, a silicon substrate, or a gold substrate. Preferably, the solid support substrate has a self-assembled monolayer (SAM) on the surface. Preferably, the capture probe has a length ranging from about 5- to about 30-mers.

To increase detection sensitivity for an uncleaved signal element, it is preferable that a label is attached to one end of the cleavable capture probe to form a label-attached cleavable capture probe structure or to one end or side of an uncleaved probe to form a label-attached uncleaved probe structure, to increase detection sensitivity for an uncleaved signal element. In this case, the label may comprise a metal

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microsphere, a conducting polymer, a fluorescent dye, a magnetic microsphere, and a streptavidin-labeled microsphere. Preferably, the metal microsphere is formed of a metal selected from the group consisting of gold, silver, nickel, platinum, chromium, and copper. Preferably, a gold microsphere has a diameter ranging from about 1 nm to about 10 μm. Preferably, the streptavidin-labeled microsphere is attached to the cleavable capture probe via biotin.

To achieve another object of the present invention, there is provided a nucleic acid hybridization assay device comprising: a solid support substrate; a plurality of cleavable signal elements according to any of the cleavable signal elements described above attached to the solid support substrate; and an internal or external detector which detects a uncleaved signal element and a cleaved signal element from the plurality of cleavable signal elements.

It is preferable that the detector comprises an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device. Preferably, the optical device detects fluorescence of the uncleaved signal element and cleaved signal element.

It is preferable that the detector detects a differential reflective signal or a differential conductive signal of the uncleaved signal element and the cleaved signal element. Preferably, the detector detects the differential reflective signal by measuring the reflectance, absorbance, or scattering of light or a laser beam incident on the uncleaved signal element and the cleaved signal element.

Alternatively, the detector may detect the differential conductive signal by measuring the capacitance and impedance of the uncleaved signal element and the cleaved signal element. In this case, preferably, the capacitance and impedance measurement device measures the

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frequency response characteristics of the uncleaved signal element and the cleaved signal element.

In the nucleic acid hybridization assay device according to the present invention, the capacitance and impedance measurement device may comprise interdigitated array electrodes having at least one digit and arranged on the solid support substrate. Preferably, the interdigitated array electrodes are substantially formed of gold. Preferably, the interdigitated array electrodes have an input port to check for the frequency response characteristics, and the input port is connected to an electronic control device which generates a frequency signal of a constant bandwidth.

In the nucleic acid hybridization assay device according to the present invention, a plurality of cleavable signal elements may be deposited on the interdigitated array electrodes, preferably only in the space between the interdigitated array electrodes.

In the nucleic acid hybridization device according to the present invention, to increase the sensitivity of the detector, it is preferable that a label-attached uncleaved probe structure is formed on the solid support substrate by attaching a label to the uncleaved signal element.

Preferably, the plurality of cleavable signal elements are deposited on the solid support substrate in a spatially-addressable pattern, more preferably, to enable a single-sample assay for multiple analytes, a multiple-sample assay for a single analyte, or a multiple sample assay for multiple analytes.

In the nucleic acid hybridization device according to the present invention, it is preferable that the solid support substrate is a plastic substrate formed of a material selected from the group consisting of polypropylenes, polyacrylates, polyvinyl alcohols, polyethylenes, polymethylmethacrylates, and polycarbonates. Among those materials for the solid support substrate, polycarbonates are more preferred.

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Preferably, the solid support substrate is formed of a circular disk or a rectangular disk. The circular disk may have a diameter of approximately 120 mm and a thickness of approximately 1.2 mm. The nucleic acid hybridization assay device according to the present invention may include a plurality of circular disks.

It is preferable that, in the nucleic acid hybridization device according to the present invention, the circular disk comprises: a central void to engage a rotational drive means; a sample injection port through which a sample is injected; and an annular and/or a spiral track in which the plurality of cleavable signal elements are deposited in the spatially-addressable pattern. Preferably, an address pattern that provides coded address information is formed on the circular disk.

Alternatively, the circular disk in the nucleic acid hybridization device according to the present invention may comprise: a central void to engage a rotational drive means; a sample injection port through which a sample is injected; and a radial assay sector in which the plurality of cleavable signal elements are deposited in the spatially-addressable pattern. Preferably, the circular disk comprises a plurality of assay sectors. The plurality of assay sectors may be connected to respective separate sample injection ports or to a common sample injection port. The plurality of cleavable signal elements are deposited in each of the plurality of assay sectors in an appropriate pattern for a single-analyte assay or a multiple-analyte assay. Therefore, the nucleic acid hybridization assay device according to the present invention is applicable for a single-sample assay for multiple analytes, a multi-sample assay for a single analyte, and a multi-sample assay for multiple analytes.

It is preferable that the circular disk includes in a central track a database associated with bioinformatics required for diagnosis and assay interpretation, and telephone numbers, web link information and

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software required for remote diagnosis.

In the nucleic acid hybridization device according to the present invention, it is preferable that a detector is mounted on the circular disk. The detector may comprise a non-contact interface through which information read from the cleaved signal element and the uncleaved signal element is transmitted to an external central controller or storage device. Preferably, the non-contact interface comprises an infrared interface and an optical interface. As an example, the infrared interface may an infrared sensor, and the optical interface may be a photosensor.

Preferably, the circular disk in the nucleic acid hybridization assay device according to the present invention simultaneously comprises at least one SNP (single nucleotide polymorphism) assay sector for SNP detection and at least one expression assay sector for expression profile analysis. In this case, the SNP assay sector and the expression assay sector may be arranged separate in an angular direction or in a radial direction.

To achieve still another object of the present invention, there is provided bio-driver apparatus comprising: a rotary disk receiver onto which any nucleic acid assay device described above is to be loaded; a motor driver which rotates the disk; a rotary connector which connects the motor driver to a central void portion of the disk such that the disk is rotatable; and an optical device to write data in or to read data from the disk.

Preferably, the bio-driver apparatus further comprises a central controller which transmits information read from the disk by the optical device to an external storage unit, transmits information to be written to the optical device, and generates and outputs a variety of control signals for the motor driver and the other elements.

In the bio-driver apparatus according to the present invention, it is preferable that the rotary connector comprises an upper rotor and/or a

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lower rotor, the upper and lower rotors being pushed close to the top and bottom surfaces, respectively, of the central void portion when the disk begins to rotate.

In the bio-driver apparatus according to the present invention, the optical device may detect fluorescence, preferably, a differential reflective signal by measuring the reflectance, absorbance, or scattering of incident light or an incident laser beam.

Alternatively, the present invention provides a bio-driver apparatus comprising: a rotary disk receiver onto which any nucleic acid assay device described above is to be loaded; a motor driver which rotates the disk; a rotary connector which rotatably connects the motor driver to a central void portion of the disk; an external power connector which powers and/or supplies a control signal to a detector mounted on the disk; and a non-contact interface through which information read by the detector is transmitted.

Preferably, the bio-driver apparatus further comprises a central controller which transmits information read from the disk by the detector to an external storage unit and generates and outputs a variety of control signals for the motor driver and the other elements.

Preferably, the bio-driver apparatus further comprises an optical device to write data in or to read data from the disk. Software including, for example, bioinformatics information, can be written in or read from the disk.

Preferably, the detector detects a differential conductive signal by measuring capacitance and impedance.

Preferably, the rotary connector comprises an upper rotor and/or a lower rotor, the upper and lower rotors being pushed close to the top and bottom surfaces, respectively, of the central void portion when the disk begins to rotate.

Preferably, the power connector comprises a brush that

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frictionally contacts the upper and/or lower rotors in connection with an external power supply unit, and each of the upper and lower rotors comprises an annular electrode plate frictionally contacting the brush. One of the upper and lower rotors may be used. In this case, two opposite nodes of the power supply unit are connected to one brush. When both of the upper and lower rotors are used, brushes contacting the upper and lower rotors may respectively connected to the opposite nodes of the power supply unit.

It is preferable that the annular electrode plate comprises at least one conductive arm connected thereto, and the central void portion of the disk comprises a hole to engage the at least one conductive arm and a circuit pattern connected to the hole to power the detector mounted on the disk and/or supply the control signal to the detector. In this case, the at least one conductive arm may a spring at its one end that is connected to the annular electrode plate.

In the bio-driver apparatus according to the present invention, it is preferable that the power connector comprises an electromagnet attached to the rotary disk receiver in connection with the external power supply unit, and the electromagnet induces an AC voltage to a wound coil on the disk so that the detector is powered in a non-contact manner. In this case, the disk further preferably comprises a rectifier for rectifying the AC voltage induced to the wound coil.

To achieve yet still another object of the present invention, there is provided a remote diagnostic system comprising: any nucleic acid hybridization assay device according to the present invention described above, an existing communication network such as the Internet; and a computer in which software capable of controlling access to the existing communication network and digitizing information read from the nucleic acid hybridization assay device is installed, wherein the digitized information from the nucleic acid assay hybridization assay device is

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transmitted to a doctor or a hospital, and a patient is provided with a prescription, through the existing communication network.

In the remote diagnostic system according to the present invention, the computer may comprise assay interpretive algorithms, bioinformatics information, and self-diagnostics related software. Preferably, the computer comprises software capable of uploading diagnostic information to remote locations and device drivers. In this case, the software may include educational information for patients on clinical assays, a variety of wet sites and links enabling a patient to directly communicate with a doctor or hospital based on his/her diagnosis result.

It is preferable that the computer comprises a camera and a microphone for viewing a patient's face and listening to his/her voice. It is preferable that the diagnostic data based on the digitized information are displayed on a computer monitor, the computer automatically or manually transmits the diagnostic data to a specialist through the existing communication network, and the patient waits for a prescription from the specialist.

To achieve another object of the present invention, there is provided a nucleic acid hybridization assay method comprising: hybridizing a capture probe to a target nucleic acid present in a liquid sample by contacting any nucleic acid hybridization assay device according to the present invention described above, with the liquid sample; contacting the cleavable capture probe with a restriction enzyme or cleavage enzyme which is specifically responsive to a cleavable signal element depending on whether the capture probe and the target nucleic acid are hybridized or not; washing the nucleic acid hybridization assay device to remove the cleavable signal element cleaved by the restriction enzyme or cleavage enzyme; and detecting whether the uncleaved signal element or the cleaved signal element exists on the

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solid support substrate.

Preferably, the nucleic acid hybridization assay method further comprises contacting the cleavage capture probe with a DNA polymerization solution before contact with the restriction enzyme. As a result, the restriction probe forms a double strand through DNA extension using the target nucleic acid hybridized to the capture probe as a primer. Preferably, the nucleic acid hybridization assay method further comprises contacting the cleavage capture probe with a 3'→5' exonuclease solution before contact with the DNA polymerization solution. As a result, a portion of the target nucleic acid that remains as a single strand without hybridization to the capture probe is cleaved, so that the target nucleic acid can act as the primer.

It is preferable that the nucleic acid hybridization assay method further comprises attaching a label to the cleavable signal element before contacting the capture probe with the liquid sample, or to an uncleaved signal element after contacting the cleavable capture probe with the restriction enzyme or cleavage enzyme. When the label attachment is applied before contacting the sample, it is preferable that the label is attached during the synthesis of the capture probe or after the immobilization of the capture probe to a solid support substrate. Preferably, simple washing is performed between contact with the restriction enzyme and the label attachment.

It is preferable that the nucleic acid hybridization assay method further comprises at least one wash step. In the nucleic acid hybridization assay method, washing may be performing by rotating the nucleic acid hybridization assay device with or without addition of a detergent solution, or by applying an external electric field.

One embodiment of the nucleic acid hybridization assay method according to the present invention comprising: (a) injecting a sample into

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a sample injection port disposed near the center of a disk in a nucleic acid hybridization assay device; (b) rotating the disk and stopping the rotation of the disk when the sample reaches an outer edge of the disk; (c) incubating the disk in a stationary state at room temperature for hybridization; (d) adding a buffer solution as a washing solution while rotating the disk at a high speed, to wash the disk; (e) adding a DNA polymerization solution containing a mixed solution of four dNTPs and a DNA polymerase and incubating the disk in a stationary state for DNA extension; (f) adding a solution of a restriction enzyme specifically responsive to a double strand of a particular sequence and incubating the disk in a stationary state, to cleave the double strand; (g) washing the disk by rotating the disk at a high speed with the addition of a buffer solution or by applying an external electric or magnetic field; and (h) drying the disk and reading information from the disk using a detector which is programmed to detect a predetermined assay site on which a cleavable signal element is deposited and comprises an optical device, an electrochemical device, or a capacitance and impedance measurement device.

It is preferable that the nucleic acid hybridization assay method further comprises adding a 3'→5' exonuclease solution before step (e) of DNA extension. As a result, a portion of the target nucleic acid that remains as a single strand without hybridization to the capture probe is cleaved, so that the target nucleic acid can act as the primer.

Another embodiment of the nucleic acid hybridization assay according to the present invention comprises: (a) injecting a sample into a sample injection port disposed near the center of a disk in a nucleic acid hybridization assay device; (b) rotating the disk and stopping the rotation of the disk when the sample reaches an outer edge of the disk; (c) incubating the disk in a stationary state at room temperature for

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hybridization; (d) adding a buffer solution as a washing solution while rotating the disk at a high speed, to wash the disk; (e) adding a solution of a cleavage enzyme specifically responsive to a double strand or single strand of nucleic acids and incubating the disk in a stationary state, to cleave the double strand or single strand; (f) washing the disk by rotating the disk at a high speed with the addition of a buffer solution or by applying an external electric or magnetic field; and (g) drying the disk and reading information from the disk using a detector which is programmed to detect a predetermined assay site on which a cleavable signal element is deposited and comprises an optical device, an electrochemical device, or a capacitance and impedance measurement device.

To increase detection sensitivity, the nucleic acid hybridization assay methods described above may further comprise attaching a label to the cleavable signal element before sample injection, or to an uncleaved signal element after strand cleavage. When the label attachment is applied before contacting the sample, it is preferable that the label is attached during the synthesis of the capture probe or after the immobilization of the capture probe to a solid support substrate. Preferably, simple washing is performed between contact with the restriction enzyme and the label attachment.

Brief Description of the Drawings

FIGS. 1A through 2C show alternative embodiments of the attachment of a plurality of cleavable signal elements to a derivatized site of a variety of substrates, in particular:

FIG. 1A is a schematic representation of the attachment of a plurality of cleavable capture probes (cleavable signal elements) at a derivatized site on the plastic (carbonate) substrate of an assay device by covalent bonds, where n of $(CH)_n$ is an integer greater than zero;

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FIG. 1B is a schematic representation of a nucleic acid hybridization assay shortly after introduction of a sample containing nucleic acids;

FIG. 1C is a schematic representation of a step after the procedure of FIG. 1B, in which oligonucleotides present in the sample have bound to complementary oligonucleiotide of a first capture probe to form a double strand, but have not bound to oligonulcleotide of a second capture probe, where the rectangular box denotes the complementary double strand formation;

FIG. 1D is a schematic representation of a step after the procedure of FIG. 1C, in which a single-stranded restriction probe ligated to the end of the first capture probe is double-stranded using a DNA polymerization solution, in particular, the restriction probe forms a double strand through DNA extension using the complementary target nucleic acid attached to the first capture probe in the step of FIG. 1C as a primer, whereas the second capture probe still remains as a single strand;

FIG. 1E is a schematic representation of a step after the assay procedure of FIGS. 1C and 1D, in which the restriction probe ligated to the end of the first capture probe that has formed the double strand is cleaved by contact with a restriction enzyme, and the cleavable first capture probe double-stranded through the complementary hybridization is removed from the substrate surface, whereas the second capture probe still remains as a single strand on the substrate surface;

FIG. 1F is a schematic representation of the removal of the first capture probe cleaved in the procedure of FIG. 1E by washing;

FIG. 1G is a schematic representation of the formation of a "label-attached uncleaved probe" structure by contacting the second capture probe with a label, for example, an SSB protein, in which the second capture probe is tethered to the substrate surface while the first

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capture probe is removed from the substrate surface through washing, which provides differential signals as well as spatially-addressable differential reflective signals to a detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device;

FIG. 2A is a schematic representation of an embodiment of the nucleic acid hybridization assay according to the present invention to increase the sensitivity of a detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, in which a plurality of cleavable capture probes (cleavable signal elements) are covalently bound to a derivarized site of the plastic substrate (polycarbonate) surface of an assay device, and metal microspheres, conducting polymers, or fluorescent labels are attached to the other free end of the cleavable capture probes;

FIG. 2B is a schematic representation of the cleavage and removal of the first capture probe by washing; and

FIG. 2C is a schematic representation of the labeling of the other free end of the cleavable capture probes with a streptavidin-labeled magnetic microbead;

FIGS. 3A through 3D show alternative embodiments of the spatially addressable arrangement of the cleavable signal elements;

FIGS. 4A through 4E show alternative embodiments of the supply of power to a rotating assay device (disk);

FIG. 4F shows an implementation of detection of analyte-specific signals generated by the assay device using an optical device;

FIGS. 5A through 5I show alternative embodiments of the detection of analyte-specific signals generated by the assay device of FIG. 3C using a capacitance and impedance measurement device having interdigitated array electrodes;

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FIGS. 6A and 6B show alternative embodiments of implementation of the differential reflection between an uncleaved signal element and a cleaved signal element;

FIGS. 6C through 6E show alternative embodiments of implementation of the differential conductance (impedance or capacitance) between the uncleaved signal element and the cleaved signal element using the interdigitated array electrodes;

FIG. 7A illustrates the arrangement of four separate assay sectors in an assay device, each containing a different cleavable signal element, to assay in parallel a single sample for four different analytes;

FIG. 7B shows an embodiment of the arrangement of the assay device of FIG. 7A on a disk;

FIG. 8 shows an embodiment of a remote diagnostic system according to the present invention, in which the information read by a detector of the assay device is digitalized as computer software and mutually transmitted to and received by a patient and a doctor through an existing communication network, for example, the Internet;

FIG. 9 illustrates a washing method by external electric field application;

FIGS. 10A through 10K illustrate embodiments of attachment of cleavable signal elements to different types of substrate surfaces for an assay device;

FIGS. 11A and 11B show alternative embodiments of an assay device using the cleavable signal element according to the present invention for diagnosing a variety of diseases through both single nucleotide polymorphism (SNP) detection and gene expression profile determination;

FIG. 12 shows the reaction mechanism of the 3'→5' exonuclease;

FIGS. 13A and 13B are photographs showing the results of an

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analysis in Example 2 optically measured by atomic force microscopy (AFM); and

FIG. 14 is a graph of the impedance measured in Example 2.

Detailed Description of the Invention

The present invention, including a nucleic acid hybridization assay method and device, using a cleavage technique specifically responsive to a complementary double strand or single strand of nucleic acids or oligonucleotide will be described in greater detail with reference to the appended drawings.

< Spatially Addressable Cleavable Signal Elements >

The reaction mechanism of a cleavable signal element according to the present invention, termed a "bio-bit", can be easily understood by reference to FIGS. 1A through 1C. Referring to FIG. 1A, a plastic substrate 20 has a substrate 21 to which are attached cleavable capture probes 34 and 35. The substrate 20 can be formed of a porous or solid substrate using a variety of materials including plastic, glass, mica, silica, and the like, but plastic is most preferred for reasons of economy, ease of derivatizaton for attaching the cleavable signal elements to the surface, and compatibility with existing laser reflection-based detectors, such as CD-ROM and DVD readers. Suitable plastics include polypropylenes, polyacrylates, polyvinyl alcohols, polyethylenes, polymethylmethacrylates, and polycarbonates, with polypropylenes and polycarbonates being preferred and polycarbonates being the most preferred.

The cleavable signal elements 34 and 35 include respective capture probes 34b and 35b and respective restriction probes 34a and 35a. The surface 21 of the substrate 21 can be derivatized to provide covalent bonding to each of the cleavable signal elements 34 and 35.

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To protect the cleavable capture probes 34 and 35 from direct contact with the surface 21 of the substrate 21, a monomer layer of non-reactive molecules, for example, an alkane chain $(CH_2)_n$, can be formed on any substrate according to the present invention. As an example, an alkane chain $((CH_2)_n)$ 31 attached to one end of the restriction probe 34a is shown.

The alkane chain 21 at one end of each restriction probes 34a and 35a is attached to the surface 21 via an amide linkage. The restriction probes 34a and 35a of the cleavable signal elements 34 and 35 have a cleavage site that is susceptible to cleavage by a restriction enzyme in double-strand formation.

Analyte specificity is conferred upon the cleavable signal element by the sequence of the capture probes 34b and 35b. The capture probes 34b and 35b includes an oligonucleotide of 5- to 20-mers, preferably 8- to 17-mers, most preferably 8- to 12-mers, but longer oligonucleotides (cDNA) can be used. A large number of cleavable signal elements 34 and 35 are present at particular derivatized sites on the surface 21 of the substrate 20 of an assay device called a "bio-disk".

In the present invention, the oligonucleotides of the capture probes 34b and 35b bind with the complementary single strands of nucleic acids present in a test sample. In other words, the complementary oligonucleiotides form double strands, each including a specific binding pair.

As shown in FIGS. 1A through 2C, the cleavable signal elements (cleavable capture probes) 34 and 35 at different sites on the assay device surface have discrete oligonucleotide sequences. In FIG. 1A, the first and second cleavable signal elements 65 and 66 have oligonucleotides 35b and 34b, respectively.

As shown in FIGS. 1B and 1C, when contacted with a test sample containing an oligonucleotide 36, the complementary oligonucleotide

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35b binds with the oligonucleotide 36 present in the test sample to form a double strand (referred to as also a "capture double strand") 37, as shown in FIG. 1C. If there no complementarity between the sample oligonucleotide 36 and the oligonucleotide 34b, there is no binding between those groups as illustrated in FIG. 1C. The capture probe 35b forms the double strand 37 with the sample oligonucleotide 36, whereas the restriction probe 35a at one end of the capture probe 35b currently remains as a single strand.

To form a double strand of the restriction probe 35a at one end of the capture probe 35b that have formed the double strand 37, as shown in FIG. 1D, a DNA polymerization solution is added. The restriction probe 35a is double-stranded via DNA extension using a target nucleic acid hybridized to the capture probe 35b as a primer. The restriction double strand 38 formed through the DNA extension is shown in FIG. 1B.

In FIG. 1E, cleavage of the restriction double strand 38 by addition of a restriction enzyme after formation of the capture and restriction double strands 37 and 38 in the steps of FIGS. 1C and 1D is shown.

After the restriction double strand 38 is cleaved at the cleavage site 38c, the first cleavable signal element 65 specifically bound with the test sample is detached and removed from the surface 21 by washing. This is illustrated in FIG. 1F. If it is desired to assay multiple samples for a single oligonucleotide, the captures probes at different sites will generally have the same oligonucleotide sequence. Presence or absence of a cleavable signal element (after cleavage) on the surface 21 may be detected from differential reflectance of incident light, in particular, incident laser light, or by a capacitance and impedance measurement device capable of measuring conductivity variations.

FIG. 1G illustrates an alternative embodiment of labeling after the washing to increase the sensitivity of a detector. The uncleaved signal element 66 remaining on the substrate 20 after the step of FIG. 1F is

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brought into contact with a label 39, for example, an SSB protein, to form a "label-attached uncleaved probe" structure and thus increase a difference in reflectivity or conductivity between the cleaved signal element 65 and the uncleaved signal element 66, thereby resulting a higher sensitivity of the detector.

FIG. 2A is a schematic representation of an embodiment of the nucleic acid hybridization assay according to the present invention to increase the sensitivity of a detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device. As shown in FIG. 2A, a plurality of cleavable capture probes (cleavable signal elements) are covalently bound to a derivarized site of the plastic substrate (polycarbonate) surface of an assay device, and a label 40, such as a metal microsphere, conductive polymer, or fluorescent label is attached to the other free end of the cleavable capture probes.

The label 40, such as the metal microsphere, act as a reflective signal generation element to permit detection of the presence of the first and second cleavable signal elements 65 and 66 coupled to the substrate 20 of the assay device. Suitable materials for the reflective signal generation element include gold (Au), silver (Ag), nickel (Ni), chromium (Cr), platinum (Pt), and copper (Cu), but Au is preferred due to its nature to easily and strongly bind to a thiol (SH)-group attached to one end of the cleavable signal elements 34 and 35. The metal microsphere may be formed of solid metal, metal-coated plastic, or glass bead. Any reflective materials, instead of metal, can be used. Au-microspheres directly bind to a thiol group attached to one end of the cleavable signal elements 34 and 35.

In FIG. 2A, the first and second cleavable signal elements 65 and 66 have the oligonucleiotides 35b and 34b, respectively. When contacted with a test sample containing an oligonucleotide, the

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complementary oligonucleotide 35b binds with the oligonucleotide present in the test sample to form a double strand (not shown). If there no complementarity between the sample oligonucleotide and the oligonucleotide 34b, there is no binding between those groups. The capture probe 35b forms the double strand with the sample oligonucleotide, whereas the restriction probe 35a at one end of the capture probe 35b currently remains as a single strand.

To form a double strand with the restriction probe 35a at one end of the capture probe 35b that have formed the double strand, a DNA polymerization solution is added. The restriction probe 35a is double-stranded via DNA extension using a target nucleic acid of the hybridized capture probe as a primer. After formation of the complete double strand, the double-stranded restriction probe is cleaved by addition of a restriction enzyme.

After the double-stranded restriction probe is cleaved at the cleavage site 38c, the first cleavable signal element 65 specifically bound with the test sample and the label 40 are detached and removed from the surface 21 by washing. This is illustrated in FIG. 2B. Presence or absence of a cleavable signal element (after cleavage) on the surface 21 may be detected from differential reflectance of incident light, in particular, incident laser light, and by a capacitance and impedance measurement device capable of measuring conductivity variations.

FIG. 2C shows an embodiment of the labeling of cleavable capture probes with the label 39, which may be a metal microsphere, conducting polymer, or fluorescent label, in FIG. 1G. In FIG. 2C, a plurality of cleavable signal elements are covalently bound to a derivatized site of the surface 21 of the assay device substrate 20, and the other end of the cleavable signal elements is labeled with the label 39, such as a metal microsphere, conducting polymer, or fluorescent

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label, via avidin 51 and biotin 50.

FIGS. 3A through 3D show alternative embodiments of the spatially addressable arrangement of the cleavable signal elements. In particular, FIG. 3A shows an address pattern 500 formed on a substrate 70 of a circular disk to provide coded address information, from which the location of a cleavable signal element 200 may be optically or fluorescently measured, and the attachment of the cleavable signal element 200 to annular tracks 351 by deposition. The circular disk has a central void 61 to engage a rotational drive means. Adjacent annular tracks are connected with each other by spiral-track bridges 352 and 353 to permit the sample injected through a sample injection port 354 to uniformly and outwardly spread by a centrifugal force generated as the disk rotates. The address information of the disk can be obtained from the address pattern 500, which is a regular pattern impressed at a fixed location. Reference numeral 350 denotes a track-to-track interval. FIG. 3A shows the deposition of the cleavable signal element in an appropriate pattern to assay in parallel a single sample for multiple analytes.

FIG. 3B shows an address pattern 401 formed on the substrate 70 of the circular disk to provide coded address information, from which the location of the cleavable signal element 200 may be optically or fluorescently measured, and the attachment of the cleavable signal element 200 to radial tracks 351 by deposition. The circular disk has a central void 61 to engage a rotational drive means. FIG. 3B shows the deposition of the cleavable signal element in an appropriate pattern to assay in parallel multiple samples. A database associated with bioinfomatics required for diagnosis and assay interpretation, and telephone numbers, web link information, and software required for remote diagnosis may be coded and stored in a central track 402. The embodiment of FIG. 3B shows assay sectors with individual sample

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injection ports 354 and segregated from one another, thereby permitting rotation of the assay device without sample mixing. In FIG. 3B, reference numeral 444 denotes a sample flow channel along which a sample flows from the sample injection port 354 to a corresponding assay sector 800. If multiple sample injection ports 354 are interconnected with each other, a single sample can be assayed for multiple analytes.

FIG. 3C shows the attachment of the cleavable signal element 200 to a radial track for each assay sector 800 by deposition. To electrically measure whether the cleavable signal element 200 is cleaved or not using the detector including a capacitance and impedance measurement device described above, an electronic control unit 63 and a circuit pattern 64 connecting each of the assay sectors 800 to the electronic control unit 63 are mounted on the substrate 70 of the circular disk. The electronic control unit 63 measures the capacitance and impedance with respect to each of the assay sectors 800 by checking for their frequency response characteristics, thereby providing information on whether the cleavable signal element 200 is cleaved or not, or information on the degree of cleavage. The frequency response characteristics measured by the electronic control unit 63 is transmitted to an external central controller (not shown) or storage device (not shown) via a non-contact interface 107, for example, an infrared interface or optical interface, designed on the disk. In FIG. 3C, reference numeral 354 denotes a sample injection port.

FIG. 3D shows an address pattern 401 formed on the substrate 70 of the circular disk to provide coded address information, from which the location of the cleavable signal element 200 may be optically or fluorescently measured, and the attachment of the cleavable signal element 200 at a constant interval over the entire surface of the disk by deposition. The circular disk has a central void 61 to engage a

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rotational drive means. The structure of FIG. 3D is suitable for a single-analyte assay with multiple samples or for a multiple-analyte assay with a single sample. To end this, samples may be injected through individual sample injection ports arranged in an ink-jet array corresponding to the location of each capture probes. Alternatively, a sample may be injected through a single sample injection port and spread over the entire substrate by a rotational force.

FIGS. 4A and 4B shows embodiments of a bio-driver, which is a mechanical device for rotating the disk of the assay devices described above. The electronic control device 63 transmits the measured frequency response characteristics to an external central controller 101 or storage device 111 through a non-contact interface, for example, an infrared interface or optical interface, which are located adjacent the central void 61 of the disk. Reference numerals 106 and 107 denote reception and transmission portions, respectively, of the non-contact interface. The reception and transmission portions 106 and 107 of the non-contact interface may be implemented with infrared sensors for infrared interfacing, or photosensors for optical interfacing.

In FIGS. 4A and 4B, embodiments of the supply of power to the electronic control unit 63 on the disk while it rotates are also shown. Reference numeral 100 denotes a driver body for supporting the bio-driver. A printed circuit board (PCB) 140 is connected to the driver body 100 below the bio-driver, and the central controller 101 for controlling the bio-driver and the storage unit 111 are mounted on the PCB 104. The central controller 101 controls a motor 102 to rotate the disk or stop rotation of the disk, controls movement of an optical device 103, and controls upper and lower rotors 104 and 105 such that they rotate adjacent the central void 651 of the disk upon rotation of the disk. The central controller 101 transmits the information read from the disk by the optical device 103 to the storage unit 111, or information to be

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written to the optical device 103, and provides a number of control signals required to read/write information to the other elements.

FIG. 4A shows an embodiment of the supply of power to the electronic control unit 63 on the disk by frictional contact between the upper and lower rotors 104 and 105 and respective brushes 108 and 109. In FIG. 4A, reference numeral 110 denotes a power supply unit for supplying a DC power to the brushes 108 and 109, and reference numerals 227 and 228 denotes arms. Alternatively, one of the upper and lower rotors 104 and 105 may be used. In this case, two opposite nodes of the power supply unit 110 are connected to one brush.

FIGS. 4C and 4D show embodiments of the supply of power to the electronic control unit 63 mounted on the disk by frictional contact between the upper rotor 104 and the brush 108, and between the lower rotor 105 and the brush 109, respectively. In particular, in FIG. 4C, an annular electrode plate 223 mounted on a top plate 222 of the upper rotor 104 to frictionally contact the brush 108 is shown. The two conductive arms 227 connected to the annular electrode plate 223 act as connectors to engage holes 302, which are described later, formed near the central void 61 of the disk. The annular electrode plate 223 has a radius of r1. Reference numeral 277 denotes a groove which supports the upper rotor 104 against the driver body 100. In FIG. 4C, an annular electrode plate 225 mounted on a bottom plate 224 of the lower rotor 105 to frictionally contact the brush 109 is shown. The two conductive arms 229 connected to the annular electrode plate 225 act as connectors to engage holes 301, which are described later, formed near the central void 61 of the disk.

FIG. 4E shows the holes 301 and 302 to engage the conductive arms 227 and 229, respectively, formed in the central void 61 of the disk. The central void 61 has a radius of r0. Reference numeral 333 denotes a hole in the central void 61. As the disk starts to rotate, the conductive

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arms 227 and 228 rotate while being engaged with the holes 301 and 302 as the upper rotor 104 and the lower rotor 105 are pushed closer together. A negative (ground) voltage is applied to the conductive arm 227 connected to the upper rotor 104, whereas a positive voltage is applied to the conductive arm 228 connected to the lower rotor 105. The holes 301 and 302 of the disk, which are engaged with the conductive arms 227 and 228, are connected to circuit patterns 303 and 304 to thereby supply power to the electronic control unit 63. To make the holes 301 and 302 engage easier with the conductive arms 227 and 118 when the upper rotor 104 and the lower rotor 105 are pushed closer together upon rotation of the disk. The conductive arms 227 and 228 have a spring 226 at its one end connected to the respective annular electrode plates 223 and 225.

FIG. 4B shows an embodiment of the supply of power to the electronic control unit 63 where an AC voltage is induced to a wound coil 152 on the disk and rectified by magnetic induction between an electromagnet 150 attached to the driver body 100 and the wound coil 152 to thereby supply power to the electronic control unit 63 in a non-contact manner. In FIG. 4B, reference numeral 110 denotes a power supply unit for supplying an AC current to the electromagnet 150.

FIG. 4F shows an implementation of detection of analyte-specific signals generated by the assay device of FIG. 3A, 3B, or 3D using the optical (or fluorescent) device 103. The optical device 103 is provided with differentially reflective (fluorescent) signals between the uncleaved signal element 66 and the cleaved signal element 65 with respect to incident light, in particular, incident laser light. The optical device 103 may include a light source, an incident light emitting portion, and a reflective light receiving portion.

FIGS. 5A through 5G show alternative embodiments of the detection of analyte-specific signals generated by the assay device of

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FIG. 3C using a capacitance and impedance measurement device having interdigitated array electrodes. In particular, FIG. 5A shows an embodiment of the capacitance and impedance measurement device implemented by interdigitated array electrodes 702 and 703 and a plurality of cleavable signal elements. The cleavable signal elements are attached to digits between the interdigitated array electrodes 702 and 703. The sensitivity of the detector increases with more digits.

Capacitance and impedance can be determined by measuring the frequency characteristics of the sample with application of AC signals having a predetermined bandwidth from the electronic control unit 63 to two input ports 704 and 705 of the interdigitated array electrodes 702 and 703.

FIG. 5B shows a state where the uncleaved signal element 34 remains between the interdigitated array electrodes 702 and 703 on the surface of a substrate 701. FIG. 5C shows a state where only a cleaved residue 38b remains after most of the cleavable signal element is has been detached. The electronic control unit 63 is provided with the differential frequency response characteristics between the uncleaved signal element 34 and the cleaved signal element 38b.

FIGS. 5D and 5E are for illustrating an embodiment of the capacitance and impedance measurement device implemented with interdigitated array electrodes and cleavable signal elements having one end labeled with a label 40 such as a metal microsphere, conducting polymer (e.g., polyaniline), or a fluorescent label. FIG. 5D shows a state where the uncleaved signal element 34 remains on the surface of the substrate 701 after cleavage of the cleavable signal elements and washing. FIG. 5E shows a state where the cleavable signal element has been detached.

FIGS. 5F and 5G is for illustrating an embodiment of the

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capacitance and impedance measurement device implemented with interdigitated array electrodes and a "label-attached uncleaved probe" structure formed through additional contact between the uncleaved signal element 34 and a label 39 after cleavage and wash steps. FIG. 5F shows a state where the uncleaved signal element 34 remains on the surface of the substrate 701 being labeled with the label 39. FIG. 5G shows a state where the cleavable signal element has been detached.

FIG. 5H shows an embodiment of arrangement of a plurality of assay sectors 800 on the disk, each including a pair of interdigitated array electrodes 702 and 703. Each of the assay sectors 800 may be constructed by combination of multiple pairs of interdigitated array electrodes 702 and 703 for multiple-analyte assay.

To enable the detector including the capacitance and impedance measurement device constructed with the interdigitated array electrodes 702 and 703 to electrically measure whether the cleavable signal element is cleaved or not, circuit patterns 64 which connect the electronic control unit 63 to each of the detectors arranged in the assay sectors 800, are imprinted in the substrate 70 of the circular disk. The electronic control unit 63 measures the capacitance and impedance with respect to each of the assay sectors 800 by checking for the frequency response characteristics from the assay sectors 800 and thereby obtains information on whether the cleavable signal element is cleaved or not or information on the degree of cleavage. In FIG. 5H, reference numeral 354 denotes a sample injection port, and reference numeral 444 denotes a sample inflow channel. Although multiple sample injection ports 354 are illustrated in FIG. 5H, only one simple injection port may be formed to assay a signal sample for multiple analytes.

FIG. 5I shows an embodiment of the capacitance and impedance measurement device in which a plurality of assay sectors 800, each including the interdigitated array electrodes 702 and 703, are arranged

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on a solid support 71 of a common shape. The electronic control unit 63 and circuit patterns 64 which connect the electronic control unit 63 to each of the detectors including the capacitance and impedance measurement device and arranged in the assay sectors 800, are mounted in the solid support 71, so that whether the cleavable signal element is cleaved or not can be measured using the converter. The electronic control unit 63 measures the capacitance and impedance with respect to each of the assay sector 800 by checking for the frequency response characteristics from the assay sectors 800 and thereby obtains information on whether the cleavable signal element is cleaved or not or information on the degree of cleavage. In FIG. 5I, reference numeral 354 denotes a sample injection port, reference numeral 444 denotes a sample inflow channel, reference numeral 356 denotes a sample exhaust port, and reference numeral 445 denotes a sample exhaust channel.

FIGS. 6A and 6B show alternative embodiments of implementation of the differential reflection between a cleaved signal element and an uncleaved signal element. Referring to FIG. 6A, a gold layer 22 and a self-assembled monolayer (SAM) 32 are sequentially formed on a substrate, and a cleavable signal element 34 is immobilized on the SAM 32. Reference numeral 65 denotes a cleaved signal element having a cleaved residue 38b left after the cleavable signal element has been detached. Reference numeral 66 denotes an uncleaved signal element. FIG. 6B illustrates the application of a label 39, such as a metal microsphere, conducting poymer, or fluorescent label, to increase the sensitivity of the detector. As shown in FIG. 6B, the gold layer 22 and the SAM 32 are sequentially formed on the substrate, and a cleavable signal element 34 is immobilized on the SAM 32. Reference numeral 65 denotes a cleaved signal element having a cleaved residue 38b left after the cleavable signal element has been

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detached. Reference numeral 66 denotes an uncleaved signal element.

FIGS. 6C through 6E show alternative embodiments of implementation of the differential conductance (impedance or capacitance) between the cleaved signal element and the uncleaved signal element using interdigitated array electrodes. Referring to FIG. 6C, the gold layer 22 and the SAM 32 for immobilization of the cleavable signal element 34 are formed on a substrate 20. The gold layer 22 constitutes the interdigitated array electrodes. A protective layer 33 is formed to protect the gold layer 22 from the cleavable signal element 34 adhering to the gold layer 22. As shown in FIG. 6C, which is a partial cross-sectional view of the assay sector 800 of FIG. 5A, only a cleaved residue 38b of a cleaved signal element 65 remains after the cleavable signal element has been detached. Reference numeral 66 denotes an uncleaved signal element.

FIG. 6D shows an embodiment of labeling one free end of the cleavable capture probe, which constitutes a cleavable signal element whose the other end is attached to the substrate, with a label 40 such as a metal microsphere, conducting polymer, or fluorescent label to increase the sensitivity of the detector. FIG. 6E shows an embodiment of formation of a "label-attached uncleaved probe" structure after washing by additional labeling of the uncleaved signal element 66 with a label 39, such as a metal microsphere, conducting polymer, or fluorescent label. As shown in FIGS. 6D and 6E, which are partial cross-sectional views of the assay sector 800 of FIG. 5A, only a cleaved residue 38b of a cleaved signal element 65 remains after the cleavable signal element has been detached. Reference numeral 66 denotes an uncleaved signal element labeled with the label 40 or 39.

FIG. 7A illustrates the arrangement of four separate assay sectors in an assay device, each containing a different cleavable signal element

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200, to assay in parallel a single sample for four kinds of anlytes. The single sample injected through the sample injection port 354 is supplied to each of the assay sectors through the sample inflow channel 444. On each of the assay sectors 800, the cleavable signal element 200 having a capture probe complementary to a different analyte is deposited. Preferably, the cleavable signal element 200 is fluorescently detected. In this case, a fluorescent label is applied to the end of the cleavable signal element 200, as illustrated in FIG. 2A. The assay device of FIG. 7A includes a sample exhaust port 356 and a sample exhaust channel 445. Alternatively, different kinds of cleavable signal elements 200 that are complementary to a plurality of discrete analytes may be deposited within one assay sector to enable multi-analyte assay in a single assay sector.

FIG. 7B shows an embodiment of the assay device according to the present invention, in which a plurality of assay devices of FIG. 7A are radially arranged on a disk.

FIG. 8 shows an embodiment of a remote diagnostic system according to the present invention, in which the information read from the assay device is digitalized as computer software and mutually transmitted to and received by a patient 151 and a doctor 125 through an existing communication network 133. In FIG. 8, reference numeral 120 denotes a detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, as described above, to detect the presence or absence of the cleavable signal element on the solid support (substrate). The detector 120 may be a bio-driver including a central controller and an assay device in the form of disk, bio-CD, or bio-DVD where analyte-specific cleavable signal elements are spatially and addressibly arranged in a variety of ways. Reference numeral 127 denotes a software-installed hard disk driver (HDD) or memory. The software may

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include assay interpretive algorithms, bioinformatics information, and self-diagnostics related information. The software may further include software capable of uploading the diagnostic information to remote locations and device drivers. The software may include educational information for patients on clinical assays, and may be modified for chosen audiences. The software may include a variety of wet sites and links, for example, enabling a patient to communicate with a doctor or hospital based on his/her diagnosis result. Reference numerals 121 and 123 denote a camera and a microphone for viewing a patient's face and listening to his/her voice, respectively. Reference numeral 151 denotes a patient. A hospital 124, a doctor 125, and a nurse 126, which provide remote diagnosis services, are also shown in FIG. 8.

< Method of Applying Sample >

A cleavable signal element according to the present invention is suitable for detecting, in particular, a nucleic acid amplified to a limited size through an amplification scheme using a variety of polymerase chain reactions (PCRs), ligase chain reactions (LCRs), and T7 and SP6RNA polymerases.

In an assay method according to the present invention, a sample to be tested is first introduced. After a dilute fluid sample is applied near the center of the substrate (solid support) of a circular, disk-type assay device, the assay device is rotated. The fluid sample evenly diffuses over and uniformly covers the surface of the substrate by a centrifugal fore generated by the rotation of the assay device.

In this method of applying the sample, 100 μL of the test sample is diluted to about 1 mL. This dilute sample is dropwise added near the center of the disk. The assay sites and the surface of the disk are hydrophilic, and the fluid sample forms a thin fluid film on the rotating

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disk. The thickness of the fluid film can be adjusted by the frequency of the dropwise addition and the frequency of disk rotation. Preferably, the thickness of the fluid film is less than 10 μm to permit all molecules in the fluid sample to react with the cleavable signal element. About 10 μL of the fluid sample is needed to fully cover the surface of the disk. This sample apply method is suitable for, in particular, the assay devices of FIGS. 3A through 3D and FIG. 5F.

Another sample apply method is available with the cleavable signal element and assay device according to the present invention, in particular, the assay devices of FIGS. 3B, 3C, and 5H, each of which includes 8 separate assay sectors 800 and is suitable to apply a single sample to each assay sector.

In other aspects of the present invention, separate samples may be applied to discrete sites of the disk-type assay device. In view of this, the assay device according to present invention can assay approximately one thousand different samples. In addition, to increase the sensitivity of the detector, approximately one million gold microspheres, conducting polymers, or fluorescent labels can be applied to label assay sites.

As an embodiment, the assay device of FIG. 3D, which has at the assay sites on the disk a plurality of cleavable signal elements with identical capture probes conferring identical analyte specificity, may be designed to concurrently assay 1024 patient samples. In other words, the assay device of FIG. 3D may include 1024 cleavable signal elements on the disk. In such an embodiment, each of the capture probes on the disk may be identical, so as to assay for the same analyte. Capture probes at particular sites on the disk have the same oligonucleotide sequence as those at other sites on the disk. This application is particularly useful in mass analysis conducted in clinical laboratories

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where a large number of patient samples are analyzed at the same time for the presence or absence of a single analyte.

Patient samples may be applied to particular assay sites on the disk by a known method, such as ink jet printing, micropippet arrays with disposable tips, or a combination thereof.

Alternatively, the assay device of FIG. 3D may be applied to assay a single sample for multiple analytes by using a plurality of diverse, cleavable signal elements specific to different analytes for each assay device.

< Hybridization >

In a nucleic acid hybridization assay according to the present invention, after the sample injection, rotation of the disk is halted, and the disk is incubated in a stationary state at room temperature for hybridization reaction between the capture probe and the complementary target nucleic acid in the sample.

< First Wash Step>

The nucleic acid hybridization assay according to the present invention involves first and second wash steps. After the even application of the sample over the disk surface and an appropriate incubation period for the hybridization, a first wash step is necessary. For example, in nucleic acid hybridization assays, at a lower salt concentration of the wash solution, washing is smooth, thus reducing mismatch as between analyte (target nucleic acid) and capture probes. In contrast, at a higher salt concentration, washing is not smooth, thereby permitting mismatch to occur. Adjusting the stringency of wash in nucleic acid hybridization assays, in terms of salt concentration, is well within the skill in the art.

In one aspect according to the present invention, the surface of

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the circular, disk-type assay device may be washed by adding a wash solution near the center of the rotating disk. The sample solution is removed as it pushes out from the periphery of the disk and is collected. Because of the rotation of the disk, the wash step may be eliminated if the fluid sample is adequately removed from the disk by centrifugal force. This centrifugal force is strong enough to mechanically denature mismatching oligonucleotides.

Alternatively, mismatching oligonucleotides may be removed with application of an external electric field. Due to the nature of its phosphate backbone which is negatively charged, the sample oligonucleotides hybridized to the cleavable signal element with a weak binding force can be denatured by applying an external negative electric field.

As is shown in FIG. 9, the external electric field is applied with an electrode plate 133, disposed directlly above the assay device, and an external voltage source 221. FIG. 9 shows an embodiment of washing away mismatching oligonucleotides 333 from the assay device of FIG. 6D with application of an external electric field.

< DNA Extension Step >

When a restriction enzyme specifically responsive to a particular sequence of a double stand is used according to the present invention, DNA extension is needed after the hybridization and first wash step. In DNA extension, a single-stranded restriction probe is double-stranded using the target nucleic acid previously hybridized to the capture probe as a primer with addition of a DNA polymerization solution containing four dNTPs and a polymerase.

Prior to addition of the DNA polymerization solution, it is preferable to contact the restriction probe with a 3'→5' exonuclease

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solution. As is shown in FIG. 12, a step of hydrolytic cleaving a single-straned target nucleic acid portion 79, which is unbound to the capture probe, by addition of the 3'→5' exonuclease solution, may be further included before the DNA extension. The result is readily applied to DNA extension using the target nucleic acid previously hybridized to the capture probe as a primer.

< Cleavage Step >

After the first wash step (or DNA extension), a solution containing a restriction enzyme or cleavage enzyme (DNAse or nuclease) is added and distributed over the surface of the disk. The disk is incubated in a stationary state at room temperature, and the complementary double strand or single strand resulting from the hybridization is specifically cleaved. This enzymetic cleavage is maintained for a few seconds.

< Second Wash Step >

After the enzymatic cleavage step, a second wash step is needed to remove the cleaved signal elements. In this second wash step, differential wash stringencies are provided to permit variation in the specificity and sensitivity of the nucleic acid hybridization assay.

The cleaved signal elements may be removed by rotating the assay device, with or without addition of wash solution, or by applying an external electric field. In this aspect, four parameters may be varied to provide differential wash stringency: label particle size (such as metal microspheres, conducting polymers, or fluorescent labels), rotational speed, the valency of capture probe attachment, and the intensity of external electric field.

Gold microspheres suitable for use in the cleavable signal element and assay device of the present invention are readily available

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in varying diameters from Aldrich Chemical Company, British BioCell International, Nanoprobes, Inc., ranging from 1 nm to and including 0.5-5 micrometers in diameter. Gold microspheres of lesser or greater diameter may be formed as needed in the present invention. At a given rotational speed, the largest gold microspheres experience larger centrifugal and drag forces and are removed before smaller micsrospheres with equal bonding. This provides a basis for differential stringency of wash, and also of quantitative analysis.

The centrifugal force affecting the gold microspheres may also be adjusted by rotation frequency so that the loose and weakly bound gold microspheres are removed. Only the capture probes which have bound to a complementary molecule from the sample will continue to bind the gold microspheres to the substrate.

Furthermore, while the above embodiments of the invention have been described with a single metal microsphere attached to the end of a single cleavable signal element, it should be appreciated that when gold microspheres are used in a preferred embodiment of the invention, thousands of cleavable signal elements may bind to one gold microsphere, depending upon its diameter. Thus, the stringency of the assay wash may be adjusted, at any given rotational speed, by varying the diameter of the gold microsphere, and by varying additionally the relative density of cleavable signal elements to gold microspheres. Thus, if virtually all cleavable signal elements under a certain gold sphere are connected by complementary molecules, the binding is very strong. If the cleavable signal elements are fixated only partially under a certain gold microsphere, the microsphere may remain or be removed depending on the radius of the microsphere and the frequency of rotation. Alternatively, ferromagnetic microspheres, gold-coated iron beads, or an iron alloy beads may be used instead of the gold microspheres. In this case, those probes detached through cleavage

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may be removed with application of a magnetic field.

< Detection Step >

After removal of cleaved signal elements through the second wash step, the disk may be read directly. Alternatively, the disk may be covered by an optically transparent plastic coating to prevent the further removal of the gold microspheres through spin coating with a polymerizable lacquer that is polymerized with UV-light. Spin coating of compact disks is well established in the art. The assay device disk is expected to have a shelf-life of over ten years.

Subsequently, the disk can be scanned by a laser reader which will detect, through reflection, the presence of a microsphere or other reflective elements at the various spatially predetermined locations. Based on the distance of the microsphere from the axis of rotation of the disk and the angular distance from an address line forming a radial line on the disk, the location of a particular metal microsphere can be specifically determined. Based on that specific location and the predetermined locations of specific binding pairs as compared to a master distribution map, the identity of the bound material can be identified. Thus, in the foregoing manner it is possible in one fluid sample to analyze for thousands, or even greater numbers, of analytes simultaneously.

< Additional Labeling Step >

In the case of forming the "label-attached uncleaved probe" structure, an additional step of labeling the cleavable signal element or uncleaved probe is included before sample injection or after cleavage. In particular, the additional labeling before sample injection follows the synthesis of the capture probe or capture probe attachment to the substrate (solid support). The additional labeling after cleavage

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requires a preceding sample washing step.

The nucleic acid hybridization assay according to the present invention involves the sample injection, hybridization, first wash and cleavage, additional labeling reaction, and second wash steps described above.

< Synthesis of Cleavable Signal Element and Attachment to Glass Substrate>

FIGS. 10A through 10C show alternative embodiments of the synthesis of cleavable signal elements and attachment to glass substrates.

1. Cleaning of Glass Substrate

A detergent (Alconox) is first dissolved in distilled water, and glass substrates are sonicated in the detergent solution for approximately 50 minutes. The glass substrates are rinsed with distilled water to remove any sticking detergent. The rinsed glass substrates are boiled or sonicated in a piranha solution (a 3:7 mixture of H_2O_2 and H_2SO_4) for 30 minutes. For glass substrates coated with, for example, gold, the glass substrates are socked in the piranha solution for washing, without sonication. Next, the glass substrates are removed from the piranha solution and rinsed copiously with distilled water to completely remove the piranha solution from the glass substrate surface (Steps 10a-1, 10b-1, and 10c-1 of FIGS. 10A, 10B, and 10C).

2. Reaction for Oligonucleotide Attachment

(a) Attachment of oligonucleotide using amine-ologinucleotide

FIG. 10A shows the procedure of attachment of oligonucleotide 34 on a cleaned glass substrate 24 using amino-oligonucleotide. The cleaned glass substrate 24 is reacted with a silanazation material, for

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example, (10-carbomethoxydecyl)dimethylchlorosilane ($ClSi(CH_3)_2$-$(CH_2)_n$-COOH), with a carboxyl-convertible functional group.

For the reaction, the cleaned glass substrate 24 is dried in a vacuum and reacted in a solution of the silanization material of about 0.5 mL in 20 mL of toluene for about 24 hours in an argon gas atmosphere. Next, the glass substrate is washed with toluene and then acetone, and dried in a vacuum or by flowing gas. The glass substrate is socked and reacted in a 1M HCl solution at 50℃ for 5 hours, thereby resulting in a carboxyl-substituted glass substrate (Step 10a-2). The glass substrate with the carboxyl group is washed with acetone and dried. The glass substrate is reacted in an aqueous solution of 0.2M N-hydroxysuccinimide (NHS), 0.2M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), and amine-oligonucleotide (H_2N-oligo) of 0.01-0.1 mg/mL for 20 hours, followed by surface washing. The result is an oligonucletide-attached glass substrate (Step 10a-3).

(b) Attachment of oligonucleotide using amine-oligo-thiol group

FIG. 10B shows the procedure of attachment of oligonucleotide on the cleaned glass substrate 24 using an amine-oligo-thiol group. The cleaned glass substrate 24 is reacted with a silanazation material, for example, (10-carbomethoxydecyl)dimethylchlorosilane ($ClSi(CH_3)_2$-$(CH_2)_n$-COOH), with a carboxyl-convertible functional group.

For the reaction, the cleaned glass substrate 24 is dried in a vacuum and reacted in a solution of the silanization material of about 0.5 mL in 20 mL of toluene for about 24 hours in an argon gas atmosphere. Next, the glass substrate is washed with toluene and then acetone, and dried in a vacuum or by flowing gas. The glass substrate is socked and reacted in a 1M HCl solution at 50℃ for 5 hours, thereby resulting in a carboxyl-substituted glass substrate (Step 10b-2). The glass substrate

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with the carboxyl group is washed with acetone and dried. The glass substrate is reacted in an aqueous solution of 0.2M NHS, 0.2M EDCI, and an amine-oligonucleotid-thiol group (H_2N-oligo-SH) of 0.01-0.1 mg/mL for 20 hours, followed by surface washing. The result is an oligonucletide-attached glass substrate (Step 10b-3).

(c) Attachment of oligonucleotide using biotin-avidin reaction

FIG. 10C shows the procedure of attachment of oligonucleotide on the cleaned glass substrate 24 using biotin-avidin reaction. The cleaned glass substrate 24 is reacted with a silanazation material, for example, (10-carbomethoxydecyl)dimethylchlorosilane ($ClSi(CH_3)_2$-$(CH_2)_n$-COOH), with a carboxyl-convertible functional group.

For the reaction, the cleaned glass substrate 24 is dried in a vacuum and reacted in a solution of the silanization material of about 0.5 mL in 20 mL of toluene for about 24 hours in an argon gas atmosphere. Next, the glass substrate is washed with toluene and then acetone, and dried in a vacuum or by flowing gas. The glass substrate is socked and reacted in a 1M HCl solution at 50℃ for 5 hours, thereby resulting in a carboxyl-substituted glass substrate (Step 10c-2). The glass substrate with the carboxyl group is washed with acetone and dried. The glass substrate is reacted in an aqueous solution of 0.2M NHS, 0.2M EDCI, and avidin (denoted by reference numeral 51) of 11 mg/mL for 20 hours, followed by surface washing with buffer (10mN Tris, pH 7.2).

Next, the glass substrate surface is reacted with a solution of biotin (denoted by reference numeral 50)-oligonucleotide in 1 XTBE for 8 hours and washed with buffer (Step 10c-4). Alternatively, the glass substrate is reacted with a solution of biotin-oligo-SH in 1 XTBE and washed with buffer (Step 10c-5).

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< Synthesis of Cleavable Signal Element and Attachment to Gold Substrate >

FIGS. 10D through 10H show alternative embodiments of the synthesis of cleavable signal elements and attachment to gold substrates.

1. Cleaning of Glod Substrate

Gold substrates are soaked in a saturated KOH solution for 1 hour and rinsed copiously with distilled water. The gold substrates are then soaked in sulfuric acid for approximately 2 hours, followed by rinsing with distilled water (Steps 10d-1, 10e-1, 10g-1, and 10h-1).

2. Reaction for Oligonucleotide Attachment

(a) Attachment of oligonucleotide using thiol group

FIG. 10D shows the procedure of attachment of the oligonucleotide 34 on a cleaned gold substrate 22 using a thiol group. The entire surface of the gold substrate 22 is spray-coated with a buffer solution into which HS-$(CH_2)_n$-oligo has been dissolved, tightly sealed to prevent coating evaporation, and reacted for approximately 5 hours. Following washing with buffer (Step 10d-2), a buffer solution containing HS-$(CH_2)_n$-OH is applied to the gold substrate surface to space out the oligonucleotides immobilized on the surface (Step 10d-3).

FIG. 10E shows another embodiment of the attachment of the oligonucleotide 34 on the cleaned gold substrate 22 using the thiol group. The entire surface of the gold substrate 22 is spray-coated with a buffer solution containing HS-$(CH_2)_n$-COOH, for example, HS-$(CH_2)_6$-COOH in 0.2M aqueous mercaptohexanoic acid solution, tightly sealed to prevent coating evaporation, and reacted for approximately 10 hours. Following washing with distilled water and drying (Step 10e-2), the resulting gold substrate is reacted in an aqueous solution of 0.2M NHS, 0.2M EDCI,

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and amine-oligonucleotide-thiol (H_2N-oligo-SH) of 0.01-0.1 mg/mL for 10 hours (Step 10e-3).

(b) Attachment of oligonucleotide using biotin-avidin reaction

FIG. 10F shows the procedure of attachment of the oligonucleotide 34 on the cleaned gold substrate 22 using biotin-avidin reaction. The entire surface of the gold substrate 22 is coated with a solution of biotin disulfide N-hydroxysuccinimide (prepared by dissolving 1 g of biotin disulfide N-hydroxysuccinimide in 200 μL of dimethylformamide (DMF) and diluting the solution with addition of 800 μL of distilled water), tightly sealed to prevent coating evaporation, and reacted for approximately 5 hours. Following washing with distilled water (Step 10f-2), the resulting gold substrate is spray-coated with an avidin solution, tightly sealed, and reacted for 10 hours (Step 10f-3, reference numeral 51 denotes an avidin molecule. Next, the glass substrate surface is reacted with a solution of biotin (denoted by reference numeral 50)-oligonucleotide in 1 XTBE for approximately 8 hours and washed with buffer (Step 10f-4).

FIG. 10G shows another embodiment of the attachment of the oligonucleotide 34 on the cleaned gold substrate 22 using the biotin-avidin reaction. The surface of the gold substrate 22 is coated with a solution of avidin (denoted by reference numeral 51) in 1 XTBE, tightly sealed to prevent coating evaporation, and reacted for approximately 5 hours (Step 10g-2). Next, the glass substrate surface is reacted with a solution of biotin (denoted by reference numeral 50)-oligonucleotide in 1 XTBE for approximately 8 hours and washed with buffer (Step 10g-3).

(c) Attachment of oligonucleotide-biotin using thiol group

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FIG. 10H shows the procedure of attachment of ologonucleotide-biotin on the cleaned gold substrate 22 using the thiol group. The entire surface of the cleaned gold substrate 22 is spray-coated with a buffer solution into which HS-$(CH_2)_n$-oligo-biotin has been dissolved, tightly sealed to prevent coating evaporation, and reacted for approximately 5 hours. Following washing with buffer (Step 10h-2), a buffer solution containing HS-$(CH_2)_n$-OH is applied to the gold substrate surface to space out the oligonucleotides immobilized on the surface (Step 10h-3).

< Synthesis of Cleavable Signal Element and Attachment to Plastic Substrate >

FIGS. 10I through 10K shows alternative embodiments of the synthesis of cleavable signal elements and attachment to plastic substrates.

1. Cleaning of Plastic Substrate

Gold substrates are soaked and sonicated in a Alconox solution for about 30 minutes and rinsed copiously with distilled water.

2. Reaction for Oligonucleotide Attachment

FIG. 10I shows an embodiment of the attachment of the ologonucleotides 34 on a cleaned plastic substrate 20. The surface of the plastic substrate 20 is aminated by ammonia plasma (Step 10i-1) and completely spray-coated with a buffer solution in which -COOH-R-COOH, where R is any amine-reactive formula, for example, 3,3-diimethylgutaric acid (-HOOC-CH_2-$C(CH_3)_2$-CH_2-COOH), has been dissolved (Step 10i-2). Preferably, R is alkane or other functional groups. The plastic substrate 20 is tightly sealed and reacted for approximately 10 hours. Following washing with distilled water and

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drying, the plastic substrate is reacted in an aqueous solution of 0.2M NHS, 0.2M EDCI, and amine-oligonucleotide (H_2N-oligo) of 0.01-0.1 mg/mL for 10 hours (Step 10i-3).

FIG. 10J shows another embodiment of the attachment of the oligonucleotides on the cleaned plastic substrate 20. The surface of the plastic substrate 20 is aminated by ammonia plasma (Step 10j-1) and completely spray-coated with a buffer solution in which -COOH-R-COOH has been dissolved (Step 10j-2). The plastic substrate 20 is tightly sealed and reacted for approximately 10 hours. Following washing with distilled water and drying, the plastic substrate is reacted in an aqueous solution of 0.2M NHS, 0.2M EDCI, and amine-oligonucleotide-thiol (H_2N-oligo-SH) of 0.01-0.1 mg/mL for 10 hours (Step 10j-3).

FIG. 10K shows still another embodiment of the attachment of the oligonucleotides to the cleaned plastic substrate 20. The surface of the plastic substrate 20 is aminated by ammonia plasma (Step 10k-1) and completely spray-coated with a solution of succinimidyl 4-maleimido butyrate (SMB), a heterobifunctional crosslinker, in a 1:10 mixture of DMF and sodium bicarbonate buffer (50mM, pH 8.5) (Step 10k-2: monolayer formation). The resulting plastic substrate is tightly sealed and reacted for approximately 3 hours. Following washing with distilled water and drying, the plastic substrate is reacted with HS-oligonucleotide-biotin in HEPES buffer (10 mM, pH 6.6, 5.0 mM EDTA) for 3 hours (Step 10k-3).

< Method to increase Detector Sensitivity >

In the nucleic acid hybridization assay according to the present invention, to increase the sensitivity of the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, after Steps 10b-3,

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10c-5, 10e-3, and 10j-e, or after the first wash step and cleavage, a metal microsphere suspension is spread over the substrate and reacted at room temperature for about .5 hours to form a metal microsphere-attached cleavable signal element or a "label-attached uncleaved probe" structure.

In the nucleic acid hybridization assay according to the present invention, to increase the sensitivity of the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, alternatively, after Steps 10a-3, 10c-4, 10d-3, 10f-4, 10g-4, and 10i-3, or after the first wash step and cleavage, a conducting polymer solution is spread over the substrate and reacted at room temperature for about 5 hours to form a conducting polymer-attached cleavable signal element or a "label-attached uncleaved probe" structure.

In the nucleic acid hybridization assay method according to the present invention, to increase the sensitivity of the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, alternatively, after Steps 10a-3, 10c-4, 10d-3, 10f-4, 10g-4, and 10i-3, or after the first wash step and cleavage, an aqueous solution of fluoreceine isothiocyanate of 0.1 mg/mL, a fluorescer, is spread over the substrate and left in a dark room for about 5 hours to form a fluorescent-labeled cleavable signal element or a "label-attached uncleaved probe" structure. For label attachment, the amine group of the oligonucleotides should extend towards the reaction solution.

In the nucleic acid hybridization assay method according to the present invention, to increase the sensitivity of the detector including an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device, alternatively, after Steps 10h-4 and 10k-3 or after the first wash step and cleavage, a

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suspension of streptavidin labeled microbeads 40 or streptavidin-labeled magnetic microbeads is spread over the substrate and reacted at room temperature for about 5 hours to form a streptavidin-labeled microbead-attached (or magnetic microbead- attached) cleavable signal element" or a "label-attached uncleaved probe" structure.

< Gold Particles as Signal Responsive Moieties >

In preferred embodiments of the present invention, particles that reflect or scatter light are used as signal responsive moieties. A light reflecting and/or scattering particle is a molecule or a material that causes incident light to be reflected or scattered without absorbing the light energy. Such light reflecting and/or scattering particles include, for example, metal particles, colloidal metal such as colloidal gold, colloidal non-metal labels such as colloidal selenium, dyed plastic particles made of latex, polystyrene, polymethylacrylate, polycarbonate or similar materials.

The size of such particles ranges from 1 nm to 10 μm, preferably from 500 nm to 5 μm, and most preferably from 1 to 3 μm. The larger the particle, the greater the light scattering effect. Metal microspheres 1 nm to 10 μm in diameter, preferably 0.5-5 μm, most preferably 1-3 μm in diameter, are presently preferred in the light reflecting/light scattering embodiment of the present invention. Metal microspheres provide a convenient signal responsive moiety for detection of the presence of an uncleaved signal element bound to the disk. Typical materials are gold, silver, nickel, chromium, platinum, copper, and the like, or alloys thereof, with gold being most preferred. The metal microspheres may be solid metal or may be formed of plastic, or glass beads or the like, upon which a coating of metal has been deposited. Metal microspheres may also be alloys.

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Gold spheres suitable for use in the cleavable reflective signal element and assay device of the present invention are readily available in varying diameters from Aldrich Chemical Company, British BioCell International, Nanoprobes, Inc., and others, ranging from 1 nm to and including 0.5 μm (500 nm) -5 μm in diameter. It is within the skill in the art to create gold microspheres of lesser or greater diameter as needed in the present invention. Much smaller spheres can be used advantageously when reading is performed with optical microscopy, UV-light, electron beam or scanning probe microscopy. Smaller spheres are preferred because more cleavable signal elements can be discriminated in a given area of a substrate.

Although spherical particles are preferred, non-spherical particles are also useful for some embodiments. In biological applications, the signal responsive moiety--particularly gold or latex microspheres--will preferably be coated with detergents or derivatized so that they have a surface charge. This is done to prevent the attachment of these particles nonspecifically with surfaces or with each other.

The preferred gold microspheres bind directly to the thiol group of the end of the cleavable signal element, yielding a very strong bond.

Furthermore, while the above embodiments of the invention have been described with a single metal microsphere attached to the end of a single cleavable signal element, it should be appreciated that when gold is used in a preferred embodiment of the invention, thousands of cleavable signal elements may bind one gold microsphere, depending upon its diameter. It is estimated that one sphere of 1-3 μm may be bound by approximately 1,000-10,000 cleavable signal elements.

As a result, the stringency of the assay wash may be adjusted to give higher assay reliability, at any given rotational speed, by varying not only the diameter of the gold sphere, but also the relative density of

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cleavable signal elements to gold microspheres.

Accordingly, if virtually all captures probes under a certain gold microsphere are connected by complementary molecules, the binding is very strong. If the capture probes are fixated only partially under a certain gold microsphere, the microsphere may remain or be removed depending on the radius of the microsphere and the frequency of the rotation.

In another preferred embodiment of the present invention, since the metal microsphere increases conductivity, it can improve the sensitivity of a detector constructed of the capacitance and impedance measurement device.

In still another preferred embodiment of the present invention, conducting polymers or fluorescent labels may be used instead of the metal microsphere. The conducting polymer or fluorescent label acts as a light reflecting (light diverging) and scattering particle or a conductivity-increasing particle, so it can improve detection sensitivity when used with a photodetector (fluorescent detector) or a detector constructed of the capacitance and impedance measurement device.

< Other Light-Responsive Signal Responsive Moieties >

In other embodiments of the cleavable signal element and assay device of the present invention, a light-absorbing rather than light-reflective material can be used as a signal responsive moiety. The approach is analogous to that used in recordable compact disks.

Although similar in concept and compatible with CD readers, information is recorded differently in a recordable compact disk (CD-R) as compared to the encoding of information in a standard CD. In CD-R, the data layer is separate from the polycarbonate substrate. The polycarbonate substrate instead has impressed upon it a continuous spiral groove as a reference alignment guide for the incident laser. An

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organic dye is used to form the data layer. Although cyanine was the first organic dye used for these disks, a metal-stabilized cyanine compound is generally used instead of "raw" cyanine. An alternative material is phthalocyanine. One such metallophthalocyanine compound is described in U.S. Patent No. 5,580,696.

In CD-R, the organic dye layer is sandwiched between the polycarbonate substrate and the metalized reflective layer, usually 24 carat gold, but alternatively silver, of the media. Information is recorded by a recording laser of appropriate preselected wavelength that selectively melts "pits" into the dye layer, it simply melts it slightly, causing it to become non-translucent so that the reading laser beam is refracted rather than reflected back to the reader's sensors. As in a standard CD, a lacquer coating protects the information layers.

A greater number of light-absorbing dyes may be used in this embodiment of the present invention than may be used in CD-R. Light-absorbing dyes are any compounds that absorb energy from the electromagnetic spectrum, ideally at wavelength(s) that correspond to the wavelength(s) of the light source. As is known in the art, dyes generally consist of conjugated heterocyclic structures, exemplified by the following classes of dyes: azo dyes, diazo dyes, triazine dyes, food colorings or biological stains. Specific dyes include: Coomasie Brilliant Blue R-250 Dye (Biorad Labs, Richmond, Calif.); Reactive Red 2 (Sigma Chemical Company, St. Lois, Mo.), bromophenol blue (Sigma); xylene cyanol (Sigma); and phenolphthalein (Sigma). The Sigma-Aldrich Handbook of Stains, Dyes and Indicators by *Floyd J. Green*, published by Aldrich Chemical Company, Inc., (Milwaukee, Wis.) provides a wealth of data for other dyes. With these data, dyes with the appropriate light absorption properties can be selected to coincide with the wavelengths emitted by the light source.

In other embodiments, the signal responsive moiety may be a

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fluorescer, such as fluorescein, propidium iodide or phycoerythrin, or a chemiluminescer, such as luciferin, which responds to incident light, or an indicator enzyme that cleaves soluble fluorescent substrates into insoluble form. Other fluorescent dyes useful in this embodiment include texas red, rhodamine, green fluorescent protein, and the like. Fluorescent dyes will prove particularly useful when blue lasers become widely available.

The present invention preferentially employs a circular assay device as the substrate for the patterned deposition of light-reflective, light-scattering, light-absorptive, or fluorescent cleavable signal elements. In a preferred embodiment, the assay device is compatible with existing optical disk readers, such as a compact disk (CD) reader or a digital versatile disk (DVD) reader, and is therefore preferentially a disk of about 120 mm in diameter and about 1.2 mm in thickness. It will be appreciated, however, that the cleavable signal elements of the present invention may be deposited in spatially-addressable patterns on substrates that are not circular but rectangular.

The maximum number of cleavable signal elements that can be spatially discriminated on an optical disk is a function of the wavelength and the numerical aperture of the objective lens. One known way to increase memory capacity in all sorts of optical memory disk, such as CD-ROMs, WORM (Write Once Read Many) disks, and magneto-optical disks, is to decrease the wavelength of the light emitted by the diode laser which illuminates the data tracks of the optical memory disks. Smaller wavelength permits discrimination of smaller data spots on the disk, that is, higher resolution, and thus enhanced data densities. Current CD-ROMs employ a laser with a wavelength of 780 nanometers (nm). Current DVD readers employ a laser with a wavelength between 635 and 650 nm. New diode lasers which emit, for example, blue light (around 481 nm) would increase the number of signal elements that

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could be spatially addressed on a single assay device disk of the present invention. Another way to achieve blue radiation is use of a second harmonic generator (SHG) that achieves frequency doubling of infrared laser by non-linear optical material.

Current CD-ROM readers employ both reflection reading and transmission reading. Both data access methods are compatible with the present invention. Gold particles are especially suitable for use as a signal responsive moiety for reflection type CD-ROM readers. Light-absorbing dyes are more suitable for transmission type readers such as the ones discussed in U.S. Patent No. 4,037,257.

< Other Signal Responsive Moieties >

It will be apparent to those skilled in the art that signal responsive moieties suitable for adaptation to the cleavable signal element of the present invention are not limited to light-reflecting or light-absorbing metal particles or dyes. Suitable signal responsive moieties include, but are not limited to, any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. In preferred embodiments, suitable signal responsive moieties include colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads, biotin-bound beads with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads™), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), and enzymes (e.g., horse radish peroxidase (HRP), alkaline phosphatase, etc.).

It will be apparent to those skilled in the art that numerous variations of signal responsive moieties may be adapted to the cleavable signal elements of the present invention. A number of patents, for example, provide an extensive teaching of a variety of techniques for

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producing detectible signals in biological assays. Such signal responsive moieties are generally suitable for use in some embodiments of the present invention. As a non-limiting illustration, the following is a list of U.S. patents teach the several signal responsive moieties suitable for embodiments of the present invention: U.S. Patent Nos. 3,646,346, radioactive signal generating means; 3,654,090, 3,791,932 and 3,817,838, enzyme-linked signal generating means; 3,996,345, fluorescer-quencher related signal generating means; 4,062,733, fluorescer or enzyme signal generating means; 4,104,029, chemiluminescent signal generating means; 4,160,645, non-enzymatic catalyst generating means; 4,233,402, enzyme pair signal generating means; 4,287,300, enzyme anionic charge label. All above-cited U.S. patents are incorporated herein by reference for all purposes.

Other signal generating means are also known in the art, for example, U.S. Patent Nos. 5,021,236 and 4,472,509, both incorporated herein by reference for all purposes. A metal chelate complex may be employed to attach signal generating means to the cleavable signal elements. In other embodiments, magnetic spheres may be used in place of reflective spheres, and magnetic poles may be vertically aligned by treating the disk with a magnetic field that is of sufficient strength. Since the empty sites will not have any magnetic material present, the presence or absence of a target nucleic acid in the test sample can be identified. The location of the uncleaved signal element can be detected using an optomagnetic sensor widely used in existing optomagnetic disks based on the Kerr effect or a magneto resistance (MR) sensor.

Paramagnetic ions might be used as a signal generating means, for example, ions such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium

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(III), holmium (III) and erbium (III), with gadolinium being particularly preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III).

Means of detecting such labels are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels are typically detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the colored label. Colloidal gold label can be detected by measuring scattered light. A preferred non-reflective signal generating means is biotin, which may be detected using an avidin or streptavidin compound. The use of such labels is well known to those of skill in the art and is described, for example, in U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241; each incorporated herein by reference for all purposes.

< Patterned Deposition of Cleavable Signal Elements on Plastic Substrate >

A photoresist may also profitably be used to pattern the deposition of cleavable signal elements. The resist is partially depolymerized by incident laser light during fabrication and can be dissolved from these areas. The exposed or metalized portion of the plastic substrate is treated chemically, for example, aminated by ammonia plasma. After the resist is removed, the cleavable signal elements are attached to the substrate. The use of photoresists for the patterning of master disks is well known in the compact disk fabrication arts.

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Alternatively, instead of using a resist, a solid mask containing small holes can be used during ammonia plasma treatment. Holes have a diameter of about 1 to 3 micrometers. The holes are located circularly in the mask, forming a spiral track or a pattern that is a combination of spiral and circular paths. The mask can be metal or plastic. Several metals, such as aluminum, nickel or gold can be used. Polycarbonate is a preferred plastic, because it will retain shape well. Plastics are reactive with the ammonia plasma, however, and a preferred method for using plastic masks therefore involves depositing a metal layer on the plastic, by evaporation, sputtering, or other methods known in the art. Holes may be made in the mask by laser. Those with skill in the art will appreciate that it is possible to create 1000 1μ-sized holes in one second in a thin metal or plastic plate. Alternatively, the holes can be etched by using conventional methods known in the semiconductor industry. In the mask approach to patterning the deposition of cleavable signal elements, the mask is pressed against the substrate and subjected to amination by ammonia plasma. The mask may be used repeatedly.

< SNP Detection in Nucleic Acid Hybridization Assay using Cleavable Signal Element >

In a nucleic acid hybridization assay according to the present invention, the capture probe of the cleavable signal element is oligonucleotides designed to hybridized to a complementary sequence of a target nucleic acid to be detected in the sample. For many applications of this methodology, cross-reactivity with sample oligonucleotides having even a single mismatched nucleotide should be minimized. In particular, nucleic acid hybridization assays adapted to use the cleavable signal element of the present invention for detection of

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point mutations, as, e.g., for detection of point mutations in the BRCA1 and BRCA2 genes that predispose to breast and ovarian cancers, must be able to discriminate between nucleic acid samples containing a single mismatched nucleotide, i.e., must be able to detect SNP.

The longer the oligonucleotides of the capture probe--and thus the longer the sequence that is complementary between the oligonucleotides and the nucleic acid sample--the greater the possibility of erroneously recognizing a mismatched sample, since the strength of hybridization, even given the presence of a mismatch, will be reasonably high.

Thus, one way to reduce erroneous recognition of mismatched nucleic acid sequences is to reduce the length of the oligonucleotides. Specificity is increased by shortening the length of the oligonucleotides to 15-20-mers. In this case, the mismatched oligonucleotides would use fewer nucleotides for pairing and will form highly unstable binding at room temperature. This unstable binding is denatured during the first wash step and removed. However, multiple SNP detections at a plurality of assay sites are required for diagnosing a certain disease.

FIGS. 11A through 11B show alternative embodiments of an assay device using the cleavable signal element according to the present invention capable of both single nucleotide polymorphism (SNP) detection and gene expression profile determination for diagnosis. Reference numeral 61 denotes a center void of the substrate (disk) 70. An assay sector with shorter capture probes for SNP detection and an assay sector with longer capture probes (cDNA) for gene expression profile analysis are arranged separate on the substrate 70. The assay devices shown in FIGS. 11A through 11B can be modified in a variety of ways and forms according to the arrangements shown in FIGS. 3A through 3D, to concurrently measure SNP and expression profile.

FIG. 11A shows that the assay sectors for SNP detection and

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expression profile analysis are arranged separate in an angular direction. FIG. 11B shows that the assay sectors for SNP detection and expression profile analysis are arranged separate in a radial direction. The concurrent determination of the DNP and expression profile doubles diagnostic reliability and reduces assay sites by appropriate combination of assay sites.

Best mode for carrying out the Invention

The present invention will be described in greater detail with reference to the following examples. The following examples are for illustrative purposes and are not intended to limit the scope of the invention.

Example 1: Detection of HIV-1

HIV-1 proviral DNA from clinical samples is amplified as follows, essentially as described in U.S. Patent No. 5,599,662, incorporated herein by reference.

Peripheral blood monocytes are isolated by standard Ficoll-Hypaque density gradient methods. Following isolation of the cells, the DNA is extracted as described in Butcher and Spadoro, *Clin. Immunol.* Newsletter 12:73-76 (1992), incorporated herein by reference. Polymerase chain reaction (PCR) is performed in a 100 μL reaction volume, of which 50 μL is contributed by the sample. The PCR contained the following reagents at the following initial concentrations:

10 mM Tris-HCl (pH 8.4)

50 mM KCl

200 μM each dATP, dCTP, dGTP, and dUTP

25 pmoles of Primer 1 (sequence: 5'-TGA GAC ACC AGG AAT TAG

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ATA TCA GTA CAA TGT-3')

25 pmoles of Primer 2 (sequence: 5'-CTA AAT CAG ATC CTA CAT ATA AGT CAT CCA TGT-3')

3.0 mM MgC_2

10% glycerol

2.0 units of Taq DNA polymerase (Perkin-Elmer)

2.0 units UNG (Perkin-Elmer)

Amplification is carried out in a TC9600 DNA Thermal Cycler (Perkin Elmer, Norwal, Conn.) using the following temperature profile: (1) pre-incubation--50℃ for 2 minutes; (2) initial cycle--denature at 94℃ for 30 seconds, anneal at 50℃ 30 seconds, extend at 72℃ for 30 seconds; (3) cycles 2 to 4--denature at 94℃ for 30 seconds, anneal for 30 seconds, extend at 72℃ for 30 seconds, with the annealing temperature increasing in 2℃ increments (from 52℃ to 58℃); (4) cycles 5 to 39--denature at 90℃ for 30 seconds, anneal at 60℃ for 30 seconds, extend at 72℃ for 30 seconds.

Following the temperature cycling, the reaction mixture is heated to 90℃ for 2 minutes and diluted to 1 mL. Alternatively, the sample is stored at -20℃, and after thawing, heated to 90℃ for 2 minutes then diluted to 1 mL.

The cleavable signal elements are attached in a uniform density to a derivatized 120-mm polycarbonate disk substrate, as described above.

First capture probe portion: 5'-TAG ATA TCA GTA CAA-3'

Second capture probe portion: 5'-TAT TCA GTA GGT ACA-3'

First restriction probe portion: 5'-CCCGGG-3'

Second restriction probe portion: 5'-CCCGGG-3'

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. A suspension of gold microspheres, 1-3 μm in diameter, is added dropwise to the disk, which is gently rotated to distribute the gold particles. Gold particles are added until the cleavable signal elements are saturated with the gold particles. Cleavable signal elements labeled with the gold particles at its end are attached in a uniform density to a derivatized 120-mm polycarbonate disk substrate, as described above.

Sample is applied at room temperature dropwise near the center of the stationary assay device, and the assay device is rotated. Rotation is halted after the sample reaches the outer edge of the disk, and the disk is incubated in a stationary state at room temperature for 3-5 minutes (Hybridization reaction).

One mL of buffer is added dropwise as a washing solution while the disk is rotated, to distribute the buffer by disk rotation. The disk was incubated in a stationary state for 1-2 minutes, then 5 ml of buffer is added dropwise during vigorous rotation of the disk to wash the disk, with or without the application of an external electric field vertically through the disk (First Wash Step).

About 0.1 L of a DNA polymerase solution (e.g., PCR Core Systems I, Promega Corporation) containing a mixed solution of the four dNTPs listed above and a DNA polymerase is added dropwise while the disk is rotated, to distribute the DNA polymerase solution. The disk is incubated in a stationary state for 1-2 minutes (DNA extension).

A restriction enzyme solution (e.g., sma 1, Promega Corporation) that recognizes the sequence CCCGGG of the restriction probe is added dropwise and distributed by disk rotation. The restriction enzyme cleaves between C and G of the restriction probe sequence. The disk is incubated in a stationary state for 1-2 minutes (Cleavage Step).

Five mL of buffer was added dropwise during vigorous rotation of

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the disk with or without the application of an external electric field (Second Wash Step). An appropriate restriction solution has a reaction temperature of 37℃ and to contain 10 mM Tris-HCl (pH7.4), 300 mM KCl, 0.1 mM EDTA (Ethylene Diamine Tetra Acetic acid), 1 mM DTT (DiThioTreitol), 0.5 mg/mL BSA (Bovine Serum Albumine), and 50% glycerol.

The disk is dried, then read directly in a detector programmed to assay each predetermined site upon which cleavable signal elements are deposited, which includes an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device.

The diagnostic data and a prescription are displayed on a computer monitor, the computer automatically or manually accesses the Internet to transmit the diagnostic data to a specialist at a remote location through the Internet. The patient waits for a prescription from the specialist.

Example 2: Detection of HIV-1

HIV-1 proviral DNA from clinical samples is amplified as follows, essentially as described in U.S. Patent No. 5,599,662, incorporated herein by reference.

Peripheral blood monocytes is isolated by standard Ficoll-Hypaque density gradient methods. Following isolation of the cells, the DNA is extracted as described in Butcher and Spadoro, *Clin. Immunol.* Newsletter 12:73-76 (1992), incorporated herein by reference. PCR was performed in a 100 μL reaction volume, of which 50 μL is contributed by the sample. The PCR contains the following reagents at the following initial concentrations:

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10 mM Tris-HCl (pH 8.4)

50 mM KCl

200 μM each dATP, dCTP, dGTP, and dUTP

25 pmoles of Primer 1 (sequence: 5'-TGA GAC ACC AGG AAT TAG ATA TCA GTA CAA TGT-3')

25 pmoles of Primer 2 (sequence: 5'-CTA AAT CAG ATC CTA CAT ATA AGT CAT CCA TGT-3')

3.0 mM MgC_2

10% glycerol

2.0 units of Taq DNA polymerase (Perkin-Elmer)

2.0 units UNG (Perkin-Elmer)

Amplification is carried out in a TC9600 DNA Thermal Cycler (Perkin Elmer, Norwal, Conn.) using the following temperature profile: (1) pre-incubation--50℃ for 2 minutes; (2) initial cycle--denature at 94℃ for 30 seconds, anneal at 50℃ 30 seconds, extend at 72℃ for 30 seconds; (3) cycles 2 to 4--denature at 94℃ for 30 seconds, anneal for 30 seconds, extend at 72℃ for 30 seconds, with the annealing temperature increasing in 2℃ increments (from 52℃ to 58℃); (4) cycles 5 to 39--denature at 90℃ for 30 seconds, anneal at 60℃ for 30 seconds, extend at 72℃ for 30 seconds.

Following the temperature cycling, the reaction mixture is heated to 90℃ for 2 minutes and diluted to 1 mL. Alternatively, the sample is stored at -20℃, and after thawing, heated to 90℃ for 2 minutes then diluted to 1 mL.

Following cleaning a polycarbonate disk substrate, the surface of the disk substrate is aminated by ammonia plasma and completely spray-coated with a solution of succinimidyl 4-maleimido butyrate (SMB),

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a heterobifunctional crosslinker, in a 1:10 mixture of DMF and sodium bicarbonate buffer (50mM, pH 8.5). The resulting polycarbonate substrate is tightly sealed and reacted for approximately 3 hours. Following washing with distilled water and drying, a HEPES buffer (10 mM, pH 6.6, 5.0 mM EDTA) containing HS-oligonucleotide-biotin is applied to the derivatized surface of the polycarbonate substrate to attach the HS-oligonucleotide-biotin in a uniform density, thereby constructing an assay device. The cleavable signal elements attached have the following sequences:

First capture probe (oligonucleotide) portion: 5'-TAG ATA TCA GTA CAA-3'

Second capture probe portion: 5'-TAT TCA GTA GGT ACA-3'

Sample is applied dropwise near the center of the stationary assay device, and the assay device is rotated. Rotation is halted after the sample reaches the outer edge of the disk, and the disk is incubated in a stationary state at room temperature for 3-5 minutes (Hybridization reaction).

One mL of buffer is added dropwise as a washing solution while the disk is rotated, to distribute the buffer by disk rotation. The disk is incubated in a stationary state for 1-2 minutes, then 5 ml of buffer is added dropwise during vigorous rotation of the disk to wash the disk, **with or without** the application of an external electric field vertically through the disk (First Wash Step).

A DNAse or nuclease solution is added dropwise and distributed by disk rotation. The disk is incubated in a stationary state for 1-2 minutes (Cleavage Step).

Following buffer addition, disk rotation, and a simple washing with the application of an external field, a suspension of streptoavidin-labeld

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gold microspheres is added drowpwise to the disk surface, and the disk is gently rotated to evenly distribute the gold particles (label-attached uncleaved probe structure formation), thereby resulting in a biotin-avidin binding structure. Next, distilled water is added dropwise during vigorous rotation of the disk to wash the disk, with or without the application of an external electric field (Second Wash Step).

The disk is dried, then read directly in a detector programmed to assay each predetermined site upon which cleavable signal elements are deposited, which includes an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device.

The diagnostic data and a prescription are displayed on a computer monitor, the computer automatically or manually accesses the Internet to transmit the diagnostic data to a specialist at a remote location through the Internet. The patient waits for a prescription from the specialist.

Experimental Example 1: Optical Measurement

Following the hybridization reaction, first wash step, cleavage step, and second wash step according to Example 2, whether hybridization to a target nucleic acid had occurred or not was determined by atomic force microscopy (AFM). As a result, the topography images by AFM are shown in FIGS. 13A and 13B.

FIG. 13A is a topography image taken after hybridization of biotin-attached cleavable signal elements to an oligonucleotide sample of a complementary sequence, in which after the hydbridization, the substrate was reacted with a mung bean-derived nuclease, washed, and additionally labeled with streptavidin-coated 40-nm metal microspheres. Since the cleavable signal elements are double-stranded, the cleavable

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signal elements are not cleaved by the nuclease, and the biotin-attached cleavable signal elements remain on the substrate and form a "label-attached uncleaved probe" structure by additionally contacting the streptoavidin-coated metal micrispheres, thereby increasing optical selectivity. Due to the streptoavidin-to-biotin coupling, the sensitivity of the detector is increased.

FIG. 13B is a topography image taken after reacting the biotin-attached cleavable signal elements with an oligonucleotide sample of a non-complementary sequence, in which after the reaction, the substrate was reacted with a mung bean-derived nuclease, washed, and additionally labeled with streptavidin-coated 40-nm metal microspheres. Since the cleavable signal elements remain as single strands, the cleavable signal elements are cleaved by the nuclease, and the biotin-attached cleavable signal elements are removed from the substrate. As a result, even after additionally contacting the streptoavidin-coated metal micrispheres, the cleavable signal elements do not form a "label-attached uncleaved probe" structure.

Apparently, the substrate is mostly covered with the metal microspheres in FIG. 13A, whereas few metal microspheres are shown in FIG. 13B. Differential signals from the metal microspheres are provided to the detector.

Experimental Example 2: Impedance Measurement

Following the hybridization reaction, first wash step, cleavage step, and second wash step according to Example 2, whether hybridization to a target nucleic acid had occurred or not was determined by measuring the impedance characteristics with respect to frequency. The result is shown in FIG. 14.

As can be inferred form FIG. 13, differential impedance signals

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between the uncleaved signal element (low impedance) and the cleaved signal element (high impedance) are provided to the detector.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

Industrial Applicability

As described above, the present invention provides a cleavage technique specifically responsive to a complementary double strand or single strand of nucleic acids, a nucleic acid hybridization assay method and device using the cleavable technique, and a diagnostic method and system capable of more accurately diagnosing many kinds of diseases through single nucleotide polymorphism (SNP) detection and expression profile determination that can be concurrently determined. In a preferred embodiment according to the present invention, the diagnostic system using the nucleic acid hybridization assay method and device based on the cleavage technique specifically responsive to the complementary double strand or single strand of nucleic acids can be modified for detection with standard laser-based detection systems, including CD-ROM readers and DVD readers, which enables self-diagnosis by patients at home without the need to go to a hospital. In addition, the present invention provides an assay device and method for detecting analytes using the nucleic acid hybridization assay device according to the present invention. This analyte assay device and method are useful in assaying for a number of discrete analytes with a test sample and a single analyte with multiple samples. The present invention also provides a remote diagnostic system providing convenience to both patients and doctors, in which information read from

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the assay device is digitized as software and transmitted to and received by patient and doctor through an existing communication network, such as the Internet.

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What is claimed is:

1. A cleavable signal element comprising:

a restriction probe of a single strand having a particular sequence cleavable by a restriction enzyme specific to a double strand of a particular sequence; and

a capture probe of a single strand having a complementary sequence to a target nucleic acid for diagnosis or assay to form a double strand through hybridization to the target nucleic acid,

wherein one end of the restriction probe is attached to a solid support substrate, and the other end of the restriction probe is ligated to the capture probe, thus forming a single-stranded, cleavable capture probe.

2. The cleavable signal element of claim 1, wherein when the capture probe contacts a sample including the target nucleic acid of the complementary sequence, the capture probe is double-stranded through hybridization to the target nucleic acid, the restriction probe is double-stranded through DNA extension using the target nucleic acid hybridized to the capture probe as a primer with the addition of a DNA polymerization solution, the double-stranded restriction probe is cleaved by the restriction enzyme, and the cleaved cleavable capture probe is removed from the solid support substrate through washing, thus resulting in a cleaved signal element; and

when the capture probe contacts a sample not including the target nucleic acid of the complementary sequence, the single-stranded, cleavable capture probe remains attached to the solid support substrate even after additions of the DNA polymerization solution and the restriction enzyme and washing, thus resulting in an uncleaved signal element.

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3. The cleavable signal element of claim 2, wherein the DNA polymerization solution comprises a solution of four dNTPs and a DNA polymerase solution.

4. A cleavable signal element comprising a capture probe of a single strand having a complementary sequence to a target nucleic acid for diagnosis or assay to form a double strand through hybridization to the target nucleic acid, wherein one end of the capture probe is attached to a solid support substrate, and the capture probe itself forms a single-stranded, cleavable capture probe which is cleavable by a cleavage enzyme specifically responsive to a double strand or single strand of nucleic acids.

5. The cleavable signal element of claim 4, wherein when the capture probe contacts a sample including the target nucleic acid of the complementary sequence, the capture probe is double-stranded through hybridization to the target nucleic acid, the double-stranded capture probe is cleaved by the cleavage enzyme specifically responsive to the double strand of nucleic acids, and the cleaved cleavable capture probe is removed from the solid support substrate through washing, thus resulting in a cleaved signal element; and

when the capture probe contacts a sample not including the target nucleic acid of the complementary sequence, the single-stranded, cleavable capture probe remains attached to the solid support substrate even after the addition of the cleavage enzyme and washing, thus resulting in an uncleaved signal element.

6. The cleavable signal element of claim 5, wherein the cleavage enzyme is a DNAse.

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7. The cleavable signal element of claim 4, wherein when the capture probe contacts a sample not containing the target nucleic acid of the complementary sequence, the capture probe remains as a single strand without hybridization, the single-stranded capture probe is cleaved by the cleavage enzyme specifically responsive to the single strand of nucleic acids, and the cleaved cleavable capture probe is removed from the solid support substrate through washing, thus resulting in a cleaved signal element; and

when the capture probe contacts a sample including the target nucleic acid of the complementary sequence, the capture probe is double-stranded through hybridization to the target nucleic acid, and the double-stranded, cleavable capture probe remains attached to the solid support substrate even after the addition of the cleavage enzyme and washing, thus resulting in an uncleaved signal element.

8. The cleavable signal element of claim 7, wherein the cleavage enzyme is a nuclease.

9. The cleavable signal element of claim 8, wherein the nuclease is derived from mung bean.

10. The cleavable signal element of claim 1 or 4, wherein the solid support substrate is a plastic substrate, a glass substrate, a silicon substrate, or a gold substrate.

11. The cleavable signal element of claim 1 or 4, wherein the solid support substrate has a self-assembled monolayer (SAM) on the surface.

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12. The cleavable signal element of claim 1 or 4, wherein the capture probe has a length ranging from about 5- to about 30-mers.

13. The cleavable signal element of claim 1 or 4, wherein a label is attached to one end of the cleavable capture probe to form a label-attached cleavable capture probe structure or to one end or side of an uncleaved probe to form a label-attached uncleaved probe structure, to increase detection sensitivity for an uncleaved signal element.

14. The cleavable signal element of claim 13, wherein the label comprises a metal microsphere, a conducting polymer, a fluorescent dye, a magnetic microsphere, and a streptavidin-labeled microsphere.

15. The cleavable signal element of claim 14, wherein the metal microsphere is formed of a metal selected from the group consisting of gold, silver, nickel, platinum, chromium, and copper.

16. The cleavable signal element of claim 15, wherein a gold microsphere has a diameter ranging from about 1 nm to about 10 μm.

17. The cleavable signal element of claim 13, wherein the streptavidin-labeled microsphere is attached to the cleavable capture probe via biotin.

18. A nucleic acid hybridization assay device comprising:

a solid support substrate;

a plurality of cleavable signal elements according to any of claims 1 through 17 attached to the solid support substrate; and

an internal or external detector which detects a uncleaved signal

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element and a cleaved signal element from the plurality of cleavable signal elements.

19. The nucleic acid hybridization assay device of claim 18, wherein the detector comprises an optical device, an electrochemical device, a mass measurement device, or a capacitance and impedance measurement device.

20. The nucleic acid hybridization assay device of claim 19, wherein the optical device detects fluorescence of the uncleaved signal element and cleaved signal element.

21. The nucleic acid hybridization assay device of claim 19, wherein the detector detects a differential reflective signal or a differential conductive signal of the uncleaved signal element and the cleaved signal element.

22. The nucleic acid hybridization assay device of claim 21, wherein the detector detects the differential reflective signal by measuring the reflectance, absorbance, or scattering of light or a laser beam incident on the uncleaved signal element and the cleaved signal element.

23. The nucleic acid hybridization assay device of claim 21, wherein the detector detects the differential conductive signal by measuring the capacitance and impedance of the uncleaved signal element and the cleaved signal element.

24. The nucleic acid hybridization assay device of claim 23, wherein the capacitance and impedance measurement device measures

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the frequency response characteristics of the uncleaved signal element and the cleaved signal element.

25. The nucleic acid hybridization assay device of claim 23, wherein the capacitance and impedance measurement device comprises interdigitated array electrodes having at least one digit and arranged on the solid support substrate.

26. The nucleic acid hybridization assay device of claim 25, wherein the interdigitated array electrodes have an input port to check for the frequency response characteristics, and the input port is connected to an electronic control device which generates a frequency signal of a constant bandwidth.

27. The nucleic acid hybridization assay device of claim 25, wherein a plurality of cleavable signal elements are deposited on the interdigitated array electrodes.

28. The nucleic acid hybridization assay device of claim 25, wherein the plurality of cleavable signal elements are deposited only in the space between the interdigitated array electrodes.

29. The nucleic acid hybridization device of claim 25, wherein the interdigitated array electrodes are substantially formed of gold.

30. The nucleic acid hybridization device of claim 18, wherein a label-attached uncleaved probe structure is formed on the solid support substrate by attaching a label to the uncleaved signal element to increase the sensitivity of the detector.

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31. The nucleic acid hybridization device of claim 18, wherein the plurality of cleavable signal elements are deposited on the solid support substrate in a spatially-addressable pattern.

32. The nucleic acid hybridization device of claim 31, wherein the spatially-addressable pattern enables a single-sample assay for multiple analytes, a multiple-sample assay for a single analyte, or a multiple sample assay for multiple analytes.

33. The nucleic acid hybridization device of claim 31, wherein the solid support substrate is a plastic substrate formed of a material selected from the group consisting of polypropylenes, polyacrylates, polyvinyl alcohols, polyethylenes, polymethylmethacrylates, and polycarbonates.

34. The nucleic acid hybridization device of claim 33, wherein the solid support substrate is formed of polycarbonates.

35. The nucleic acid hybridization device of claim 31, wherein the solid support substrate is formed of a circular disk.

36. The nucleic acid hybridization device of claim 31, wherein the solid support substrate is formed of a rectangular disk.

37. The nucleic acid hybridization device of claim 35, wherein the circular disk has a diameter of approximately 120 mm and a thickness of approximately 1.2 mm.

38. The nucleic acid hybridization device of claim 35, wherein the circular disk comprises:

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a central void to engage a rotational drive means;

a sample injection port through which a sample is injected; and

an annular and/or a spiral track in which the plurality of cleavable signal elements are deposited in the spatially-addressable pattern.

39. The nucleic acid hybridization device of claim 38, wherein an address pattern that provides coded address information is formed on the circular disk.

40. The nucleic acid hybridization device of claim 35, wherein the circular disk comprises:

a central void to engage a rotational drive means;

a sample injection port through which a sample is injected; and

a radial assay sector in which the plurality of cleavable signal elements are deposited in the spatially-addressable pattern.

41. The nucleic acid hybridization device of claim 40, wherein the circular disk comprises a plurality of assay sectors.

42. The nucleic acid hybridization device of claim 41, wherein the plurality of assay sectors are connected to respective separate sample injection ports.

43. The nucleic acid hybridization device of claim 41, wherein the plurality of assay sectors are connected to a common sample injection port.

44. The nucleic acid hybridization device of claim 41, wherein the plurality of cleavable signal elements are deposited in each of the plurality of assay sectors in an appropriate pattern for a single-analyte

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assay or a multiple-analyte assay.

45. The nucleic acid hybridization device of claim 35, comprising a plurality of circular disks.

46. The nucleic acid hybridization device of claim 35, wherein the circular disk includes in a central track a database associated with bioinformatics required for diagnosis and assay interpretation, and telephone numbers, web link information and software required for remote diagnosis.

47. The nucleic acid hybridization device of claim 35, wherein a detector is mounted on the circular disk.

48. The nucleic acid hybridization device of claim 47, wherein the detector comprises a non-contact interface through which information read from the cleaved signal element and the uncleaved signal element is transmitted to an external central controller or storage device.

49. The nucleic acid hybridization assay device of claim 48, wherein the non-contact interface comprises an infrared interface and an optical interface.

50. The nucleic acid hybridization assay device of claim 49, wherein the infrared interface is an infrared sensor, and the optical interface is a photosensor.

51. The nucleic acid hybridization assay device of claim 35, wherein the circular disk simultaneously comprises at least one SNP (single nucleotide polymorphism) assay sector for SNP detection and at

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least one expression assay sector for expression profile analysis.

52. The nucleic acid hybridization assay device of claim 51, wherein the SNP assay sector and the expression assay sector are arranged separate in an angular direction or in a radial direction.

53. A bio-driver apparatus comprising:

a rotary disk receiver onto which the nucleic acid assay device according to any of claims 18 through 52 is to be loaded;

a motor driver which rotates the disk;

a rotary connector which connects the motor driver to a central void portion of the disk such that the disk is rotatable; and

an optical device to write data in or to read data from the disk.

54. The bio-driver apparatus of claim 53, further comprising a central controller which transmits information read from the disk by the optical device to an external storage unit, transmits information to be written to the optical device, and generates and outputs a variety of control signals for the motor driver and the other elements.

55. The bio-driver apparatus of claim 53, wherein the rotary connector comprises an upper rotor and/or a lower rotor, the upper and lower rotors being pushed close to the top and bottom surfaces, respectively, of the central void portion when the disk begins to rotate.

56. The bio-driver apparatus of claim 53, wherein the optical device detects a differential reflective signal by measuring the reflectance, absorbance, or scattering of incident light or an incident laser beam.

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57. The bio-driver apparatus of claim 53, wherein the optical device detects fluorescence.

58. A bio-driver apparatus comprising:

a rotary disk receiver onto which the nucleic acid assay device according to any of claims 18 through 52 is to be loaded;

a motor driver which rotates the disk;

a rotary connector which rotatably connects the motor driver to a central void portion of the disk;

an external power connector which powers and/or supplies a control signal to a detector mounted on the disk; and

a non-contact interface through which information read by the detector is transmitted.

59. The bio-driver apparatus of claim 58, further comprising a central controller which transmits information read from the disk by the detector to an external storage unit and generates and outputs a variety of control signals for the motor driver and the other elements.

60. The bio-driver apparatus of claim 58, further comprising an optical device to write data in or to read data from the disk.

61. The bio-driver apparatus of claim 58, wherein the detector detects a differential conductive signal by measuring capacitance and impedance.

62. The bio-driver apparatus of claim 58, wherein the rotary connector comprises an upper rotor and/or a lower rotor, the upper and lower rotors being pushed close to the top and bottom surfaces, respectively, of the central void portion when the disk begins to rotate.

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63. The bio-driver apparatus of claim 58, wherein the power connector comprises a brush that frictionally contacts the upper and/or lower rotors in connection with an external power supply unit, and each of the upper and lower rotors comprises an annular electrode plate frictionally contacting the brush.

64. The bio-driver apparatus of claim 63, wherein the annular electrode plate comprises at least one conductive arm connected thereto, and the central void portion of the disk comprises a hole to engage the at least one conductive arm and a circuit pattern connected to the hole to power the detector mounted on the disk and/or supply the control signal to the detector.

65. The bio-driver apparatus of claim 64, wherein the at least one conductive arm has a spring at its one end that is connected to the annular electrode plate.

66. The bio-driver apparatus of claim 58, wherein the power connector comprises an electromagnet attached to the rotary disk receiver in connection with the external power supply unit, and the electromagnet induces an AC voltage to a wound coil on the disk so that the detector is powered in a non-contact manner.

67. The bio-driver apparatus of claim 66, wherein the disk further comprises a rectifier for rectifying the AC voltage induced to the wound coil.

68. A remote diagnostic system comprising:

the nucleic acid hybridization assay device according to any of

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claims 18 through 52;

an existing communication network; and

a computer in which software capable of controlling access to the existing communication network and digitizing information read from the nucleic acid hybridization assay device is installed,

wherein the digitized information from the nucleic acid assay hybridization assay device is transmitted to a doctor or a hospital, and a patient is provided with a prescription, through the existing communication network.

69. The remote diagnostic system of claim 68, wherein the computer comprises assay interpretive algorithms, bioinformatics information, and self-diagnostics related software.

70. The remote diagnostic system of claim 68, wherein the computer comprises software capable of uploading diagnostic information to remote locations and device drivers.

71. The remote diagnostic system of claim 70, wherein the software comprises educational information for patients on clinical assays, a variety of wet sites and links enabling a patient to directly communicate with a doctor or hospital based on his/her diagnosis result.

72. The remote diagnostic system of claim 68, wherein the computer comprises a camera and a microphone for viewing a patient's face and listening to his/her voice.

73. The remote diagnostic system of claim 68, wherein the diagnostic data based on the digitized information are displayed on a

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computer monitor, the computer automatically or manually transmits the diagnostic data to a specialist through the existing communication network, and the patient waits for a prescription from the specialist.

74. A nucleic acid hybridization assay method comprising:

hybridizing a capture probe to a target nucleic acid present in a liquid sample by contacting the nucleic acid hybridization assay device according to any of claims 18 through 52 with the liquid sample;

contacting the cleavable capture probe with a restriction enzyme or cleavage enzyme which is specifically responsive to a cleavable signal element depending on whether the capture probe and the target nucleic acid are hybridized or not;

washing the nucleic acid hybridization assay device to remove the cleavable signal element cleaved by the restriction enzyme or cleavage enzyme; and

detecting whether the uncleaved signal element or the cleaved signal element exists on the solid support substrate.

75. The nucleic acid hybridization assay method of claim 74, further comprising contacting the cleavage capture probe with a DNA polymerization solution before contact with the restriction enzyme.

76. The nucleic acid hybridization assay method of claim 75, further comprising contacting the cleavage capture probe with a 3'→5' exonuclease solution before contact with the DNA polymerization solution.

77. The nucleic acid hybridization assay method of claim 74, further comprising attaching a label to the cleavable signal element

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before contacting the capture probe with the liquid sample, or to an uncleaved signal element after contacting the cleavable capture probe with the restriction enzyme or cleavage enzyme.

78. The nucleic acid hybridization assay method of claim 74, further comprising at least one wash step.

79. The nucleic acid hybridization assay method of claim 74, wherein washing is performing by rotating the nucleic acid hybridization assay device with or without addition of a detergent solution, or by applying an external electric field.

80. A nucleic acid hybridization assay method comprising:

(a) injecting a sample into a sample injection port disposed near the center of a disk in a nucleic acid hybridization assay device;

(b) rotating the disk and stopping the rotation of the disk when the sample reaches an outer edge of the disk;

(c) incubating the disk in a stationary state at room temperature for hybridization;

(d) adding a buffer solution as a washing solution while rotating the disk at a high speed, to wash the disk;

(e) adding a DNA polymerization solution containing a mixed solution of four dNTPs and a DNA polymerase and incubating the disk in a stationary state for DNA extension;

(f) adding a solution of a restriction enzyme specifically responsive to a double strand of a particular sequence and incubating the disk in a stationary state, to cleave the double strand;

(g) washing the disk by rotating the disk at a high speed with the addition of a buffer solution or by applying an external electric or magnetic field; and

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(h) drying the disk and reading information from the disk using a detector which is programmed to detect a predetermined assay site on which a cleavable signal element is deposited and comprises an optical device, an electrochemical device, or a capacitance and impedance measurement device.

81. The nucleic acid hybridization assay method of claim 80, further comprising adding a 3'→5' exonuclease solution before step (e) of DNA extension.

82. A nucleic acid hybridization assay method comprising:

(a) injecting a sample into a sample injection port disposed near the center of a disk in a nucleic acid hybridization assay device;

(b) rotating the disk and stopping the rotation of the disk when the sample reaches an outer edge of the disk;

(c) incubating the disk in a stationary state at room temperature for hybridization;

(d) adding a buffer solution as a washing solution while rotating the disk at a high speed, to wash the disk;

(e) adding a solution of a cleavage enzyme specifically responsive to a double strand or single strand of nucleic acids and incubating the disk in a stationary state, to cleave the double strand or single strand;

(f) washing the disk by rotating the disk at a high speed with the addition of a buffer solution or by applying an external electric or magnetic field; and

(g) drying the disk and reading information from the disk using a detector which is programmed to detect a predetermined assay site on which a cleavable signal element is deposited and comprises an optical

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Curio v Prognosys

device, an electrochemical device, or a capacitance and impedance measurement device.

83. The nucleic acid hybridization assay method of claim 80 or 82, further comprising attaching a label to the cleavable signal element before sample injection, or to an uncleaved signal element after strand cleavage, to increase detection sensitivity.

Curio EX1010-p. 709
Curio v Prognosys

FIG. 1A

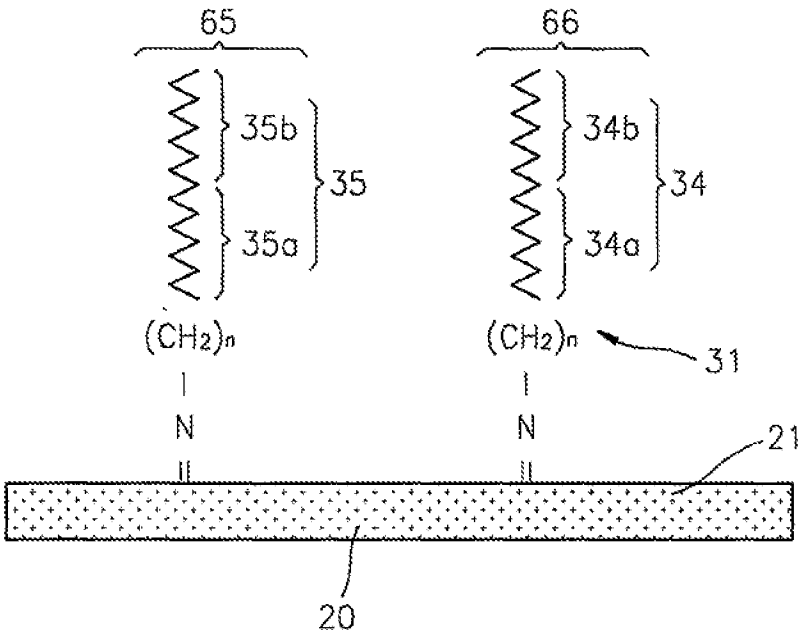

65
66
35b
35
35a
34b
34
34a
(CH2)n
(CH2)n
31
N
N
21
20

FIG. 1B

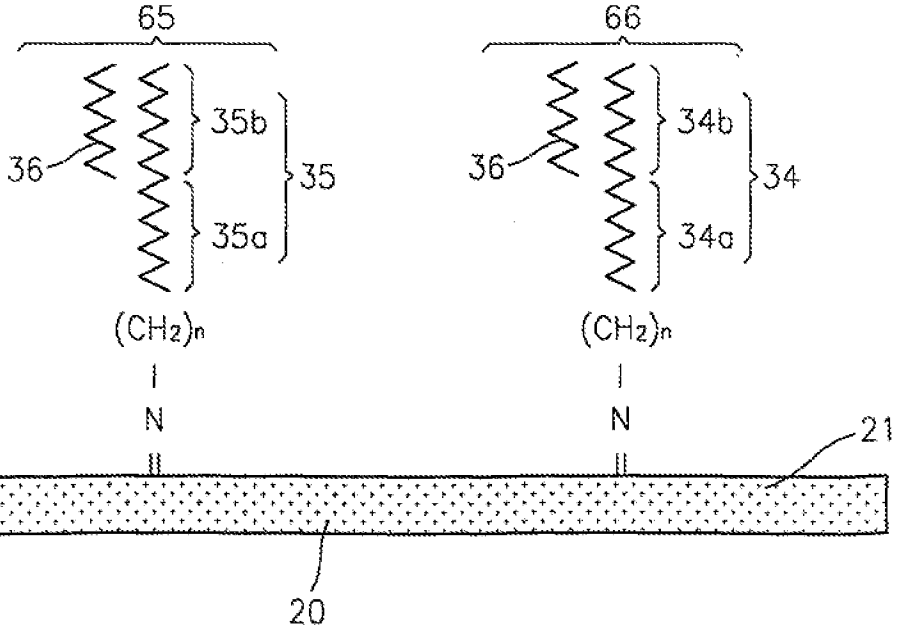

65
66
36
35b
35
35a
36
34b
34
34a
(CH2)n
(CH2)n
N
N
21
20

FIG. 1C

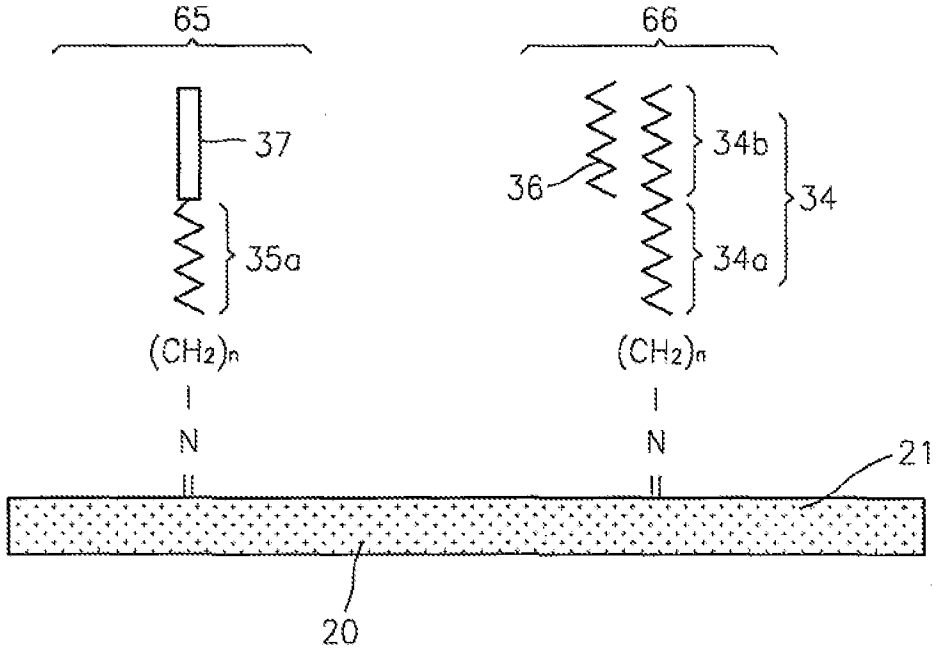

65
66
37
34b
36
34
35a
34a
(CH2)n
(CH2)n
N
N
21
20

FIG. 1D

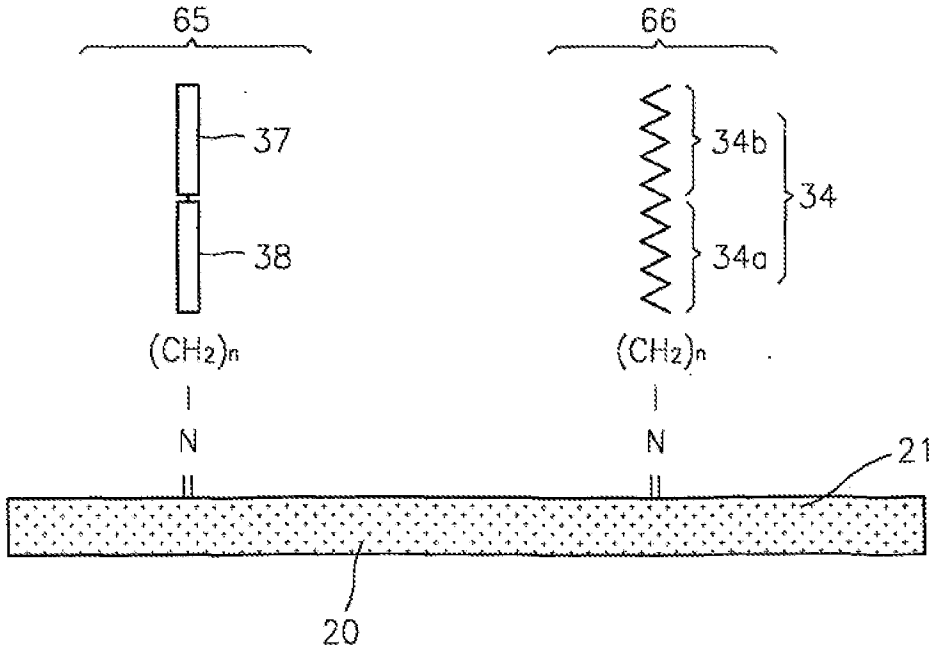

65
66
37
34b
34
38
34a
(CH2)n
(CH2)n
N
N
21
20

FIG. 1E

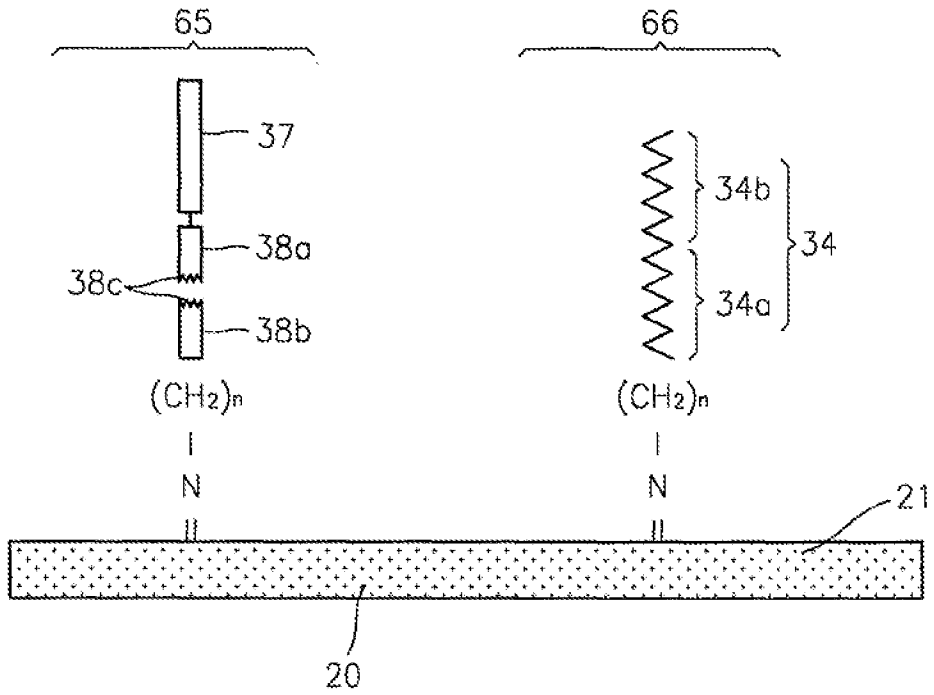

65
66
37
38a
38c
38b
34b
34
34a
(CH2)n
(CH2)n
N
N
21
20

FIG. 1F

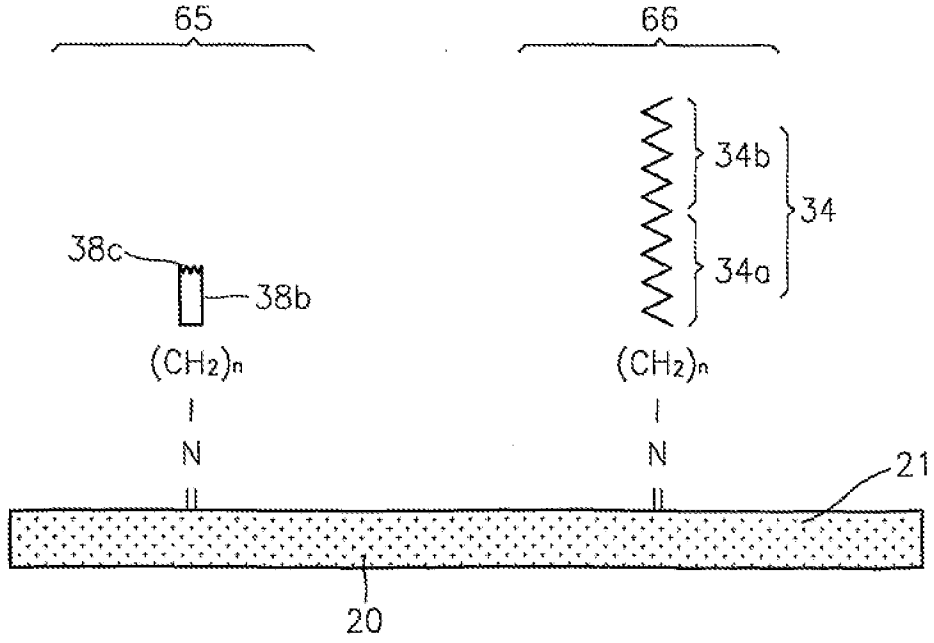

65
66
38c
38b
34b
34
34a
(CH2)n
(CH2)n
N
N
21
20

FIG. 1G

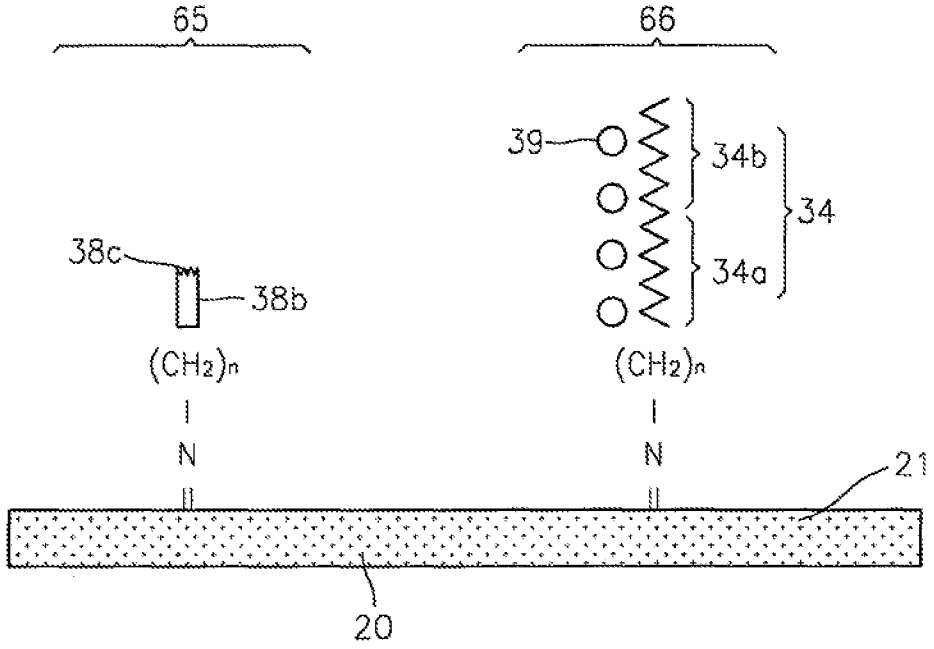

65
66
39
34b
34
34a
38c
38b
$(CH_2)_n$
$(CH_2)_n$
I
N
I
N
21
20

FIG. 2A

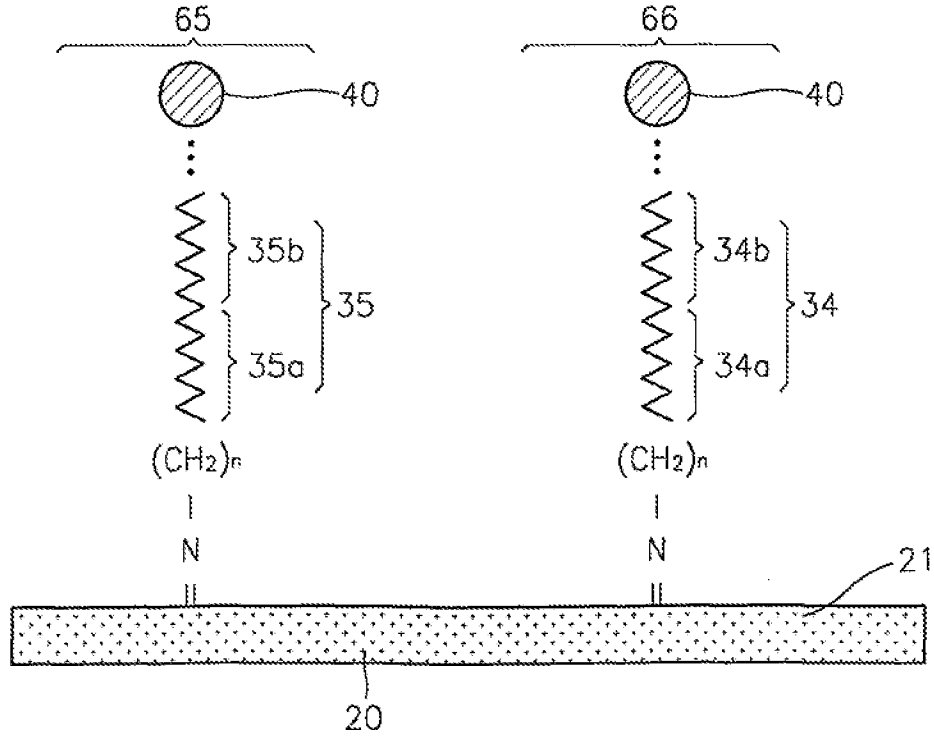

65
66
40
40
35b
35
35a
34b
34
34a
$(CH_2)_n$
$(CH_2)_n$
I
N
I
N
21
20

FIG. 2B

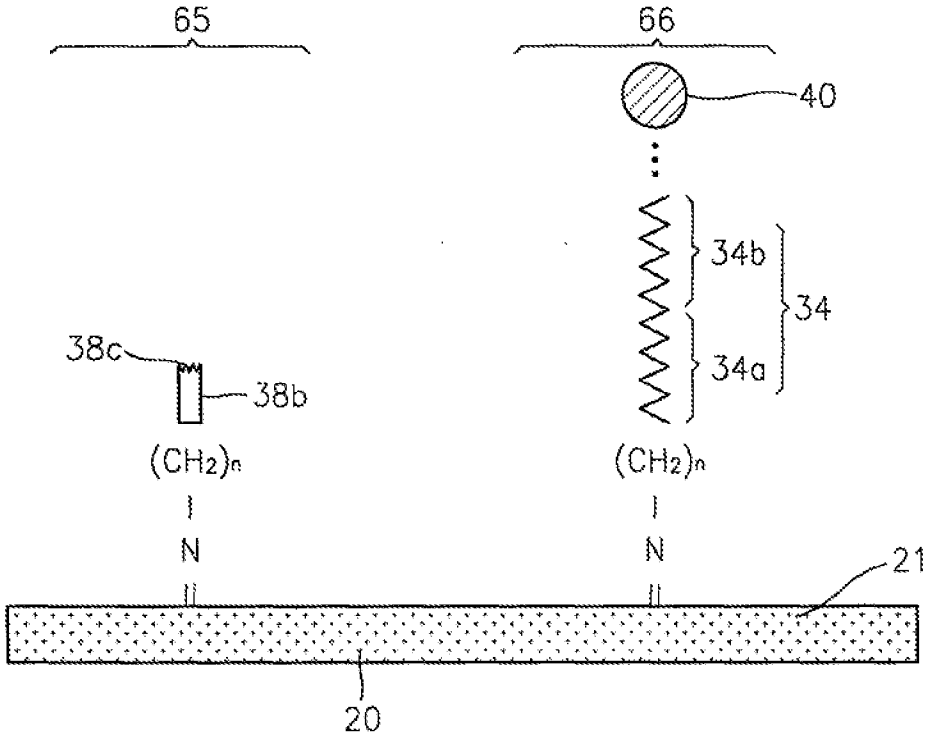

65
66
40
34b
34
34a
38c
38b
(CH2)n
(CH2)n
N
N
21
20

FIG. 2C

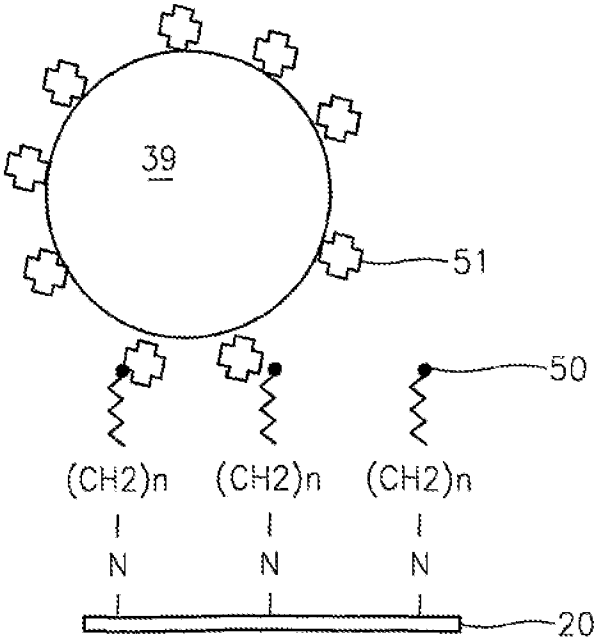

39
51
50
(CH2)n
(CH2)n
(CH2)n
N
N
N
20

FIG. 3A

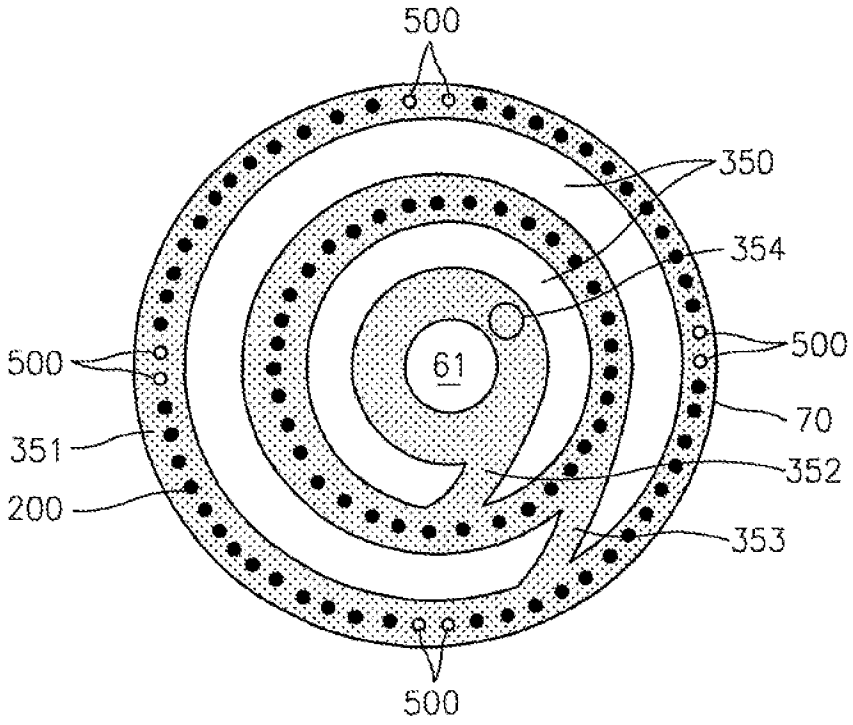

500
350
354
500
61
500
70
351
352
200
353
500

FIG. 3B

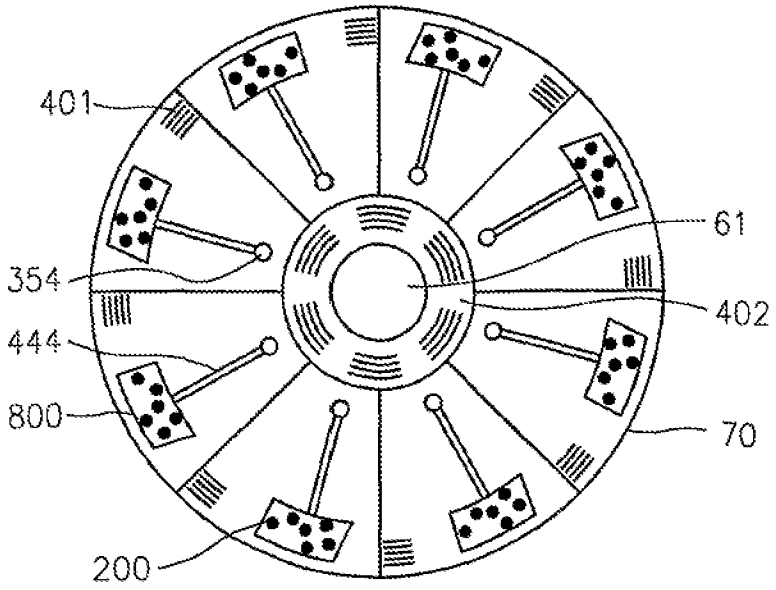

401
61
354
402
444
800
70
200

FIG. 3C

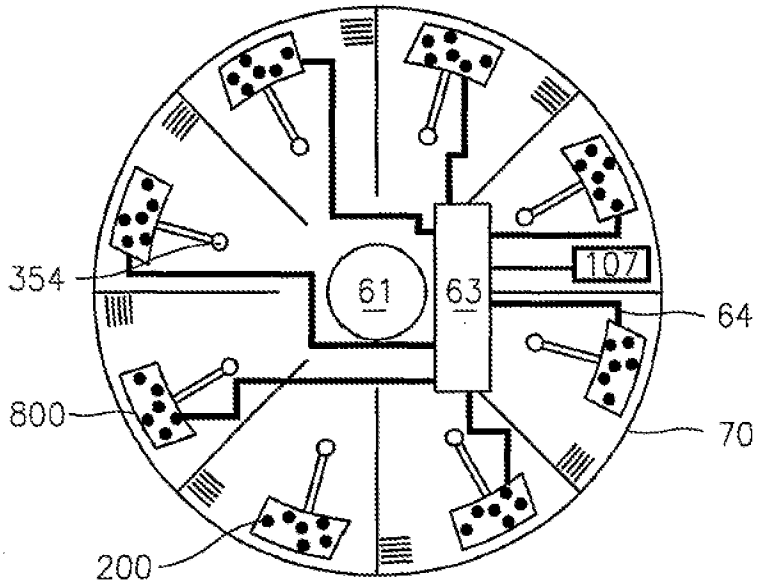


FIG. 3D

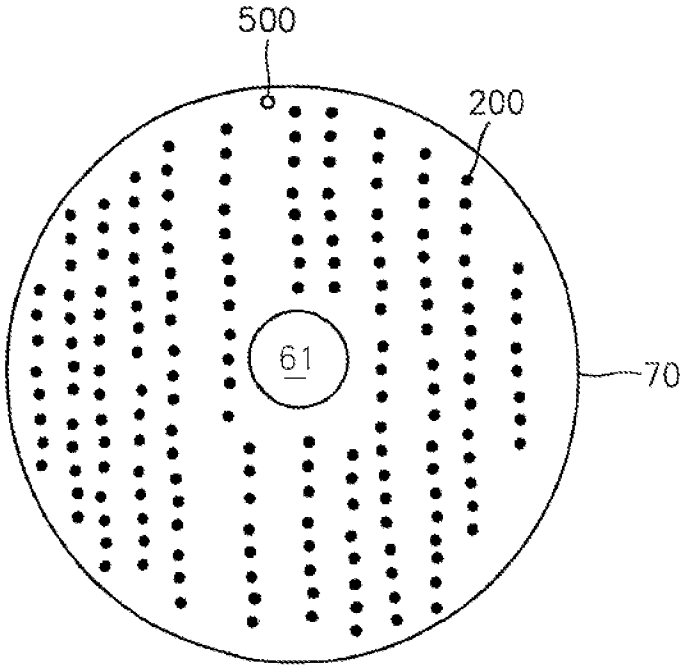


FIG. 4A

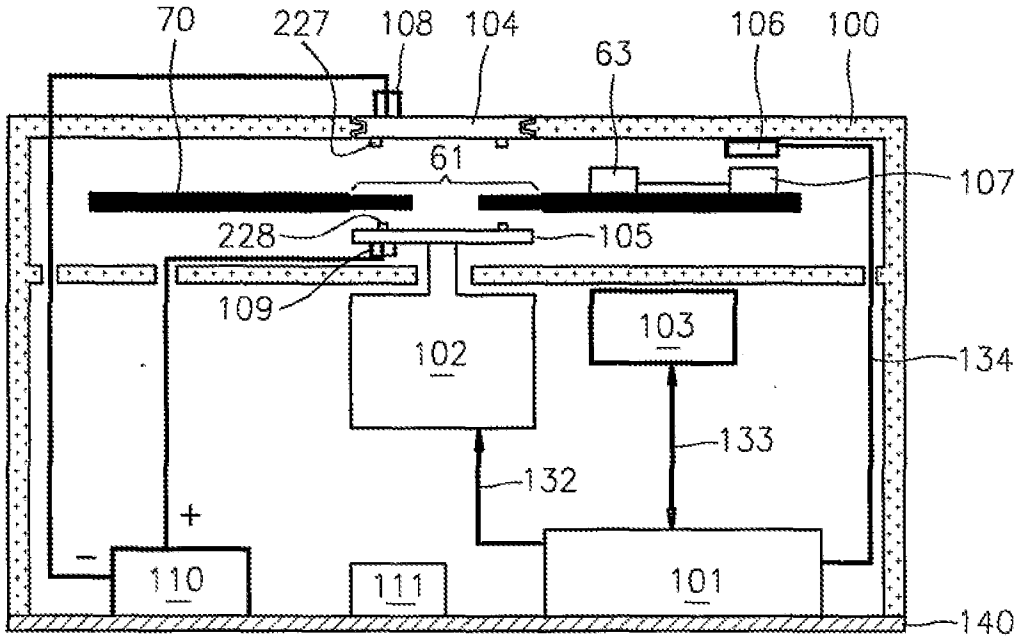

70
227
108
104
63
106
100
61
107
228
105
109
103
102
134
133
132
+
−
110
111
101
140

FIG. 4B

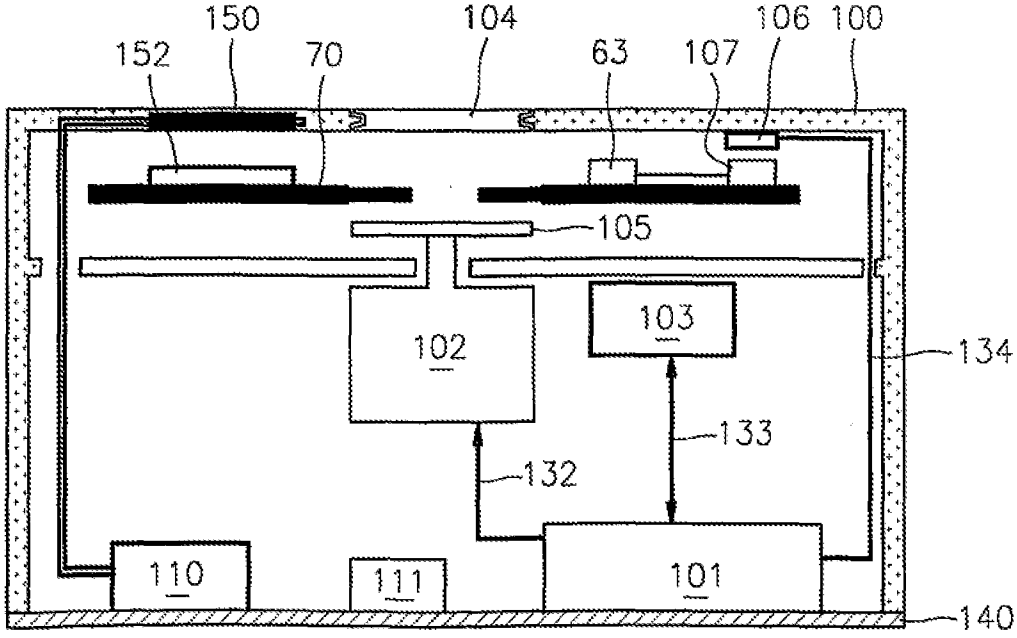

150
104
106
100
152
70
63
107
105
103
102
134
133
132
110
111
101
140

FIG. 4C

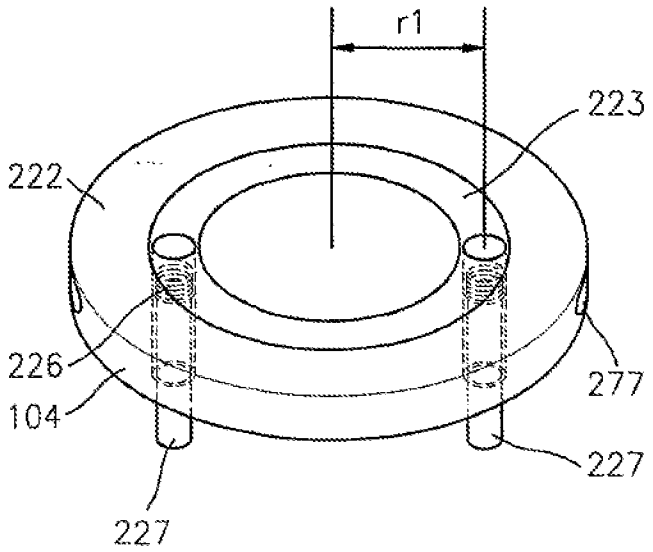


FIG. 4D

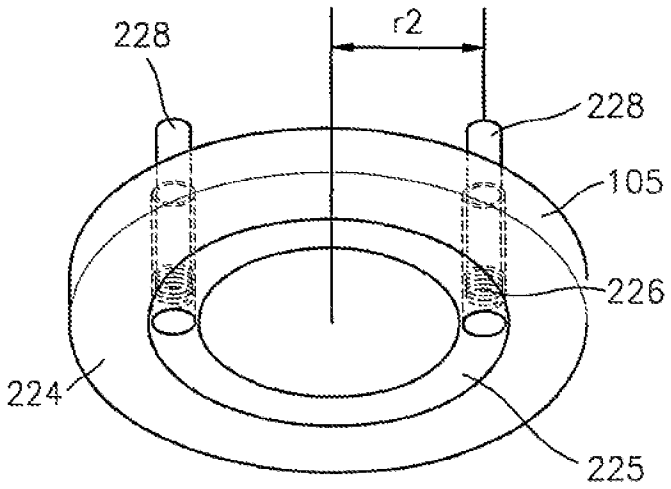


FIG. 4E

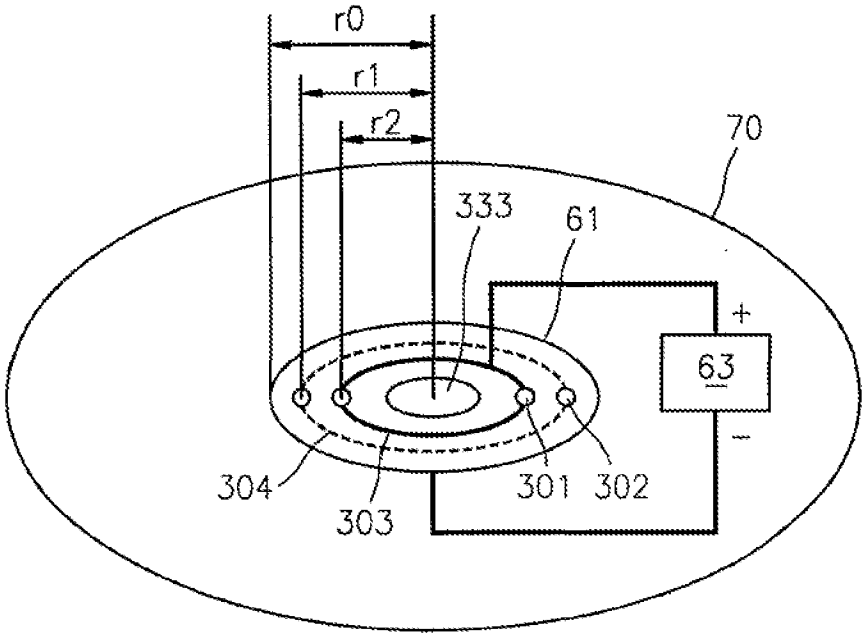

r0
r1
r2
70
333
61
+
63
−
304
303
301
302

FIG. 4F

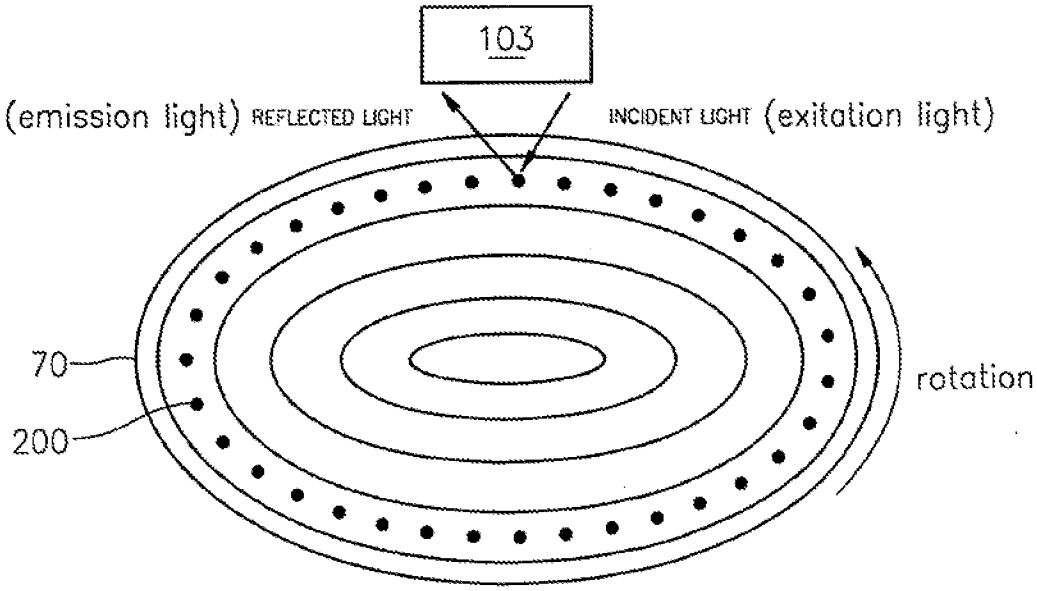

103
(emission light)
REFLECTED LIGHT
INCIDENT LIGHT
(exitation light)
70
200
rotation

FIG. 5A

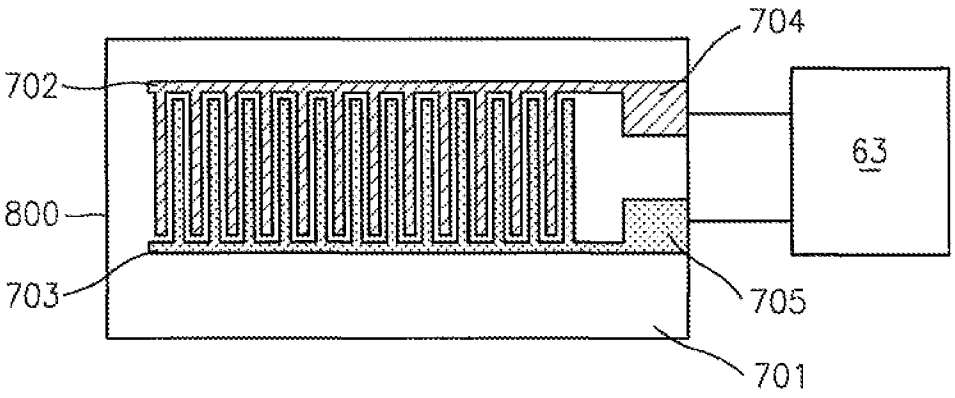


FIG. 5B

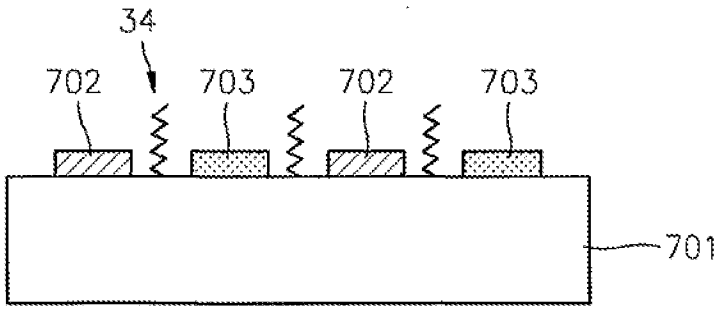


FIG. 5C

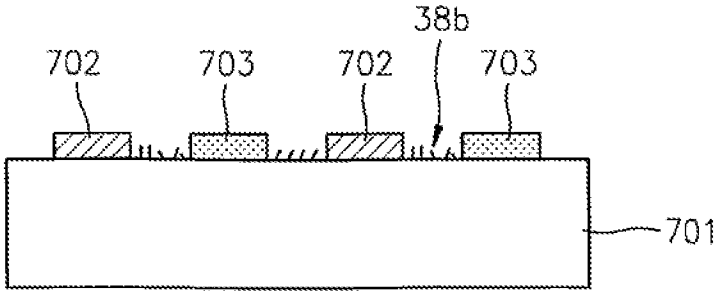


FIG. 5D

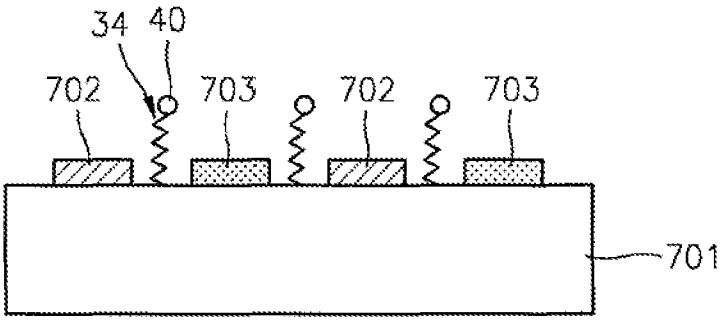


FIG. 5E

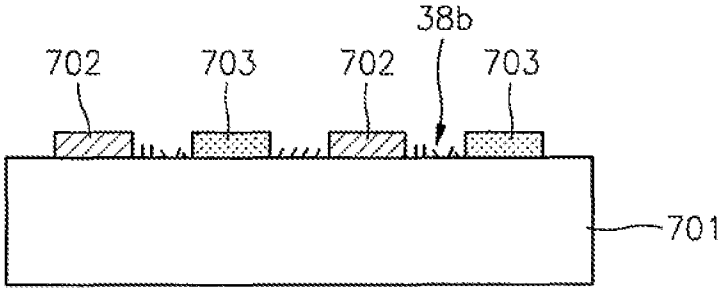


FIG. 5F

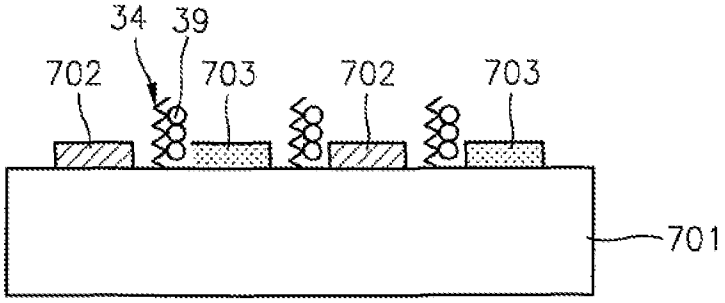


FIG. 5G

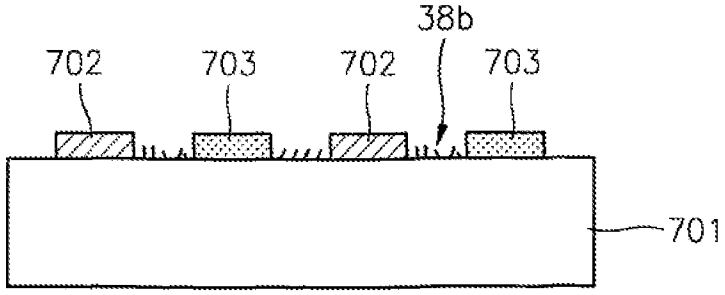


FIG. 5H

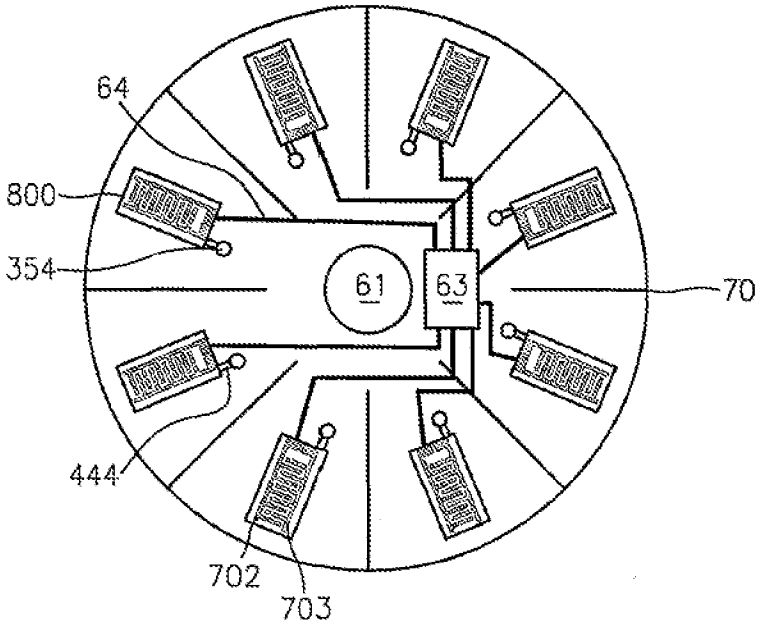

64
800
354
61
63
70
444
702
703

FIG. 5I

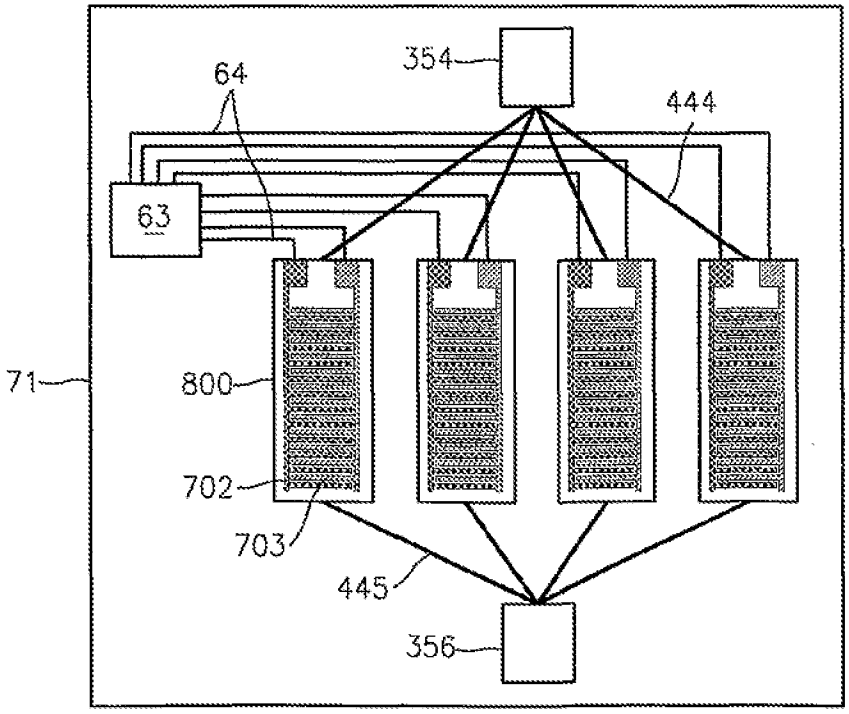

354
64
444
63
71
800
702
703
445
356

FIG. 6A

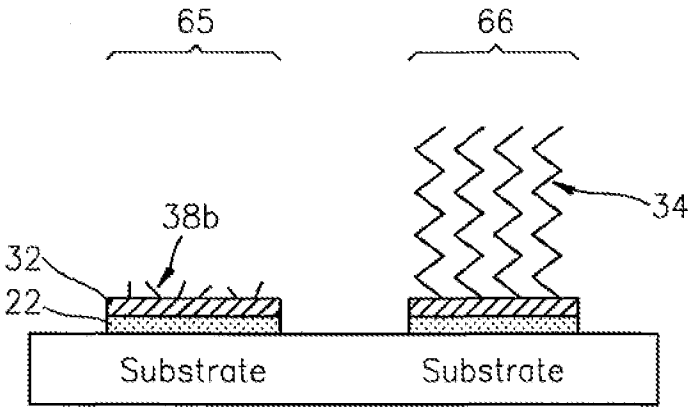

65
66
34
38b
32
22
Substrate
Substrate

FIG. 6B

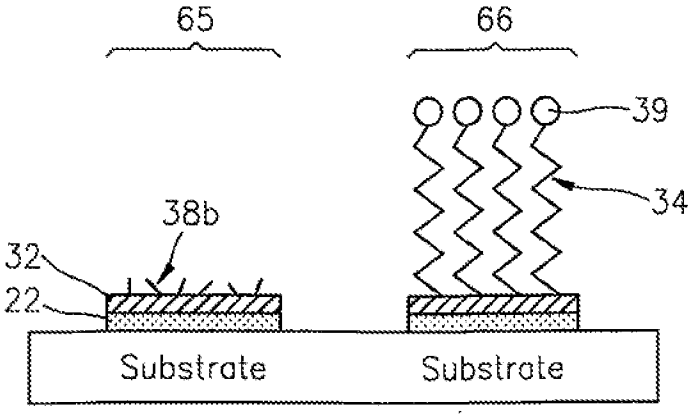

65
66
39
34
38b
32
22
Substrate
Substrate

FIG. 6C

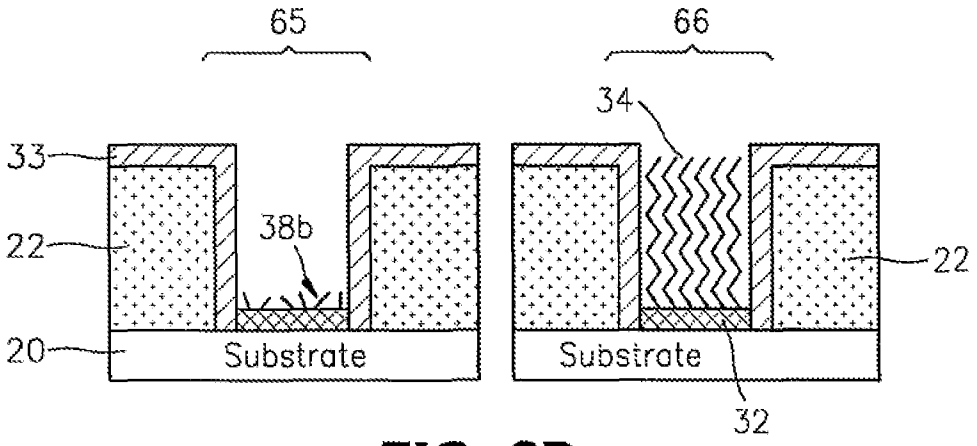


FIG. 6D

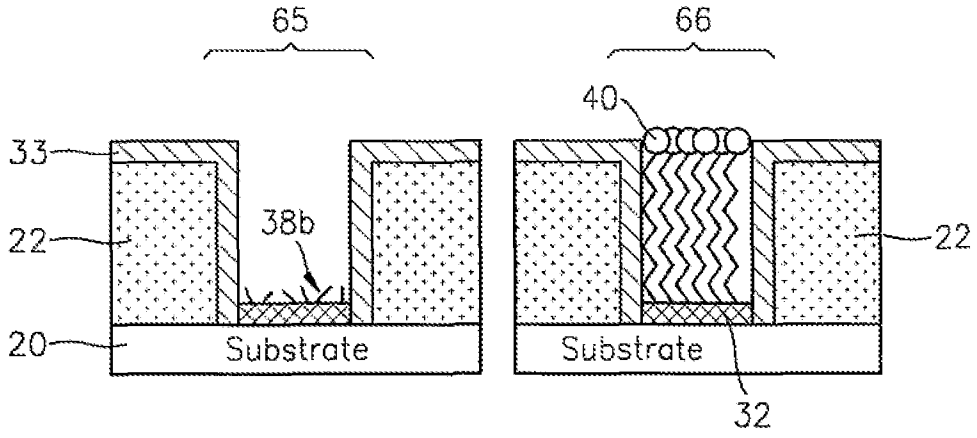


FIG. 6E

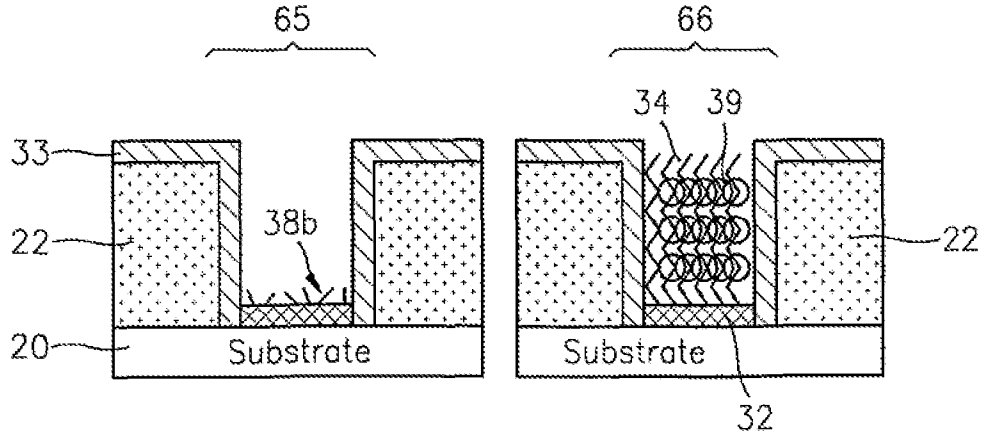


FIG. 7A

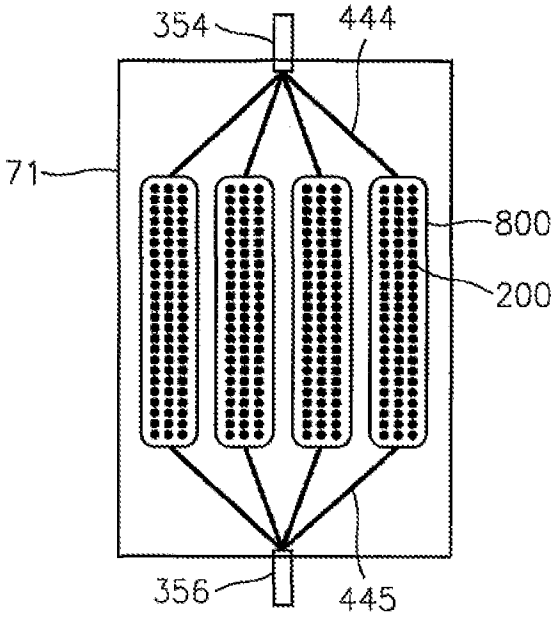
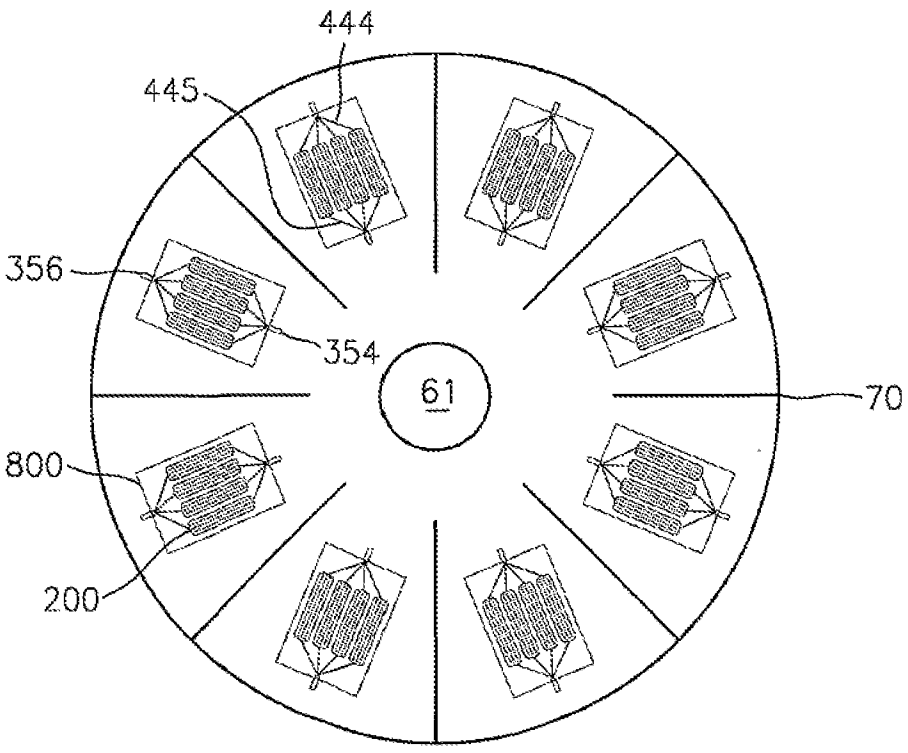
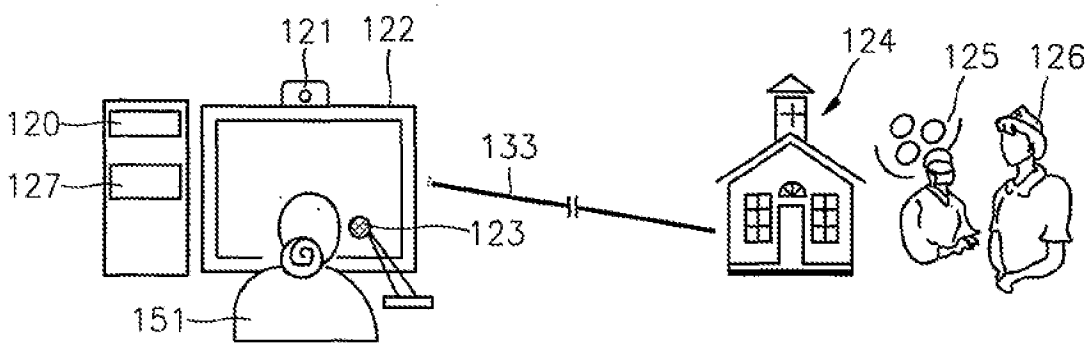


FIG. 7B



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FIG. 8



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FIG. 9

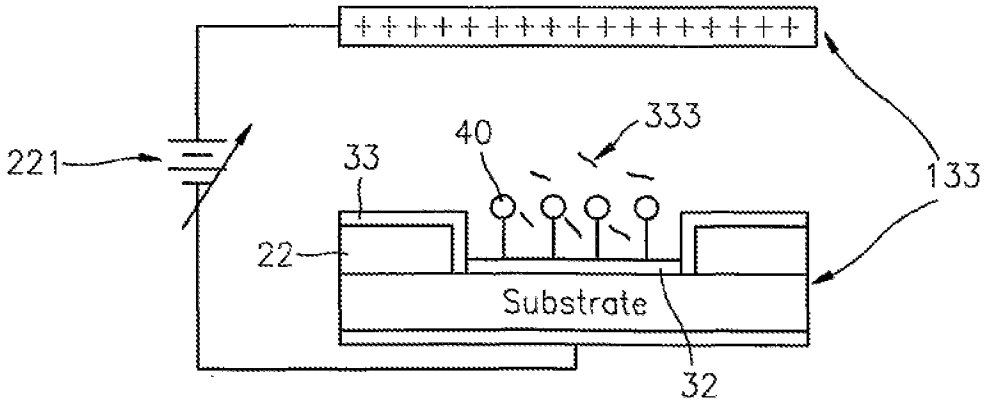


FIG. 10A

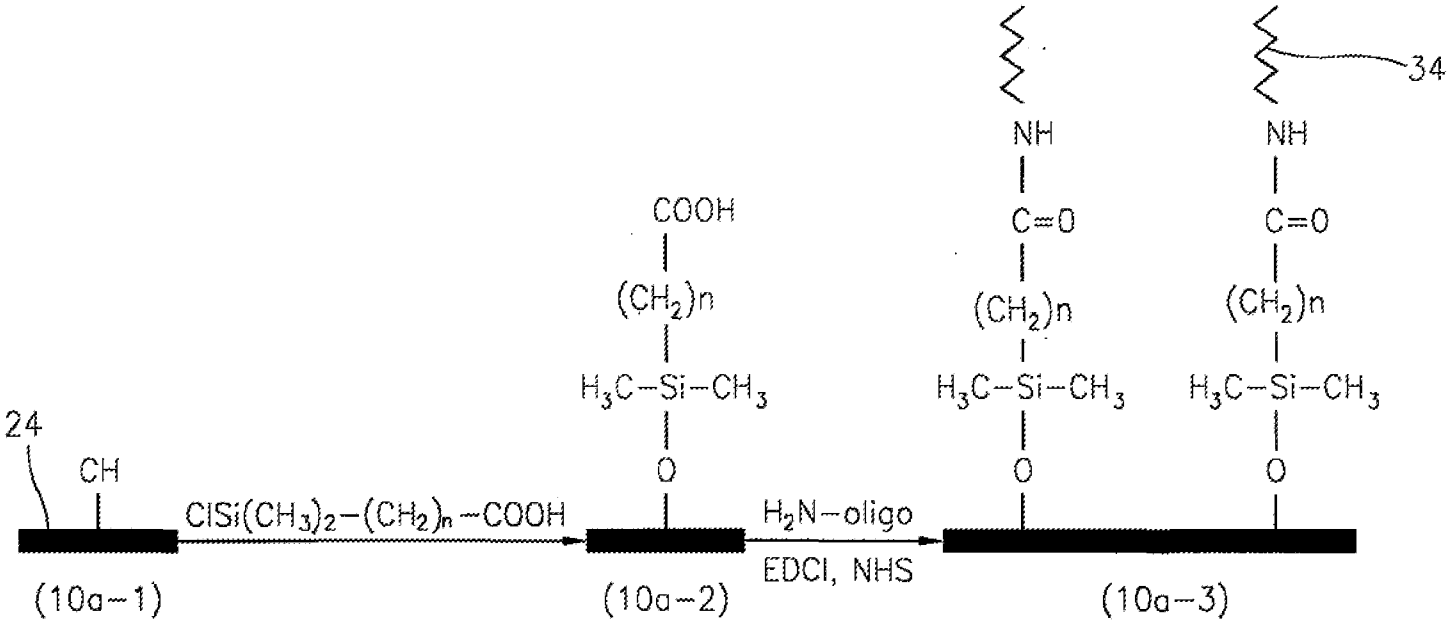

24
CH
ClSi(CH3)2–(CH2)n–COOH
COOH
(CH2)n
H3C–Si–CH3
O
H2N–oligo
EDCI, NHS
NH
C=O
(CH2)n
H3C–Si–CH3
O
34
NH
C=O
(CH2)n
H3C–Si–CH3
O
(10a–1)
(10a–2)
(10a–3)

FIG. 10B

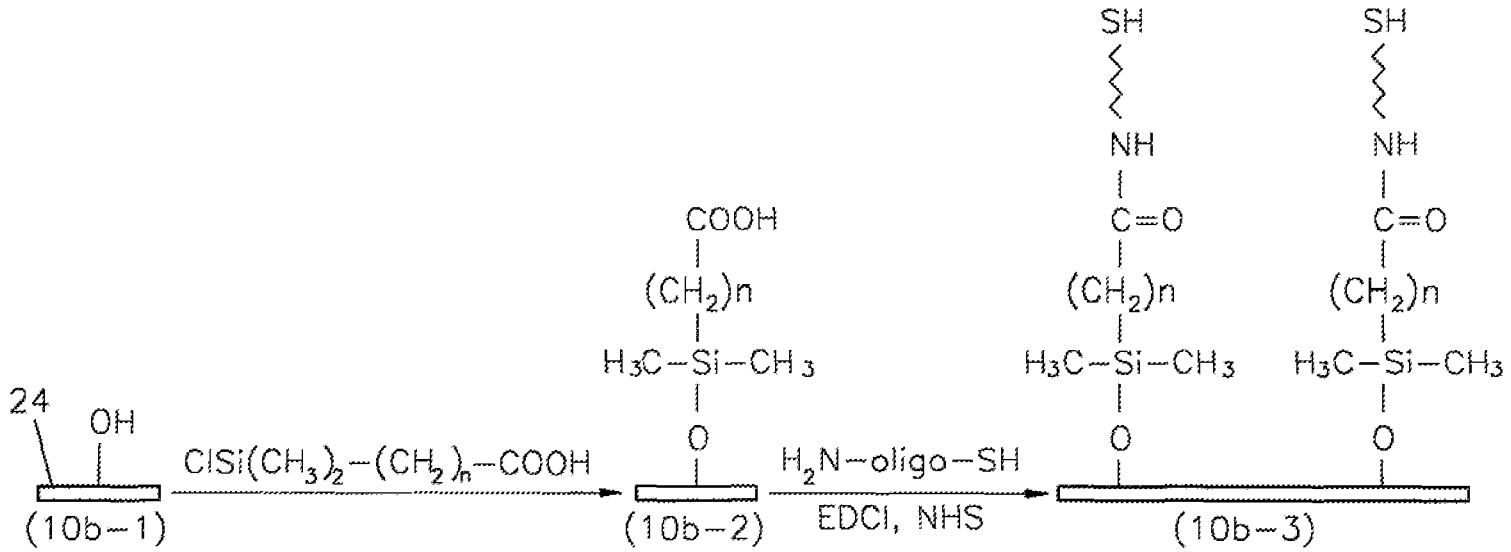

24
OH
(10b-1)
ClSi(CH3)2-(CH2)n-COOH
COOH
(CH2)n
H3C-Si-CH3
O
(10b-2)
H2N-oligo-SH
EDCI, NHS
SH
NH
C=O
(CH2)n
H3C-Si-CH3
O
(10b-3)

FIG. 10B

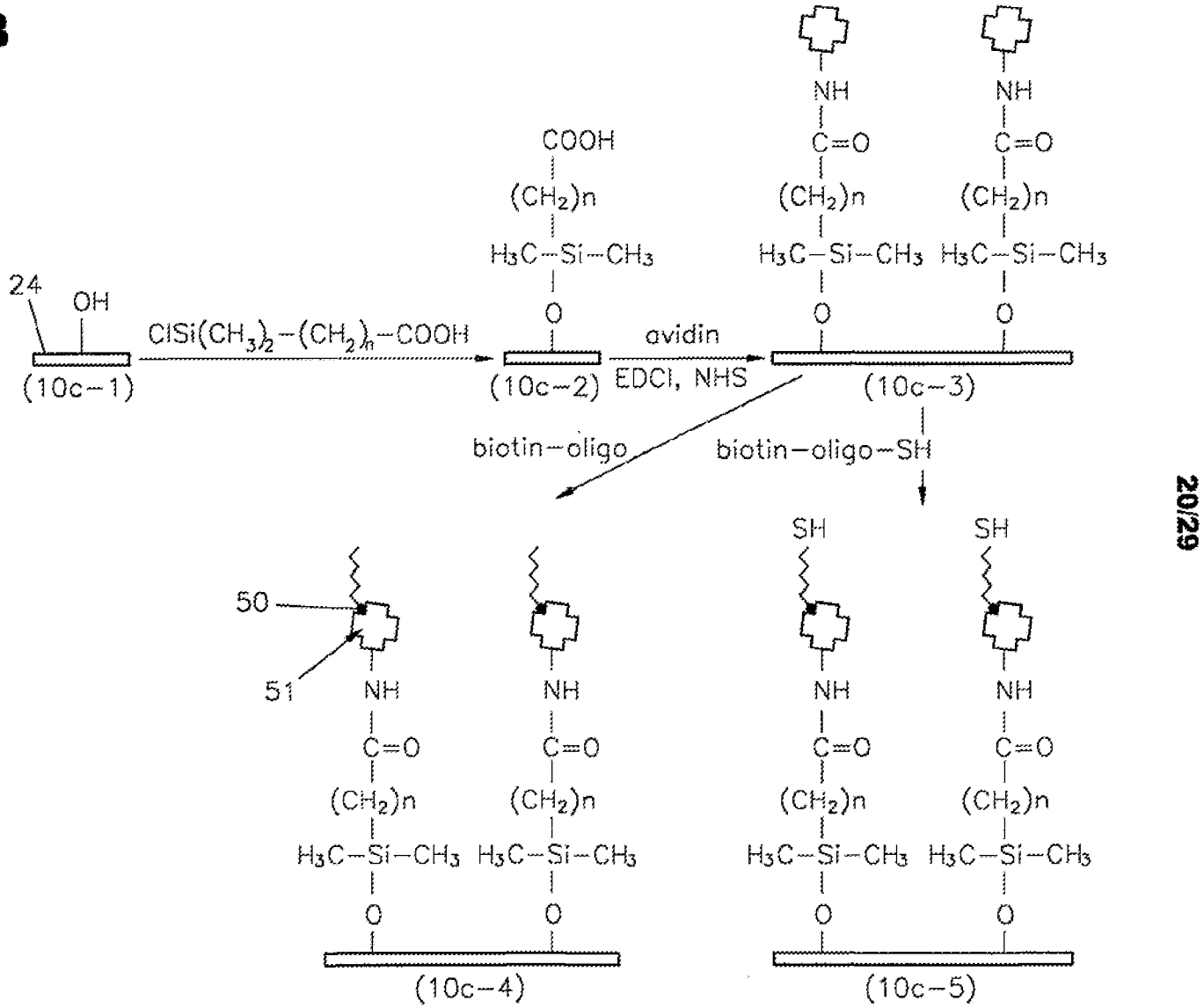

24
OH
(10c-1)
ClSi(CH3)2-(CH2)n-COOH
COOH
(CH2)n
H3C-Si-CH3
O
(10c-2)
avidin
EDCI, NHS
NH
C=O
(CH2)n
H3C-Si-CH3
O
(10c-3)
biotin-oligo
biotin-oligo-SH
50
51
SH
(10c-4)
(10c-5)

FIG. 10C

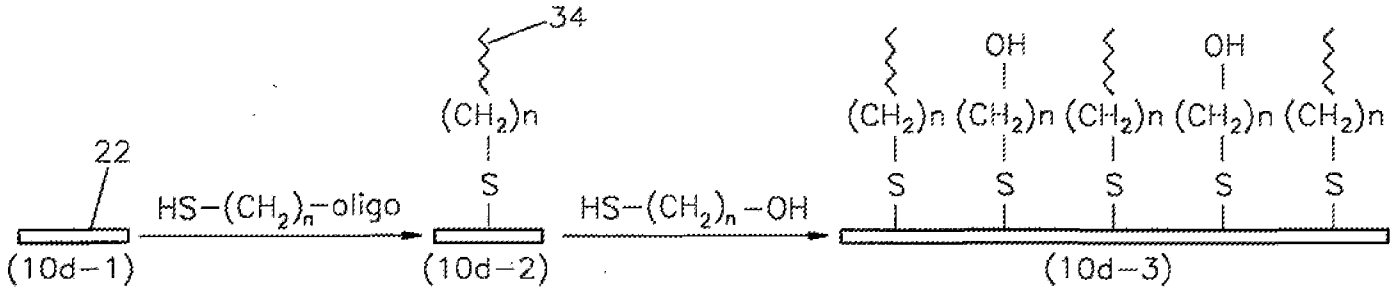


FIG. 10D

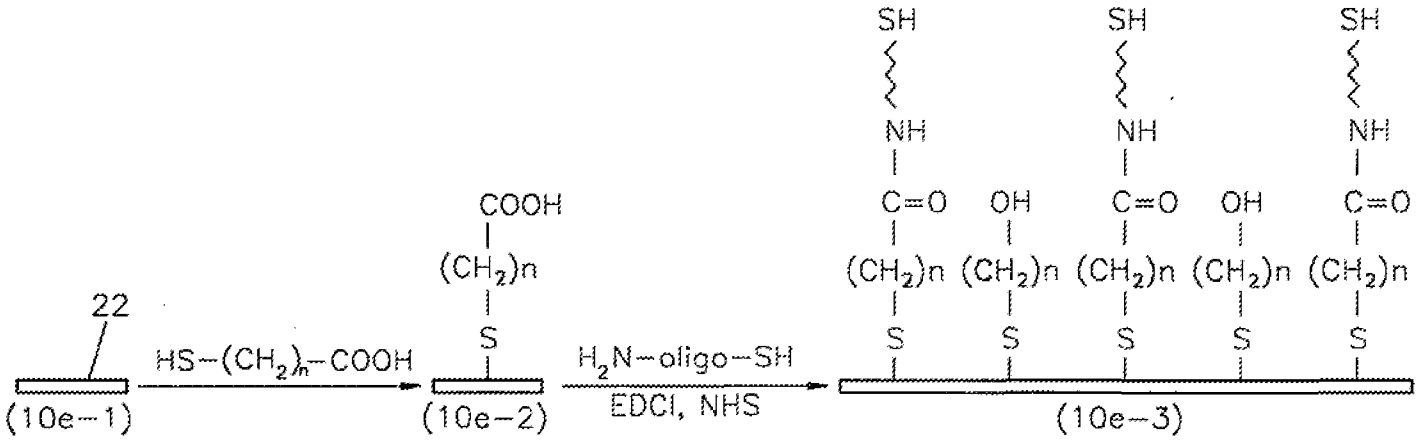


FIG. 10F

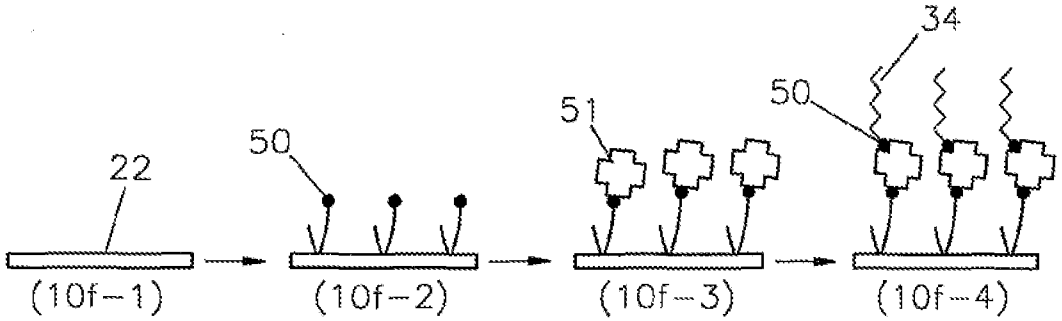


FIG. 10G

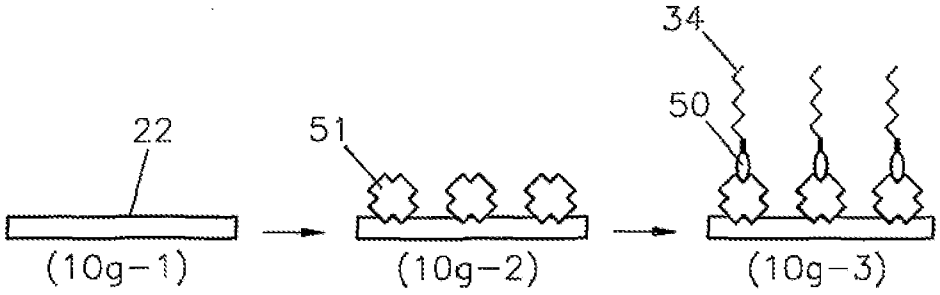


FIG. 10H

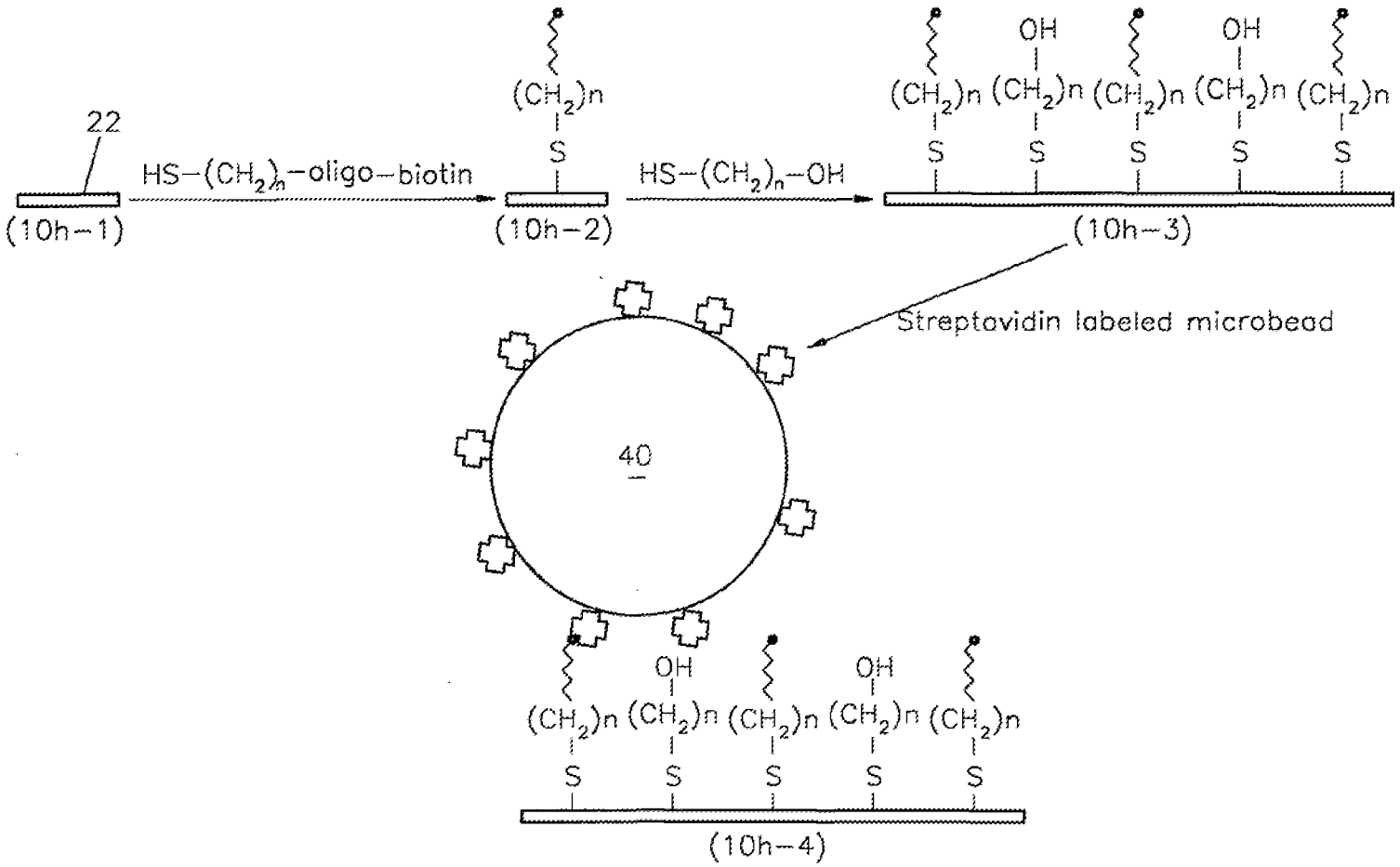

22
HS-(CH2)n-oligo-biotin
HS-(CH2)n-OH
(10h-1)
(10h-2)
(10h-3)
Streptavidin labeled microbead
40
(10h-4)

FIG. 10I

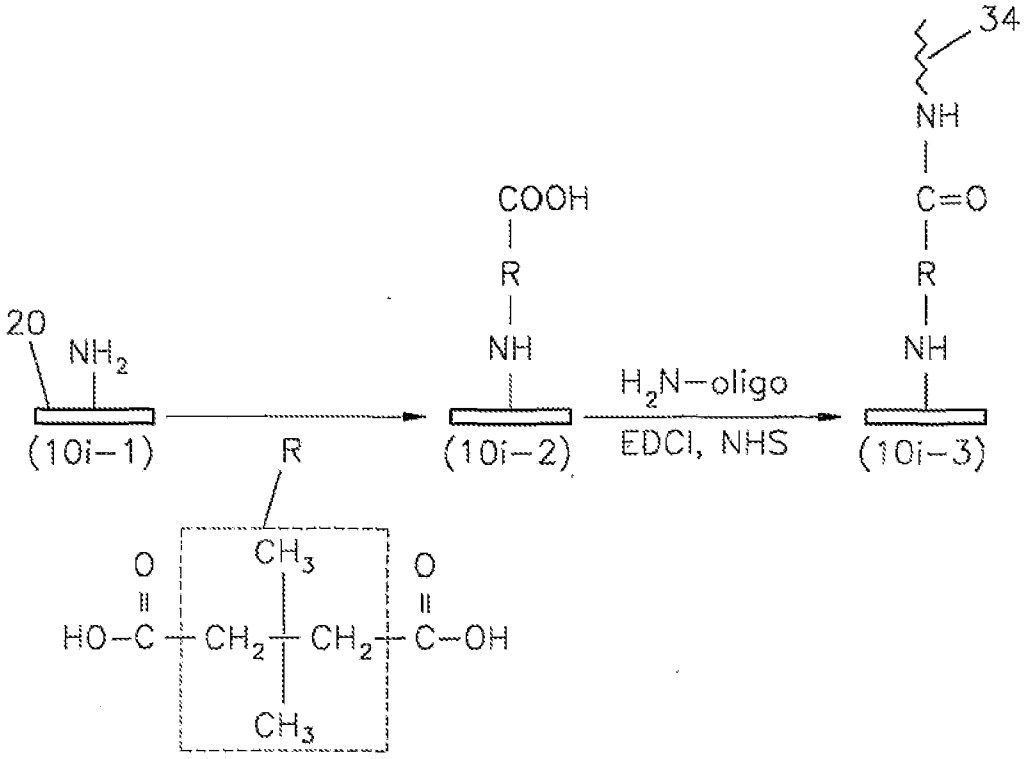
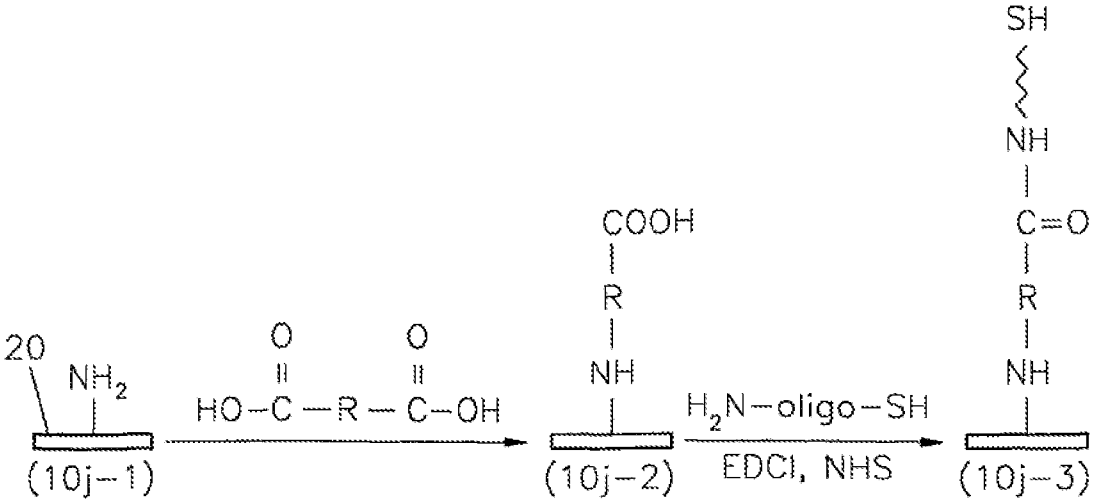


FIG. 10J



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FIG. 10K

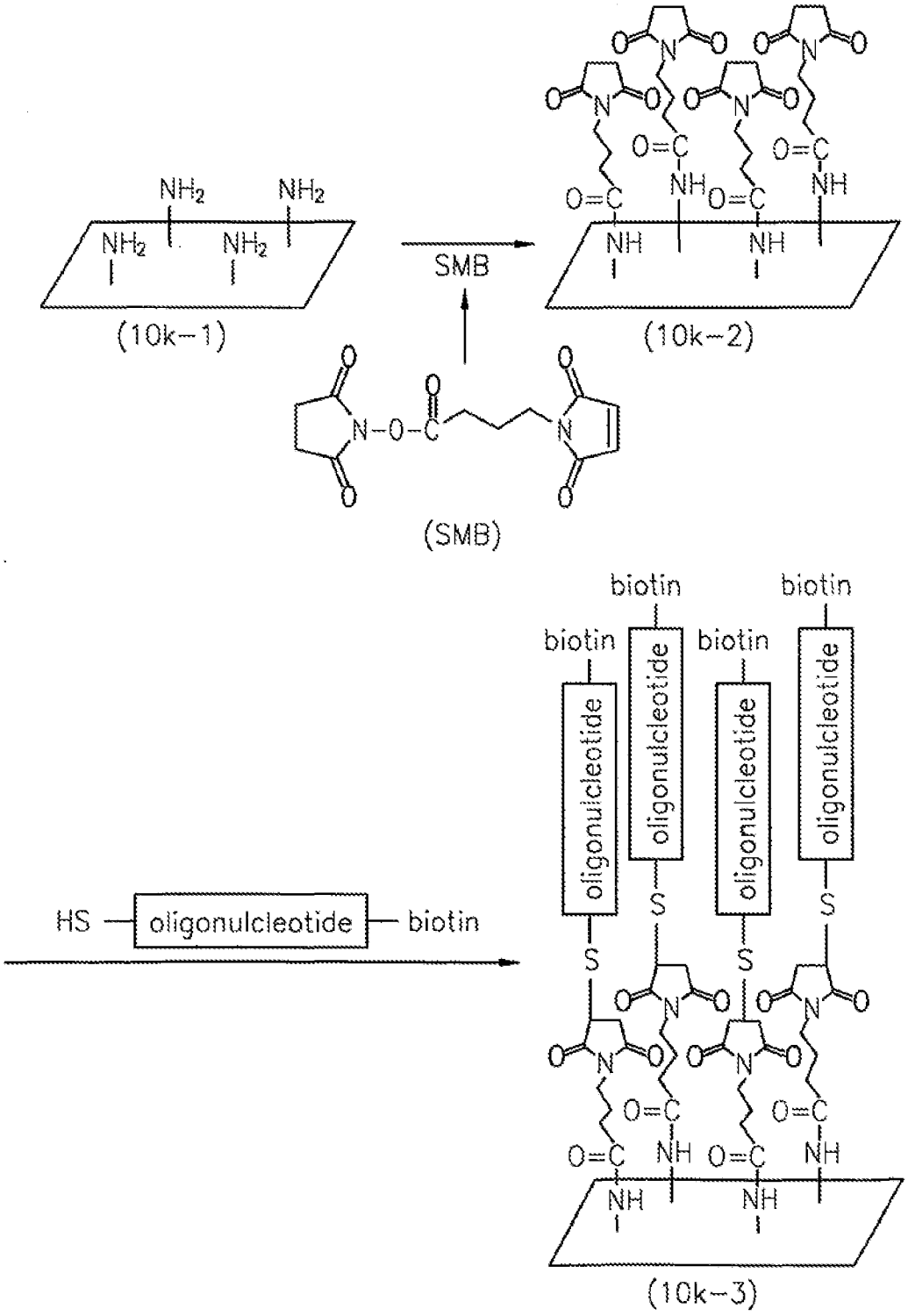

NH2
NH2
NH2
NH2
(10k-1)
SMB
(10k-2)
N-O-C
(SMB)
biotin
biotin
biotin
biotin
oligonulcleotide
oligonulcleotide
oligonulcleotide
oligonulcleotide
HS
oligonulcleotide
biotin
S
S
S
S
O=C
NH
NH
(10k-3)

FIG. 11A

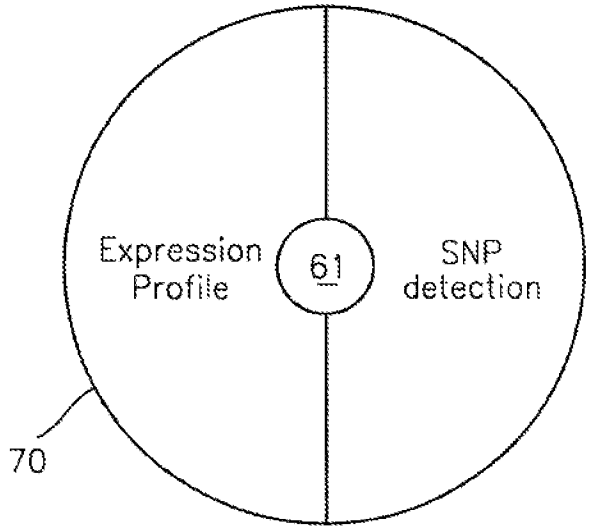
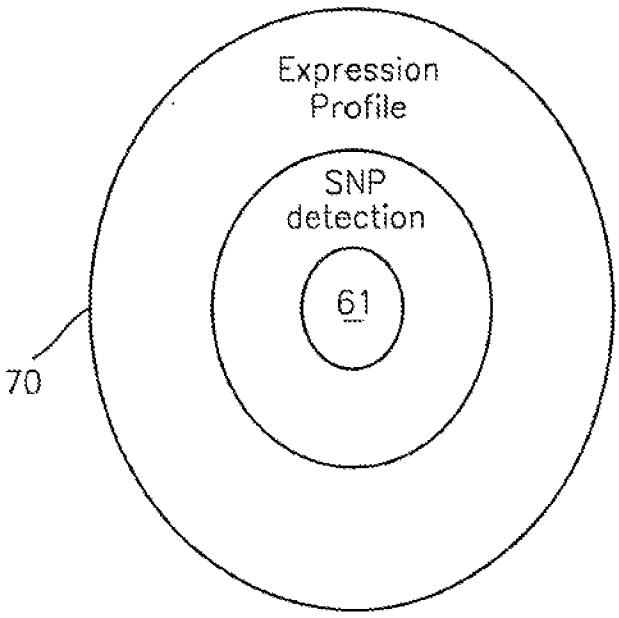


FIG. 11B



27/29

FIG. 12

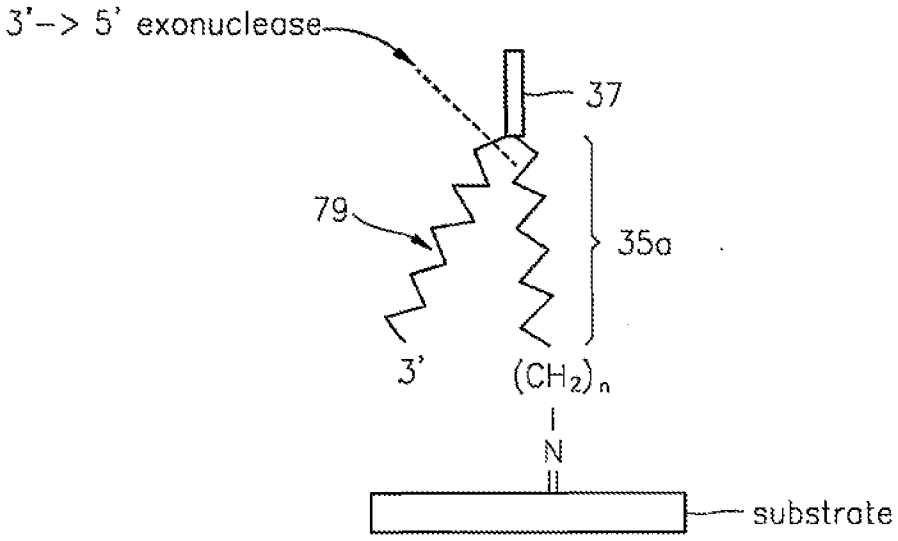


FIG. 13A

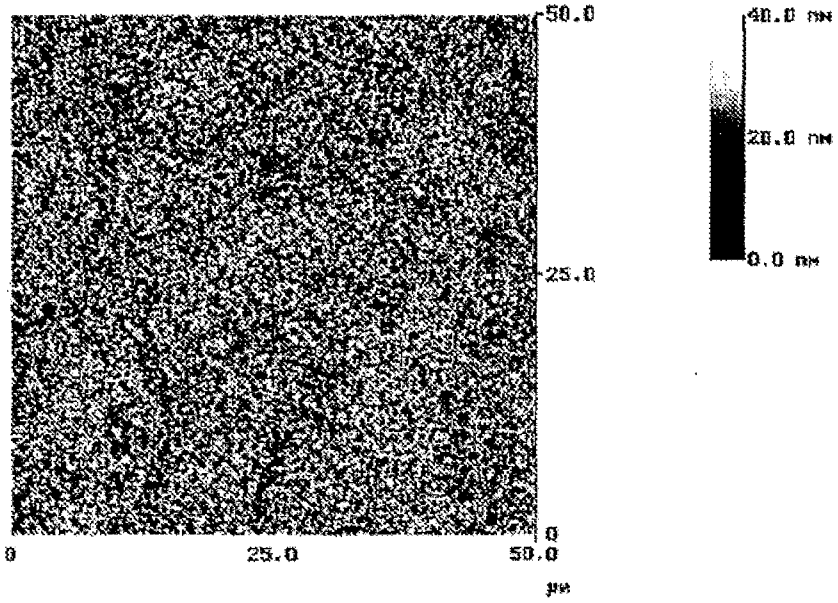
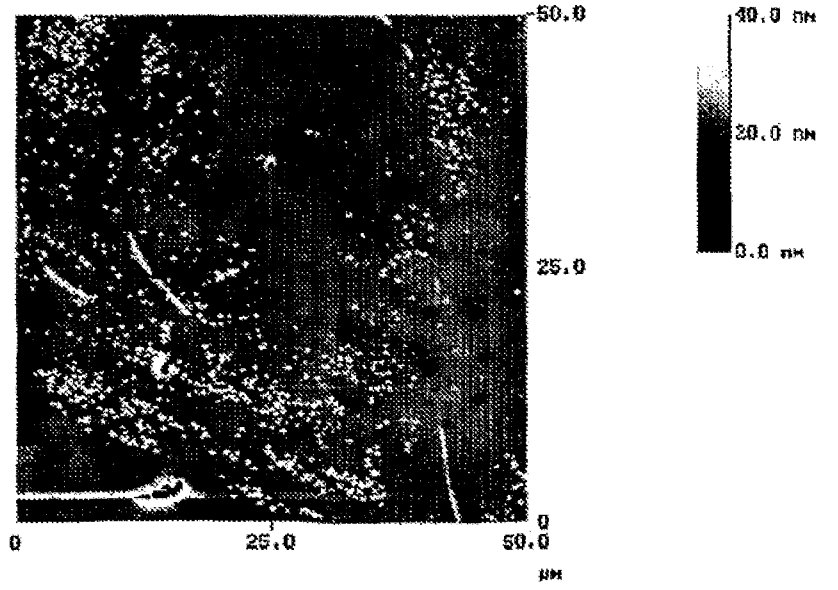
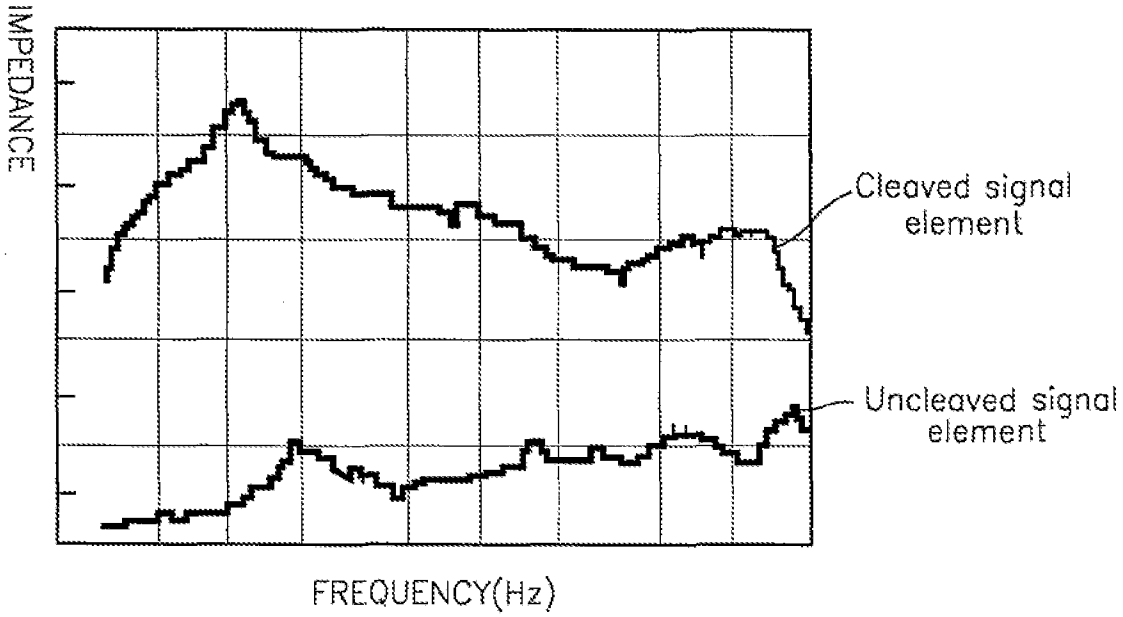


FIG. 13B



29/29

FIG. 14


IMPEDANCE
Cleaved signal element
Uncleaved signal element
FREQUENCY(Hz)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR02/00126

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12Q 1/68

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)

IPC7 C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4775619 A (CHIRON CORP.) 4 OCTOBER 1988	1
A	KR 2001-0004339 (LG ELECTRONICS INC.) 15 JANUARY 2001	18-67, 80-83
A	US 5118605 A (CHIRON CORP.) 2 JUN 1992	1 - 83

☐ 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 relevence

"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 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 relevence; 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 relevence; 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
18 JUNE 2002 (18.06.2002)	19 JUNE 2002 (19.06.2002)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	HAN, Hyun Sook Telephone No. 82-42-481-5596

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR02/00126

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4775619 A	04.10.88	US 5545730 A	13.08.96
		US 5578717 A	26.11.96
		US 5118605 A	02.06.92
		CA 1340231	15.12.98

Form PCT/ISA/210 (patent family annex) (July 1998)

Electronic Patent Application Fee Transmittal

Application Number:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Kirsten Neuman
Attorney Docket Number:	47706-0004003

Filed as Small 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($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	2806	1	120	120
		Total in USD ($)		**120**

Electronic Acknowledgement Receipt

EFS ID:	36032827
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Kirsten Neuman
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	16-MAY-2019
Filing Date:	14-FEB-2019
Time Stamp:	14:27:13
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$120
RAM confirmation Number	051719INTEFSW00013730061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	71331 cd64fb596483db8793cdc5d287682893b7fcd15c	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	PTO_1449.pdf	122556 6e018addc0cb385bb374b542e8a65cd0f3257928	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	KR1020090081260.pdf	87034 4a8d7e6243ee97016c494651c8aa442e8a4761fc	no	2
Warnings:					
Information:					
4	Foreign Reference	WO200190415_Part2.pdf	4623087 36370f7281d742af41344fee74ad3becc98dfc60	no	94
Warnings:					
Information:					
5	Foreign Reference	WO2002059364A1.pdf	9359118 3cf27a29ac898c82e767b2a52fa3ffcca9578901	no	133
Warnings:					
Information:					
6	Foreign Reference	WO200190415_Part1x.pdf	25924553 4c12eb782d78578d09d158b7651e03e0d7438747	no	279

Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	30540 549e56862ab3cea8178662859bece04f321445ac	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	40218219	

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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.

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

Application Number	16/276,235
Filing Date	February 14, 2019
First Named Inventor	Mark S. Chee
Title	SPATIALLY ENCODED BIOLOGICAL ASSAYS
Art Unit	1634
Examiner Name	James Martinell
Attorney Docket Number	47706-0004003

SIGNATURE of Applicant or Patent Practitioner

Signature	/Cameron Luitjens/	Date (Optional)	June 3, 2019
Name	Cameron M. Luitjens, Ph.D., J.D.	Registration Number	58,674
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 _____ forms are submitted.

61938662.doc

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 the Transmittal.)

☒ 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, which may be an application filed after the date of this power of attorney, or identified above:

159367

OR

☐ I hereby appoint Practitioner(s) named in the attached list 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, which may be an application filed after the date of this power of attorney, or identified above.

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

☐ The address associated with the above-mentioned Customer Number

OR

☒ The address associated with Customer Number: **159367**

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):

Prognosys Biosciences, Inc.

☐ Inventor or Joint Inventor (title not required below)

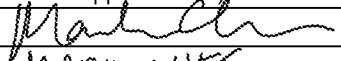
☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

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

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

SIGNATURE of Applicant for Patent

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

Signature		Date (Optional)	
Name	MARK CHEE		
Title	CEO		

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 _____ forms are submitted.

61883609.doc

Electronic Acknowledgement Receipt

EFS ID:	36188447
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Judith Witzel
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	03-JUN-2019
Filing Date:	14-FEB-2019
Time Stamp:	18:51:27
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	Power of Attorney	TransPOA.pdf	145625 0db64bab9aa231363ec317ecda2f027e5d9dcb27	no	2

Warnings:

Information:	
Total Files Size (in bytes):	145625

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003

CONFIRMATION NO. 7944

159367
FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

POA ACCEPTANCE LETTER

OC000000108672209

Date Mailed: 06/05/2019

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 06/03/2019.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/thaile/

Doc Code: DIST.E.FILE **Document Description: Electronic Terminal Disclaimer - Filed**	PTO/SB/25 PTO/SB/26 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 AND TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT**
Application Number	16276235
Filing Date	14-Feb-2019
First Named Inventor	Mark CHEE
Attorney Docket Number	47706-0004003
Title of Invention	Spatially Encoded Biological Assays

☒ 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
Prognosys Biosciences, 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)

16276260 filed on 02/14/2019

16414213 filed on 05/16/2019

16402098 filed on 05/02/2019

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.

The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

10308982

9371598

as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:
- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

(●) Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.

( ) I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.

Applicants 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 58674

( ) 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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*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:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Attorney Docket Number:	47706-0004003

Filed as Small 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	2814	1	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16276235

Filing Date: 14-Feb-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on June 6, 2019

☒ 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:	36223122
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	06-JUN-2019
Filing Date:	14-FEB-2019
Time Stamp:	13:58:09
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$160
RAM confirmation Number	060719INTEFSW00000195061050
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	37920 49688898d5d533397d44cfaa198553b6395077fc	no	3
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30654 0a4977213b22e814f4890fd78b797649df7bc7c8	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	68574		

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New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

SUPPLEMENTAL RESPONSE TO AMENDMENT(S) FILED ON MAY 15, 2019

Please consider the following reply.

Amendments to the Specification:

Please replace paragraph [00123] with the following amended paragraph:

[00123] ~~Fig. 1~~ Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system ~~101~~ 401, two reagents ~~120~~ 420, ~~122~~ 422 that specifically bind to a biological molecule of interest are associated with coding identifiers ~~106~~ 406, ~~108~~ 408 that encode for a spatial location in the sample. These coding identifiers ~~106~~ 406, ~~108~~ 408 are optionally associated with sites that assist in their identification in the assay format, *e.g.,* universal priming sites ~~104~~ 404, ~~110~~ 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section ~~116~~ 416 is encoded using the combination of the patterns ~~112~~ 412, ~~114~~ 414 created using the separate coding identifiers ~~106~~ 406, ~~108~~ 408 which provide a two dimensional code ~~118~~ 418 that shows the location of any positive detection of the biological molecule ~~102~~ 402 as well as quantifying the biological molecule ~~102~~ 402 at each location assayed in the tissue.

Please replace paragraph [00140] with the following amended paragraph:

[00140] In ~~Figure 2~~ Figure 3, two target-specific assay oligonucleotides are ligated together ~~202~~ 302 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides containing tag sequence sets X and Y are ligated ~~204~~ 304 to the target specific oligonucleotides. Oligonucleotides containing X ligate specifically to one side of the targeting construct and oligonucleotides containing Y ligate to the other. The oligonucleotides contain

universal primer sites Pl and P2. Following ligation, the constructs are eluted and, optionally, sequencing adapters can be attached ~~206~~ 306, *e.g.,* via PCR.

REMARKS

The specification has been amended to comport with the figure numbers and reference characters in the drawings.

Additionally, Applicant submits herewith marked up and clean copies of a substitute specification that corrects paragraph numbering errors. Previous paragraph number [00123] amended herein is now correctly numbered as paragraph [0114]. Previous paragraph number [00140] amended herein is now correctly numbered as paragraph [0122].

No new matter has been added.

Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: June 11, 2019

/Cameron Luitjens/

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SPATIALLY ENCODED BIOLOGICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Patent Application No. 61/321,124, filed April 5, 2010 and is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] This invention relates to assays of biological molecules, and more particularly to assays for determining spatial distributions of a large number of biological molecules in a solid sample simultaneously.

BACKGROUND OF THE INVENTION

[0003] In the following discussion certain articles and methods will be described for background and introductory purposes. Nothing contained herein is to be construed as an "admission" of prior art. Applicant expressly reserves the right to demonstrate, where appropriate, that the articles and methods referenced herein do not constitute prior art under the applicable statutory provisions.

[0004] Comprehensive gene expression analysis and protein analysis have been useful tools in understanding mechanisms of biology. Use of these tools has allowed the identification of genes and proteins involved in development and in various diseases such as cancer and autoimmune disease. Conventional methods such as *in situ* hybridization and other multiplexed detection of different transcripts have revealed spatial patterns of gene expression and have helped shed light on the molecular basis of development and disease. Other technologies that have enabled the quantitative analysis of many RNA sequences per sample include microarrays (see Shi, et al., Nature Biotechnology, 24(9):1151-61 (2006); and Slonim and Yanai, Plos Computational Biology, 5(10):e1000543 (2009)); serial analysis of gene expression (SAGE) (see Velculescu, et al, Science, 270(5235):484-87 (1995)), high-throughput implementations of qPCR (see Spurgeon, et al., Plos ONE, 3(2):e1662 (2008)) and *in situ* PCR (see Nuovo, Genome Res., 4:151-67 (1995)). As useful as these methods are, however, they do not enable simultaneous measurement of the expression of many genes or the presence and/or activity of multiple proteins at many spatial locations in a sample. Laser capture microdissection has permitted the analysis of many genes at a small number of locations, but it is very expensive, laborious, and does not scale well. Certain PCR assays in a 2D format preserve spatial

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information (see Armani, et al., Lab on a Chip, 9(24): 3526-34 (2009)), but these methods have low spatial resolution because they rely on physical transference of tissue into wells, which also prevents random access to tissue samples and high levels of multiplexing.

[0005] At present, no practical method exists to analyze at high resolution the spatial expression patterns of large numbers of genes, proteins, or other biologically active molecules simultaneously. There is thus a need for reproducible, high-resolution spatial maps of biological molecules in tissues. The present invention addresses this need.

SUMMARY OF THE INVENTION

[0006] This Summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This Summary is not intended to identify key or essential features of the claimed subject matter, nor is it intended to be used to limit the scope of the claimed subject matter. Other features, details, utilities, and advantages of the claimed subject matter will be apparent from the following written Detailed Description including those aspects illustrated in the accompanying drawings and defined in the appended claims.

[0007] The invention encompasses assay systems that provide high-resolution spatial maps of biological activity in tissues. The assay system comprises an assay capable of high levels of multiplexing where encoded probes are provided to a biological sample in defined spatial patterns; instrumentation capable of controlled delivery of reagents according to the spatial patterns; and a decoding scheme providing a readout that is digital in nature. In short, the present invention provides the ability to look at many biological targets in many locations, providing the resolution of in situ hybridization with the highly- parallel data analysis of sequencing.

[0008] Thus, in some embodiments, the invention provides an assay system to determine spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered; allowing the encoded probes to interact with the biological targets; separating encoded probes that interact with the biological targets from encoded probes that do not interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[0009] In particular aspects of the invention the biological targets comprise nucleic acids and the

encoded probes are oligonucleotides, and in some aspects, there are two encoded probes for each of the multiple nucleic acid targets. In some aspects, the multiple biological targets comprise proteins, the probe regions of the encoding probes are proteins and the coding tags comprise oligonucleotides. In some aspects the multiple biological targets comprise enzymes. In some aspects the probe regions of the encoded probes comprise antibodies, aptamers or small molecules.

[0010] Some aspects of the assay system further comprise an amplification step between the separating step and the determining step. In some aspects, the determining step is performed by nucleic acid sequencing, and in preferred aspects, the sequencing is high- throughput digital nucleic acid sequencing.

[0011] In some aspects of the invention, the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 20, in some aspects product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 50, in some aspects the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 75, 100, 150, 500, 750, 1,000, 5,000, 10,000, 25,000, 50,000, 100,000, 500,000, or 1,000,000 or more. In other aspects, the sequence of at least fifty thousand encoding probes are determined in parallel, in other aspects the sequence of at least one hundred thousand encoding probes are determined in parallel, in some aspects the sequence of at least five hundred thousand encoding probes are determined in parallel, and in some aspects the sequence of at least one million, ten million, one hundred million, one billion, ten billion, one hundred billion or more encoding probes are determined in parallel.

[0012] In some aspects, the known spatial pattern is determined by histological features of the sample. Also in some aspects, software programmed hardware performs at least two steps of the delivering step, the separation step, the determining step and the associating step.

[0013] In some aspects, the probe regions of the encoded probes are proteins and the separating step is accomplished by encoded probes that interact with the biological targets being captured by an affinity capture agent. In some aspects the probe regions of the encoding probes are nucleic acids and the separating step is accomplished by a washing of the sample.

[0014] In other embodiments there is provided an assay system to determine spatial patterns of abundance or activity or both of multiple nucleic acid targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering oligonucleotide probes for multiple nucleic acid targets to the multiple sites in the sample in a known spatial pattern; allowing the oligonucleotide probes to hybridize with the nucleic acid targets; washing unhybridized encoded oligonucleotide probes from the sample; delivering one or more encoding agents to locations of the multiple sites in the sample according to a known spatial pattern, where the

combination of encoding agents delivered to each site is different; coupling the encoding agents and the oligonucleotide probes to form encoded probes; determining all or a portion of a sequence of the encoded probes using high-throughput sequencing, and associating the abundance or activity or both of multiple biological targets to the locations of multiple sites in the sample.

[0015] Other embodiments of the invention provide an assay system to determine spatial patterns of abundance or activity or both of multiple protein targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple protein targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a protein probe region that may interact with the protein targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and the protein probe region of the encoding probe of which the coding tag is part; allowing the encoded probes to interact with the protein targets; separating encoded probes that interact with the protein targets from encoded probes that do not interact with the protein targets; determining all or a portion of a sequence of the encoded probes by high throughput sequencing, and associating the abundance or activity or both of the multiple protein targets to the locations of the multiple sites in the sample.

[0016] Other embodiments provide an assay system to determine spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and identifies the biological target; allowing the encoded probes to interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[0017] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

DESCRIPTION OF THE FIGURES

[0018] Figure 1 provides a simplified overview of the assay system of the present invention.

[0019] Figure 2 provides a simplified overview of one embodiment of the assay system of the present invention for detecting nucleic acids.

[0020] Figure 3 is a representational depiction of one embodiment of the assay overviewed in Figure 2.

[0021] Figure 4 illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention.

[0022] Figure 5 provides a simplified, specific example of the embodiment of a combinatorial encoding scheme shown in Figure 4.

DEFINITIONS

[0023] The terms used herein are intended to have the plain and ordinary meaning as understood by those of ordinary skill in the art. The following definitions are intended to aid the reader in understanding the present invention, but are not intended to vary or otherwise limit the meaning of such terms unless specifically indicated.

[0024] The term "antibody" as used herein is intended to refer to an entire immunoglobulin or antibody or any functional fragment of an immunoglobulin molecule which is capable of specific binding to an antigen (antibodies and antigens are "binding partners" as defined herein). "Antibody" as used herein is meant to include the entire antibody as well as any antibody fragments capable of binding the antigen or antigenic fragment of interest. Examples of such peptides include complete antibody molecules, antibody fragments, such as Fab, F(ab')2, CDRS, VL, VH, and any other portion of an antibody which is capable of specifically binding to an antigen. Antibodies for assays of the invention are immunoreactive or immunospecific for, and therefore specifically and selectively bind to, proteins either detected *(i.e.,* biological targets) or used for detection *(i.e.,* probes) in the assays of the invention.

[0025] The term "binding agent" as used herein refers to any agent that specifically binds to a biological molecule of interest

[0026] "Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double-stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single-stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the other strand, usually at least about 90% to about 95%, and even about 98% to about 100%).

[0027] "Hybridization" refers to the process in which two single-stranded polynucleotides bind non-covalently to form a stable double-stranded polynucleotide, The resulting (usually) double-stranded polynucleotide is a "hybrid" or "duplex," "Hybridization conditions" will typically include salt concentrations of approximately less than 1M, often less than about 500 mM and may be less than about

200 mM. A "hybridization buffer" is a buffered salt solution such as 5% SSPE, or other such buffers known in the art. Hybridization temperatures can be as low as 5°C, but are typically greater than 22°C, and more typically greater than about 30°C, and typically in excess of 37°C. Hybridizations are often performed under stringent conditions, *i.e.,* conditions under which a primer will hybridize to its target subsequence but will not hybridize to the other, non-complementary sequences. Stringent conditions are sequence-dependent and are different in different circumstances. For example, longer fragments may require higher hybridization temperatures for specific hybridization than short fragments. As other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one parameter alone. Generally stringent conditions are selected to be about 5°C lower than the T_m for the specific sequence at a defined ionic strength and pH. Exemplary stringent conditions include a salt concentration of at least 0.01 M to no more than 1M sodium ion concentration (or other salt) at a pH of about 7.0 to about 8.3 and a temperature of at least 25°C. For example, conditions of 5xSSPE (750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA at pH 7.4) and a temperature of approximately 30°C are suitable for allele-specific hybridizations, though a suitable temperature depends on the length and/or GC content of the region hybridized.

[0028] "Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, *e.g.,* oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon terminal nucleotide of one oligonucleotide with a 3' carbon of another nucleotide.

[0029] "Nucleic acid", "oligonucleotide", "oligo" or grammatical equivalents used herein refers generally to at least two nucleotides covalently linked together. A nucleic acid generally will contain phosphodiester bonds, although in some cases nucleic acid analogs may be included that have alternative backbones such as phosphoramidite, phosphorodithioate, or methylphophoroamidite linkages; or peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids, positive backbones, non-ionic backbones and non-ribose backbones. Modifications of the ribose-phosphate backbone may be done to increase the stability of the molecules; for example, PNA:DNA hybrids can exhibit higher stability in some environments.

[0030] "Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed.

The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Primers usually are extended by a DNA polymerase.

[0031] The term "SNP" or "single nucleotide polymorphism" refers to a genetic variation between individuals; *e.g.,* a single nitrogenous base position in the DNA of organisms that is variable. SNPs are found across the genome; much of the genetic variation between individuals is due to variation at SNP loci, and often this genetic variation results in phenotypic variation between individuals. SNPs for use in the present invention and their respective alleles may be derived from any number of sources, such as public databases (U.C. Santa Cruz Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway) or the NCBI dbSNP website (http://www.ncbi.nlm.nih.gov/SNP/), or may be experimentally determined as described in U.S. Pat No. 6,969,589; and US Pub. No. 2006/0188875 entitled "Human Genomic Polymorphisms." Although the use of SNPs is described in some of the embodiments presented herein, it will be understood that other biallelic or multi-allelic genetic markers may also be used. A biallelic genetic marker is one that has two polymorphic forms, or alleles. As mentioned above, for a biallelic genetic marker that is associated with a trait, the allele that is more abundant in the genetic composition of a case group as compared to a control group is termed the "associated allele," and the other allele may be referred to as the "unassociated allele." Thus, for each biallelic polymorphism that is associated with a given trait *(e.g.,* a disease or drug response), there is a corresponding associated allele. Other biallelic polymorphisms that may be used with the methods presented herein include, but are not limited to multinucleotide changes, insertions, deletions, and translocations. It will be further appreciated that references to DNA herein may include genomic DNA, mitochondrial DNA, episomal DNA, and/or derivatives of DNA such as amplicons, RNA transcripts, cDNA, DNA analogs, etc. The polymorphic loci that are screened in an association study may be in a diploid or a haploid state and, ideally, would be from sites across the genome.

[0032] The term "selectively binds", "selective binding" and the like as used herein, when referring to a binding partner *(e.g.*. protein, nucleic acid, antibody or other affinity capture agent, etc.), refers to a binding reaction of two or more binding partners with high affinity and/or complementarity to ensure selective hybridization under designated assay conditions. Typically, specific binding will be at least three times the standard deviation of the background signal. Thus, under designated conditions the binding partner binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

[0033] "Sequencing", "sequence determination" and the like means determination of information relating to the nucleotide base sequence of a nucleic acid. Such information may include the identification or determination of partial as well as full sequence information of the nucleic acid.

Sequence information may be determined "with varying degrees of statistical reliability or confidence. In one aspect, the term includes the determination of the identity and ordering of a plurality of contiguous nucleotides in a nucleic acid, "High throughput digital sequencing" or "next generation sequencing" means sequence determination using methods that determine many (typically thousands to billions) of nucleic acid sequences in an intrinsically parallel manner, *i.e.* where DNA templates are prepared for sequencing not one at a time, but in a bulk process, and where many sequences are read out preferably in parallel, or alternatively using an ultra-high throughput serial process that itself may be parallelized. Such methods include but are not limited to pyrosequencing (for example, as commercialized by 454 Life Sciences, Inc., Branford, CT); sequencing by ligation (for example, as commercialized in the SOLiD™ technology, Life Technology, Inc., Carlsbad, CA); sequencing by synthesis using modified nucleotides (such as commercialized in TruSeq™ and HiSeq™ technology by Illumina, Inc., San Diego, CA, HeliScope™ by Helicos Biosciences Corporation, Cambridge, MA, and PacBio RS by Pacific Biosciences of California, Inc., Menlo Park, CA), sequencing by ion detection technologies (Ion Torrent, Inc., South San Francisco, CA); sequencing of DNA nanoballs (Complete Genomics, Inc., Mountain View, CA); nanopore-based sequencing technologies (for example, as developed by Oxford Nanopore Technologies, LTD, Oxford, UK), and like highly parallelized sequencing methods.

[0034] The term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation, T_m=81.5+0.41 (% G+C), when a nucleic acid is in aqueous solution at 1M NaCl (see *e.g.,* Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985)). Other references *(e.g.,* Allawi and SantaLucia, Jr., Biochemistry, 36:10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

DETAILED DESCRIPTION OF THE INVENTION

[0035] The practice of the techniques described herein may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and sequencing technology, which are within the skill of those who practice in the art. Such conventional techniques include polymer array synthesis, hybridization and ligation of polynucleotides, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the examples

herein. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Green, et al., Eds., *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (1999); Weiner, Gabriel, Stephens, Eds., *Genetic Variation: A Laboratory Manual* (2007); Dieffenbach, Dveksler, Eds., *PCR Primer: A Laboratory Manual* (2003); Bowtell and Sambrook, *DNA Microarrays: A Molecular Cloning Manual* (2003); Mount, *Bioinformatics: Sequence and Genome Analysis* (2004); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); Stryer, *Biochemistry* (4th Ed.) (1995) W.H, Freeman, New York N.Y.; Gait, *"Oligonucleotide Synthesis: A Practical Approach"* (2002) IRL Press, London; Nelson and Cox, *Lehninger, Principles of Biochemistry* (2000) 3rd Ed., W. H. Freeman Pub., New York, N.Y,; and Berg, et al., *Biochemistry* (2002) 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

[0036] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" refers to one or more nucleic acids, and reference to "the assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0037] 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 this invention belongs. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing devices, formulations and methodologies that may be used in connection with the presently described invention.

[0038] Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention,

[0039] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been

described in order to avoid obscuring the invention.

The Invention in General

[0040] The assay systems of the invention provide spatially-encoded, multiplexed assays comprising 1) an assay capable of high levels of multiplexing with an efficient spatial encoding scheme; 2) instrumentation capable of delivering reagents according to a spatial pattern; and 3) decoding determined by a readout that is digital in nature. The assay systems of the invention detect the presence or absence and relative amount of a biological target or biological activity indicative of a biological target, as well as the location of the biological target or activity in a biological sample, *e.g.*, a tissue section or other biological structure disposed upon a support such as a microscope slide or culture dish.

[0041] The assay system further provides instrumentation with an ability to deliver reagents in a spatially-defined pattern. This instrumentation, together "with software, reagents and protocols, provides a key component of the highly innovative assay system of the invention, allowing for measurement of numerous biological targets or activities in a meaningful spatial environment, including gene expression and peptide localization, An encoding scheme used in these assay systems allows one to determine the location of biological targets or activity (or lack thereof) in the biological samples after the products of the multiplexed assay are removed from the biological sample and pooled for analysis. Decoding of the encoding scheme can be performed by, *e.g.*, next-generation sequencing, which easily provides millions to trillions of data points at low cost. The assay results such as the amount or activity of biological targets can then be mapped back to specific location in the biological sample. The assay systems open a new analytical window into the complex spatial patterns of cellular function and regulation in biological samples.

[0042] A simplified overview of the assay system 100 of the present invention is provided at Figure 1, At step 110, a biological sample affixed to a support is provided. The biological sample contains biological targets of interest. Biological targets can include any molecule of interest, such as nucleic acids (including, *e.g.* RNA transcripts, genomic DNA sequences, cDNAs, amplicons, or other nucleic acid sequences) and proteins, enzymes and the like. At step 120, encoded probes are delivered to the biological sample according to a known spatial pattern. Encoded probes comprise probes, which can interact "with biological targets of interest, and coding tags, which identify the positions in the sample of the biological targets being assayed, and thus can be used to link assay results back to locations in the sample. Coding tags in most embodiments are oligonucleotides. However, coding tags may also be mass tags, fluorescent labels, or other moieties.

[0043] In some embodiments, the probe and coding tag portions of the encoded probe are pre-coupled before being delivered to the biological sample, For example, in the case where the encoded probes are oligonucleotides, both the probe and coding tag sequence can be synthesized as a single oligonucleotide. Alternatively, the probe and coding tag portions of the encoding probes can be synthesized or obtained separately and combined before delivery to the biological sample *(e.g.,* two separate oligonucleotides can be synthesized and coupled by, *e.g.,* ligation; or an antibody and an oligonucleotide can be prepared separately and conjugated before delivery to the biological sample). Also, as is described in Figures 2-5, the probes and the coding tags (in encoding oligonucleotides) are synthesized separately, and are delivered to the biological sample at different steps (*e.g.,* probes first and coding tags thereafter, or vice versa) in the assay.

[0044] At step 130, the encoded probes are allowed to react or interact with the biological targets, *i.e.,* conditions are provided to allow *e.g.,* oligonucleotides to hybridize to nucleic acid targets, enzymes to catalyze reactions with protein targets, antibodies to bind epitopes, etc. In the case where the biological targets are nucleic acids, the encoded probes are typically oligonucleotides and hybridize to the target nucleic acids. In the case that the biological targets are proteins, the encoded probes typically are aptamers, small molecules, or oligonucleotide-conjugated proteins that interact with target proteins by binding to them or by reacting with them (that is, one of the proteins is a substrate for the other). Encoding oligonucleotides may be coupled to the probes (proteins) by conjugation, chemical or photo-crosslinking via suitable groups and the like.

[0045] Once encoded probes interact with the biological targets, the encoded probes that interacted with the biological targets must be separated from the encoded probes that did not interact with the biological targets at step 140. In the case where the biological targets are nucleic acids and the encoded probes are oligonucleotides, the separation can be accomplished by, *e.g.,* washing the unhybridized encoded probes from the sample. Similarly, for other assays that are based on affinity binding, including those using aptamer, small molecule, and protein probes, washing steps can be used to remove low affinity binders. In the case where the probe is transformed via interaction with the target, *e.g.,* in the case of a peptide, *e.g.,* via cleavage by a protease or phosphorylation by a kinase, it is convenient to collect, all encoded probes--both encoded probes that interacted with the biological targets and were transformed and encoded probes that were not transformed. After collection or pooling, an antibody or other affinity capture agent can be used to capture probes that were transformed by addition of a moiety (*e.g.,* a phosphate group). In cases where probes have been transformed via cleavage, the transformed probes can be separated, *e.g.,* by capturing the non-transformed probes via a tag that is removed from the transformed probes during the transformation *(e.g.,* by cleavage), or by adding a new tag at the site of cleavage.

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[0046] Once the reacted (transformed) or interacted encoded probes are separated from the unreacted or un-interacted encoded probes, the sequence of the reacted and/or interacted encoded probes is determined by, preferably, sequencing. The sequence of the encoded probes allows the mapping of the assay results back to locations in the biological sample.

[0047] Figure 2 provides a simplified overview of an assay system of the present invention embodying an efficient implementation of a combinatorial coding scheme for the encoding of spatial information. For purposes of this overview, the probes are oligonucleotides, but as explained elsewhere, other types of probes can also be used. In step 210, a biological sample affixed to a support, *e.g.,* a tissue sample or other biological structure, is provided. In step 220, one or more oligonucleotide probes are delivered to the biological sample, where the oligonucleotide probes are capable of hybridizing with biological targets in the biological sample. In step 230, the oligonucleotide probes are allowed to interact with (hybridize to) the nucleic acid targets; that is, appropriate conditions are provided where oligonucleotide probes can hybridize to the target nucleic acids.

[0048] In step 240, the oligonucleotide probes that did not hybridize to target nucleic acids are removed, and thereby separated from oligonucleotide probes that did hybridize to target nucleic acids. In this embodiment, separation can be accomplished by, *e.g.,* washing the sample to remove unhybridized oligonucleotide probes. Next, in step 250, encoding oligonucleotides (the encoding agents) are delivered to the biological sample according to a chosen spatial pattern, where the encoding oligonucleotides comprise coding tags that are used to encode the location of biological targets in the biological sample. Note that in contrast to the assay system of Figure 1, here the probes and encoding agents (encoding oligonucleotides) are delivered in separate steps. In step 260, the encoding oligonucleotides are coupled to the oligonucleotide probes to create encoded probes. In this case where the probes are oligonucleotides, the encoding oligonucleotides may be coupled to the oligonucleotides probes by, *e.g.,* ligation. Alternatively, the information in the encoding oligonucleotides can be transferred by using a DNA polymerase to extend a probe oligonucleotide that acts as a primer, and thereby copy and incorporate the sequence of the encoding oligonucleotides.

[0049] In step 270, the sequence of the coding tags in the encoded probes as well as the sequence or a portion of the sequence of the probe itself is determined, and in step 280, the target nucleic acids are mapped back to the biological sample. In some embodiments, the abundance of sequences reveals the relative quantity of biological targets at the location. Although this embodiment shows the individual steps in a particular order, so as to better explain the invention,

the precise order of the steps can be varied. For example, steps 220 and 250 can be combined, so that a mixture of the probes and encoding oligonucleotides is delivered according to a chosen spatial pattern. Coupling step 260 can then be carried out immediately after the combined steps 220 and 250, or concomitantly with them. In this case, step 240 would then occur after step 260. It can therefore be appreciated that the two key results of this series of steps, *i.e.,* the location-specific encoding of probe molecules and the separation of probe molecules based on their ability to interact with corresponding target molecules, can be accomplished with some flexibility in the implementation of the particular steps. Similarly, there is considerable flexibility in the design of the coding scheme. As described *infra,* the assays of the invention are particularly amenable to combinatorial methods.

[0050] Thus, the present invention provides an ability to look at many different biological targets in many locations, providing the resolution of in situ hybridization with the highly-parallel data analysis of sequencing. In some embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 20, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 50, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 100, greater than 500, 1,000, 10,000, 25,000, 100,000, 500,000, 1,000,000. It will be appreciated that, due to the spatial encoding dimension of the invention, even much larger numbers can be contemplated. For example, assaying l 0,000 targets per location x 10,000 locations would generate $1O^{8}$ different assays, and even larger numbers than these can easily be contemplated, particularly if spatial locations with resolution on the order of that of single cells are utilized. Further, in embodiments where high-throughput digital sequencing is employed, the sequences of at least 1,000 encoding probes are typically determined in parallel. More typically, using a digital readout, it is desirable to obtain multiple sequence reads for each assay (defined by a probe and a spatial location code). It is desirable to obtain an average of at least 3 copies per assay, and more typically at least 10 or at least 30 copies per assay, depending on the design of the experiment and requirements of the assay. For a quantitative readout with suitable dynamic range, it may be desirable to obtain at least 1,000 reads per assay. Therefore, if 1,000,000 assays are carried out, the number of sequence reads may be 1 billion or more. With high-throughput digital sequencing, and allowing for redundancy, the sequence of at least 10,000 encoding probes are determined in parallel, or the sequence of at least 100,000, 500,000, 1,000,000, 10,000,000, 100,000,000, 1,000,000,000 or more encoding probes are determined in parallel.

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Assays

[0051] The assay portion of the assay systems of the present invention comprise the following general steps: delivering probes and encoding agents where the encoding agents (in some embodiments pre-coupled to the probes) are delivered to the sample according to a known spatial pattern, allowing the probes to interact or react with biological targets in the sample, and, if the probes and encoding agents have not been pre- coupled, coupling the encoding agents to probes.

[0052] The samples of the present invention include virtually any biological sample or samples that can be affixed to a support or provided essentially in a two-dimensional manner, where the ability to tie an assayed biological target or activity back to the location within the biological sample is important. Exemplary biological samples include tissue sections (e.g., including whole animal sectioning and tissue biopsies), cell populations on slides or culture dishes, and the like. The assay systems of the invention are particularly advantageous in that they are compatible with numerous biological sample types, including fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the biological samples are immobilized on a substrate surface having discrete, independently measurable areas.

[0053] The biological targets to be detected can be any biological molecules including but not limited to proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Examples of subcellular targets include organelles, e.g., mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplasts, endocytic vesicles, exocytic vesicles, vacuoles, lysosomes, etc.

[0054] In some particular embodiments, the assay system is used to analyze nucleic acids, *e.g.,* by genotyping, quantitation of DNA copy number or RNA transcripts, localization of particular transcripts within samples, and the like. Figure 3 illustrates an overall scheme for an exemplary assay for, *e.g.,* detecting single nucleotide polymorphisms (SNPs) that can be used with the assay system of the invention. In Figure 3, two oligonucleotide probes are provided. Each oligonucleotide probe comprises a target-specific region (located on either side of the SNP to be analyzed) seen at 305 and 307, and ligation regions, seen at 301 and 303, The oligonucleotide probes are allowed to hybridize to a target nucleic acid (not shown) in the biological sample. At step 302, one of the oligonucleotide probes is extended to incorporate the SNP sequence and ligated to the other probe to form an extended probe comprising target nucleic acid region 309 and ligation regions 301 and 303.

[0055] Two encoding agents, both comprising a coding tag (seen at 315 and 317), a ligation region

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(seen at 311 and 313), and a primer region (seen at 319 and 321) are combined with and ligated to the extended probe at step 304 to form an encoded target- specific oligonucleotide. Again, in contrast with Figure 1, the probes and encoding agents are delivered at separate steps. Doing so allows use of the combinatorial embodiments described *infra.* In preferred embodiments, the encoding oligonucleotides within a pair of encoding oligonucleotides ligate specifically to one side of the target sequence or the other *(i.e., 5'* or 3' of the target sequence). Also, typically, the ligation and primer regions of the encoding oligonucleotides and probes are universal; that is, the set of ligation and primer regions used in constructing the probes and encoding oligonucleotides are constant, and only the target-specific regions of the probes and the coding tags of the encoding oligonucleotides differ. However, again in alternative embodiments, the ligation and primer regions are not universal and differ between probes and encoding agents.

[0056] Following ligation, the encoded probes are eluted, pooled, and, optionally, sequencing adapters are added to the encoded probes via PCR. In alternative embodiments, sequencing primers may be ligated to the encoding oligonucleotides, or sequencing primer sequences can be included as part of the encoding oligonucleotide. As seen in Figure 3, each sequencing adapter comprises primer region 319 or 321, compatible with the primer regions 319 and 321 on the encoded probes. The final construct comprising first adapter 327, first primer region 319, first coding tag 315, ligation regions 311 and 301, target region 309, ligation regions 313 and 303, second coding tag 317, second primer region 325 and second adapter 329 is now ready for input into a digital high-throughput sequencing process.

[0057] A combination of extension and ligation reactions are exemplified in Figure 3, but it should be appreciated that a variety of reactions may be used to couple the encoding oligonucleotides to the target-specific oligonucleotides, including ligation only (e.g., for oligonucleotides that hybridize to contiguous portions of the target nucleic acid sequence). Alternatively, an assay utilizing an additional oligonucleotide, such as in the GOLDENGATE® assay (see Fan, et al., Cold Spring Symp. Quant. Biol., 68:69-78 (2003); (Ilumina, Inc., San Diego, CA)), may be employed.

[0058] In other embodiments, the assay system of the invention also can be used to analyze peptides or proteins, the presence of antibodies, enzymatic and other protein activities, posttranslational modifications, active and non-active forms of peptides, as well as peptide isoforms in a biological sample. Accordingly, the probes may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, aptamers and the like.

[0059] In certain aspects, the probes are substrates for enzymes or proenzymes, *e.g.,* kinases, phosphatases, zymogens, proteases, or fragments thereof. In certain aspects, the probes are

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phosphorylation substrates used to detect proteins involved in one or more signal transduction pathways, *e.g.,* a kinase or a phosphatase. In another specific aspect of the invention, the probes are specific protease substrates that associate only with individual proteases or classes of proteases. In other aspects, the probes are different processed forms, isoforms and/or domains of an enzyme. Protein-based probes are typically conjugated or otherwise linked to oligonucleotide encoding agents. The oligonucleotide encoding agents in this case would also include a nucleotide sequence component that allows for identification of the protein probe.

[0060] In certain aspects, the present invention provides assays for evaluating differences in the amount and/or activity of biological targets between different locations in a sample and/or between samples. The method includes determining a plurality of encoded results from the biological sample and evaluating the differences in quantity of the biological targets at each location in the biological sample.

Combinatorial Embodiments

[0061] To maximize the efficiency of encoding, a combinatorial approach using pairs of coding tags in the encoding oligonucleotides can be used. By de-coupling the target-specific information and the coding tags, the number of oligonucleotides required is dramatically reduced, with a concomitant decrease in cost.

[0062] Figure 4 illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention, where nucleic acids in a representative tissue section (shown at 416) are assayed. Figure 4 at A shows two target-specific/encoding oligonucleotide constructs 420 and 422 *(e.g.,* formed between steps 302 and 304 of Figure 3) specifically bound to a target nucleic acid 402 of interest, The first encoded probe 420 comprises coding tag 408, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 404. The second encoded probe 422 comprises coding tag 406, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 410.

[0063] Figure 4 at B shows the spatial pattern that may be used for twenty different coding tags, al through a10 (coding tag 406 on encoded probe 420) and b1 through b10 (coding tag 408 encoded probe 422). Coding tag al., for example, is deposited on the biological sample in ten discrete areas or spots (shown as the first horizontal line of spots in 412). Coding tag a2 is deposited on the biological sample in ten spots on the second horizontal line in 412. Coding tag a3 is deposited on the biological sample in ten spots on the third horizontal line in 412, and so on. Whereas the "a" tags are deposited

in ten horizontal rows, the "b" tags are deposited in ten vertical rows as shown in 414. For example, coding tag b1 is deposited on the biological sample in ten discrete spots in the first vertical row of 414, coding tag b2 is deposited on the biological sample in ten discrete spots in the second vertical row of 414, and so on. Using such a configuration allows for twenty coding tags to uniquely define 100 different locations on the biological sample,

[0064] Figure 4 at C shows a representative tissue section 416 coincident with coding tag grid 418. The arrows show how the "a" coding tags and the "b" coding tags are deposited on grid 418 that is coincident with tissue section 416. If, once sequenced, coding tags al and b4, *e.g.,* are associated with a target nucleic acid sequence, then that target nucleic acid sequence *(i.e.,* biological target) was present in the tissue section at location al, b4.

[0065] Figure 5 provides a simplified, specific example of the encoding scheme of the assay systems of the invention. Figure 5 shows encoding oligonucleotides 510, comprising al, a2, a3, a4 and b1, b3, b3 and b4. Target-specific oligonucleotides (TSOs) (probes) 1 and 2 are shown at 520. A deposit or dispensing scheme is shown at 530. Like the grid exemplified in Figure 4, encoding oligonucleotides al through a4 are deposited in spots in a pattern (here, in a vertical pattern), and encoding oligonucleotides bl through b4 are deposited in spots in a pattern (here, a horizontal pattern). The grid though shown as a square with spots is actually a deposition pattern on a biological sample (not shown) such as tissue section 416 shown in Figure 4.

[0066] The target-specific oligonucleotides are delivered to the biological sample, where the target-specific oligonucleotides hybridize to target nucleic acids in the biological sample if target nucleic acids are present. Unhybridized target-specific oligonucleotides are then removed, *e.g.,* by washing. The encoding oligonucleotides are then delivered to the biological sample according to the spatial pattern shown at 530. The encoding oligonucleotides are ligated (or, *e.g.,* extended and ligated) to any target-specific oligonucleotides that hybridized to the target nucleic acid in the biological sample, the ligated constructs are then eluted from the biological sample, pooled, and sequencing adapters are added through, *e.g.,* PCR or ligation, if the sequences were not previously included in the encoding oligonucleotides. The ligated constructs are sequenced by, *e.g.,* high throughput or "next generation" sequencing.

[0067] The pool of resulting sequences is shown at 540. A sequence readout was obtained for target-specific oligonucleotide 1 only at a4bl, a4b2, alb3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at a1b1 (position shown with vertical lines). A sequence readout was obtained for both target-specific oligonucleotides 1 and 2 at positions a2b1, a3b1, a1b2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at alb4, a2b4

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or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample, and neither target nucleic acid was detected in the upper right portion of the biological sample. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample.

[0068] In addition to location information, information relating to relative abundance of the encoded tags can be obtained. For example, if it is found that there are ten times as many a4T1b1 sequences occurring in the data set as compared to a4T1b2 sequences, this would indicate that target nucleic acid sequence 1 is ten times more abundant at the a4T1b1 location than at the a4T1b2 location.

[0069] In the case of nucleotide analysis as shown in Figure 3, by ligating the coding tags directly to target-specific oligonucleotides, only 2n target-specific oligonucleotides are needed for n targets. For example, using the combinatorial approach outlined in Figure 2, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 target-specific oligonucleotides and 2 x 100 encoding oligonucleotides. The total count of assay oligonucleotides would be only 400 (200 target-specific and 200 encoding), not counting universal primers. In contrast, if the coding oligonucleotides were not decoupled from the target-specific oligonucleotides, (n x X positional codes) + (n x Y positional codes) would be needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences. Moreover, though the embodiments shown in Figures 2- 5 depict a combinatorial scheme using two encoding agents (coding tags), three, four or more encoding agents and coding tags may be used, and attached to the probe or one another by varying means and in varying combinations of steps.

[0070] Due to the spatial encoding aspect of the assay system of the invention, a large amount of information can be generated with even a modest number of assays. For example, five or more biological targets assayed at five or more positions in the sample generates 25 or more combinations. Using digital sequencing as a readout, the optimum number of sequence reads per combination depends on the sensitivity and dynamic range required, and can be adjusted, For example, if for each combination on average 100 reads are sampled, the total for 25 combination is 25,000 reads. If 1,000 targets are assayed at 1,000 locations with an average sampling depth of 1,000, then 10^9 reads are required. These numbers, although large, are within the capacity of intrinsically parallel digital sequencing methods, which can generate datasets of billions or even trillions of reads in a reasonable timeframe and at a very low cost per read. Therefore, by varying the numbers of positions interrogated or biological targets assayed, or both, and using digital sequencing, large

amounts of information can be obtained. In specific aspects, multiple locations are interrogated for two or more biological molecules.

Reagent Delivery Systems

[0071] The reagent delivery system of the invention includes instrumentation that allows the delivery of reagents to discrete portions of the biological sample, maintaining the integrity of the spatial patterns of the encoding scheme. Reagent delivery systems of the assay systems of the invention comprise optional imaging means, reagent delivery hardware and control software. Reagent delivery can be achieved in a number of different ways. It should be noted that reagent delivery may be to many different biological samples at one time. A single tissue section has been exemplified herein; however, multiple biological samples may be affixed and analyzed simultaneously. For example, pions of a tissue sample can be analyzed in parallel and the data combined to build a 3D map.

[0072] Integral to the assay system of the invention is instrumentation that allows for spatial patterning of reagents onto the biological sample. Technologies for formulating and delivering both biological molecules *(e.g.* oligonucleotides or antibodies) and chemical reagents (e.g., small molecules or dNTPs) are known in the art, and uses of these instrument systems are known to one skilled in the art and easily adaptable to the assay systems of the invention. One example of a suitable reagent delivery system is the Labcyte™ Echo acoustic liquid handier, which can he used to deliver nanoliter scale droplets containing biological molecules with high precision and reproducibility. One skilled in the art could incorporate this reagent delivery device into the overall system, using software to specify the locations to which reagents should be delivered.

[0073] Other instruments that can be used for the deposition of agents and/or coding identifiers onto biological samples include, but are not limited to, ink jet spotting; mechanical spotting by means of pin, pen or capillary; micro contact printing; photochemical or photolithographic methods; and the like. For several applications, it may be preferred to segment or sequester certain areas of the biological samples into one or more assay areas for different reagent distributions and/or biological target determination. The assay areas may be physically separated using barriers or channels.

[0074] In one exemplary aspect, the reagent delivery system may be a flow-based system. The flow-based systems for reagent delivery in the present invention can include instrumentation such as one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Reagent delivery systems are configured to move fluid to contact a discrete section of the biological sample. Movement

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of the reagents can be driven by a pump disposed, for example, downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, reagents may be driven through the fluid by gravity. US Pub. Nos. 20070166725 and 20050239192 disclose certain general-purpose fluidics tools that can be used with the assay systems of the invention, allowing for the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

[0075] In a more specific example, one or more flow-cells can be attached to the substrate-affixed biological sample from above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump is used to deliver reagents to the flow-cell and across the biological sample. The flow cells are configured to deliver reagents only to certain portions of the biological sample, restricting the amount and type of reagent delivered to any specific section of the biological sample.

[0076] In another aspect, a microfluidic system can be integrated into the substrate upon which the biological sample is disposed or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid may be formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected and delivered according to selective opening and closing of valves disposed between reagent reservoirs.

[0077] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes *(i.e.,* microfluidic structures). The pump can operate mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among other means. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. Mechanical pumps may be micromachined, molded, etc. Exemplary electrical pumps may include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[0078] Valves generally include any mechanism for regulating the passage of fluid through a channel. Valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into a channel to partially or completely block a channel, an electrocapillary structure, and/or the like.

[0079] An open gasket can be attached to the top of the biological sample and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber may be formed by a gasket sandwiched between the biological sample on the substrate and a chemically inert, water

resistant material such as, but not limited to, black-anodized aluminum, thermoplastics *(e.g.,* polystyrene, polycarbonate, etc), glass, etc.

[0080] In an optional embodiment, the assay system comprises imaging means to determine features and organization of the biological sample of interest. The images obtained, *e.g.,* may be used to design the deposition pattern of the reagents, Imaging means are optional, as an individual can instead view the biological sample using, *e.g.,* a microscope, analyze the organization of the biological sample, and specify a spatial pattern for delivery assay reagents. If included, the delivery system can comprise a microcircuit arrangement including an imager, such as a CCD or IGFET-based *(e.g.,* CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Pub. No. 20090197326, which is incorporated herein by reference. Also, it should be noted that although Figures 4 and 5 illustrate using a x,y grid configuration, other configurations can be used, such as, *e.g.,* following the topology of a tissue sample; targeting certain groups of cells, cell layers and/or cell types in a tissue, and the like.

[0081] In yet another alternative, the reagent delivery system controls the delivery of reagents to specific patterns on a biological sample surface using semiconductor techniques such as masking and spraying. Specific areas of a biological sample can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the biological sample using conventional techniques such as spraying or fluid flow. The use of masked delivery results in a patterned delivery scheme on the substrate surface.

[0082] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms (*e.g.,* thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions are be achieved.

[0083] In order to target specific sites of interest, an informative image of the biological sample to be assayed may be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions of the biological sample can be identified using image processing (e.g., images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with other features of the assay system. In some aspects, software is used to automatically translate image information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample for reagent delivery is thus an important component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and/or other very accurate physical positioning systems can be adapted to this purpose.

[0084] The invention preferably comprises a complete suite of software tailored to the assay

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system. Optionally, oligonucleotide design software is used to design the encoding nucleotides (and in embodiments where nucleic acids are assayed, the target-specific oligonucleotides) for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for reagent delivery and data analysis (i.e., sequence analysis) may be integrated to determine assay results. Integrated data analysis is particularly useful, as the type of dataset that is generated may be massive as a consequence of scale. Algorithms and software tools that are specifically designed for analysis of the spatially-associated data generated by the assay systems, including pattern-analysis software and visualization tools, enhance the value of the data generated by the assay systems.

[0085] In certain aspects, the assay system comprises processes for making and carrying out the quality control of reagents, *e.g.,* the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents are formulated according to factors such as volatility, stability at key temperatures, and chemical compatibility for compatibility with the reagent delivery instrumentation and may be analyzed by instrumentation integrated within the assay system.

Sequencing

[0086] Numerous methods can be used to identify the coding tags and probe sequences in the encoded probes of the assay systems of the invention. The coding tags can be detected using techniques such as mass spectroscopy *(e.g.,* Maldi-T of, LC-MS/MS), nuclear magnetic resonance imaging, or, preferably, nucleic acid sequencing. Examples of techniques for decoding the coding tags of the present invention can be found, for example, in US Pub. No. 20080220434, which is incorporated herein by reference. For example, the coding tags may be oligonucleotide mass tags (OMTs or massTags). Such tags are described, *e.g.,* in US Pub. No. 20090305237, which is incorporated by reference in its entirety. In yet another alternative, the encoded probes can be amplified and hybridized to a microarray. This would require separate amplification reactions to be carried out, in which each amplification is specific to a particular spatial code or subset of codes, accomplished by using code-specific primers. Each amplification would also incorporate a different resolvable label (e.g. fluorophor). Following hybridization, the relative amounts of a particular target mapping to different spatial locations in the sample can he determined by the relative abundances of the resolvable labels.

[0087] In one particularly preferred aspect, the resulting coding tags according to the assay system are substrates for high-throughput, next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of the coding tags, for example, with SOLiD™ technology (Life Technologies, Inc.) or Genome Ananlyzer (Illumina, Inc.). Such next-

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generation sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in *e.g.,* Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et al, U.S. patent publication 2005/0191656; sequencing-by-synthesis methods, *e.g.,* U.S. Pat Nos. 6,210,891; 6,828,100; 6,969,488; 6,897,023; 6,833,246; 6,911,345; 6,787,308; 7,297,518; 7,462,449 and 7,501,245; US Publication Application Nos. 20110059436; 20040106110; 20030064398; and 20030022207; Ronaghi, et al, Science, 281: 363-365 (1998); and Li, et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); ligation-based methods, *e.g.,* U.S, Patent Nos. 5,912,148 and 6,130,073; and U.S. Pat. Appln Nos. 20100105052, 20070207482 and 20090018024; nanopore sequencing *e.g.,* U.S. Pat. Appln Nos. 20070036511; 20080032301; 20080128627; 20090082212; and Soni and Meller, Clin Chem 53: 1996-2001 (2007)), as well as other methods, *e.g.,* U.S. Pat. Appln Nos. 20110033854; 20090264299; 20090155781; and 20090005252; also, see, McKernan, et al., Genome Res., 19:1527-41 (2009) and Bentley, et al., Nature 456:53-59 (2008), all of which are incorporated herein in their entirety for all purposes.

Applications of Assay System

[0088] It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous important areas of biological research, diagnostics, and drug development that will benefit from a high throughput multiplexed assay system that can measure simultaneously the amount and spatial location of a biological target in a biological sample. For example, combining the ability to estimate the relative abundance of different RNA transcripts with the ability to reconstruct an image of spatial patterns of abundance across many locations, which may be as small as or even smaller than individual cells, in a tissue enables many different areas of basic research. The following are exemplary uses and are by no means meant to be limiting in scope.

[0089] In one example, 3-dimensional patterns of gene expression are determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning, Such a method can be used to measure changes in gene expression in disease pathology, *e.g.,* in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues is obtained, leading to new insights into the function and regulation both in normal and diseased states, and provides new hypotheses that can be tested. For example, an assay system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney, et al., Nature, 447:799-816 (2007)) and modENCODE to be integrated at the tissue level. The assay

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systems also aid computational efforts to model interacting networks of gene expression in the field of systems biology.

[0090] The assay systems also provide a novel approach to analysis of somatic variation, *e.g.*, somatic mutations in cancer or variability in response to infectious organisms. For example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer-related processes in both cancer and genetically normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers. In another example, individuals have varying susceptibility to infectious organisms, and the assay systems of the invention can be used to study the interaction between microbes and tissues or the various cell types within the tissue.

~~[00100]~~**[0091]** Importantly, in addition to providing spatially-associated information, the invention allows a great increase in the sensitivity of detecting rare mutations, as signal to noise can he dramatically increased since only a small location is assayed in any given reaction. In a typical assay for rare mutations in a mixed sample, the sample is treated in bulk, *i.e.*, nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in one cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the assay systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, in the assay systems of the present invention, background is reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed, which may be particularly important in detecting key mutations in tissue sections in cancer. Already molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics. The technology of the invention promises to greatly increase the power of such approaches.

~~[00101]~~**[0092]** The present invention provides assays, assay systems, and methods of using such assays in spatially encoded biological assays. The invention provides an assay system comprising one or more agents provided in defined spatial patterns on a substrate surface, and a detection system for identifying the presence or absence, relative amount, and location of a biological molecule. Such biological molecules include, but are not limited to, nucleic acids, peptides, carbohydrates, cellular components, and the like. The assay system is a novel multiplexing approach, as it allows multiple molecules and their respective multiple locations to be identified in a single system using a unique

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encoding scheme. This encoding scheme uses both molecule-specific binding agents and coding identifiers to provide a practical and cost-effective determination of information on multiple biological molecules, including specific positional information of such molecules in a biological sample, e.g., a tissue section. The single molecule detection analysis using the encoding system also allows relative amounts of biological molecules to be detected, thus providing information on expression levels, sequestering in specific locales, and the like.

~~[00102]~~**[0093]** The assay systems detect the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. In addition, the assays provide methods for doing this for multiple biological molecules simultaneously. The assay systems utilize one or more binding agents that specifically bind to the biological molecule of interest and unique coding identifiers associated with specific binding agents. The detection system utilizes a method for identifying the presence and spatial address of the agent binding based on the positive and/or negative results that are obtained using detection of the agent and identifier and the encoding scheme of the spatial patterns on the substrate surface. In a specific aspect, the encoding scheme employs limited reagent delivery to the spatial patterns on the substrate surface, and access of the coding identifiers and/or binding agents to portions of the sample is controlled through such limited delivery.

~~[00103]~~**[0094]** In one aspect, the assay system detects the presence or absence and spatial location of a biological molecule based on the positive and/or negative results that are obtained using limited reagent delivery and the encoding scheme of the spatial patterns on the substrate surface.

~~[00104]~~**[0095]** The assay system and methods of the invention are based on relational, solid-state substrates with positions that represent specific spatial locations within a biological sample, e.g., a cell, organelle or tissue. The ability to use encoding features to represent locations allows high-throughput analysis of the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. The encoding features also allow provide assaying of multiple biological molecules at these multiple locations simultaneously.

~~[00105]~~**[0096]** A primary feature of the invention is the preservation of the spatial organization of elements in a sample of interest through the use of an encoding scheme. For example, the assay may be designed to preserve the relative position of cells in a tissue, and the assay may interrogate the individual cells for genomic DNA variation (including epigenetic modifications), and RNA and protein expression.

~~[00106]~~**[0097]** In one specific aspect, the encoding scheme of the assay system comprises the use of two or more coding patterns, each comprising regions defined by spatial patterns on the substrate surface. For example, the assay system can utilize an encoding scheme that comprises a 2-dimensional grid format based on the discrete positioning of the binding agents in the substrate surfaces. In another

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example, the spatial patterns may be based on more randomized cell locations, e.g., the patterns on the substrate surface follow an underlying biological structure rather than a strict, x,y grid pattern. This aspect includes systems with two or more substantially identical spatial patterns using different binding agents and/or coding identifiers, as well as systems having different patterns for different agents and/or coding identifiers. The encoding scheme of the systems can be controlled by delivery of different reagents to discrete regions on the substrate surfaces, which allows different reactions to take place on substantially similar agents of known location on the substrate surfaces.

~~[00107]~~**[0098]** In one specific aspect, the invention provides high resolution, high- throughput analysis of nucleic acids and/or expression levels that provides both detection and spatial identification of large numbers of nucleic acids, e.g., DNA or RNA.

~~[00108]~~**[0099]** In another specific aspect, the invention provides high resolution, high- throughput analysis of proteins that provides both detection and spatial identification of large numbers of such proteins, e.g., kinases or proteases.

~~[00109]~~**[0100]** Numerous reagent delivery systems can be used with the assay system of the invention. The primary criteria of such reagent delivery systems is the ability to direct delivery of specific agents based on spatial patterns on the substrate surface.

~~[00110]~~**[0101]** In one preferred aspect, the encoding scheme utilizes a reagent delivery system based on printing and informatics technologies to implement the spatial patterns used for identification and localization of the biological materials. For example, the patterns found in the encoding scheme may be created using ink jet printing technology to provide reagents at specific locations on one or more substrate surfaces. The desired patterns are set out in specific coding patterns on the substrate surface.

~~[00111]~~**[0102]** In certain aspects of the invention, the binding agents are immobilized directly to the substrate surface, and the location of the binding agents is known or determined prior to use of the substrate surface in the assay system. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, e.g., provided in wells or channels.

~~[00112]~~**[0103]** In specific aspects of the invention, the binding agents are nucleic acids immobilized directly or indirectly to the substrate surface, e.g., directly through the use of amino groups on the substrate surface or indirectly through the use of a linker. The location of the nucleic acid sequences is known or determined prior to use of the substrate surface in the assay system. In another specific aspect, the nucleic acids may be immobilized directly or indirectly onto beads that are then provided in known locations on the substrate surface. In yet another aspect, the nucleic acids may be provided in or on features of the substrate surface, e.g., provided in wells.

~~[00113]~~[0104] In these aspects involving nucleic acid agents, any methods of sequence determination can be used, e.g., sequencing, hybridization and the like. In a preferred aspect, nucleic acid sequencing, and preferably next-generation sequencing, is used to decode the spatial encoding scheme in the assay system of the invention. This provides a very wide dynamic range for very large numbers of assays, allowing for efficient multiplexing.

~~[00114]~~[0105] In some aspects, the assay utilizes two or more oligonucleotides, the oligonucleotides comprising a universal primer region and a region that correlates specifically to a single spatial pattern within the spatial encoding scheme. In a specific aspect, the assay comprises two allele specific oligonucleotides and one locus specific oligonucleotides. These oligonucleotides allow the identification of specific SNPs, indels or mutations within an allele. This is useful in the identification of genetic changes in somatic cells, genotyping of tissues, and the like.

~~[00115]~~[0106] In other specific aspects of the invention, the binding agents are peptides. In one aspect, these peptides are associated directly or indirectly to known locations on a substrate surface, e.g., using binding protein pairs or through oligonucleotide linkers complementary to oligonucleotides on the substrate surface. In another aspect, the binding agents are peptides are immobilized directly or indirectly onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the peptides may be provided in or on features of the substrate surface, e.g., provided in wells.

~~[00116]~~[0107] In yet other specific aspects of the invention, the binding agents are chemical entities (e.g., small molecules) that are coded, e.g. using sequence tags or mass spectroscopy tags as coding identifiers. In one aspect, these chemical entities can be are immobilized directly to the substrate surface. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, e.g., provided in wells.

~~[00117]~~[0108] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

~~[00118]~~[0109] **The Invention in General**

~~[000119]~~[0110] The assay system and methods of the invention are based on relational methods that allow extraction of data to detect the presence or absence and relative amount of a biological molecule, and the location of this molecule in a sample having a distinct structure, *e.g.,* a tissue section or other biological structure with distinct locations of specific biological molecules. The encoding

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scheme used in these systems corresponds to the structural elements of the sample, and the information obtained using a two-dimensional coding system is indicative of the spatial addresses of these molecules in a sample of interest.

~~[00120]~~[0111] Integral to the assay system of the invention is a method for spatial patterning of reagents. Technologies for formulating and delivering both biological molecules *(e.g.* DNA or antibodies) and chemical reagents *(e.g.,* small molecules or dNTPs) have already been demonstrated, and use of these systems will be available to one skilled in the art and easily adaptable upon reading this specification.

~~[00121]~~[0112] The assay design of the invention provides an accurate and easily scalable spatial encoding system. The ability to deliver reagents in a spatially defined pattern together with software, reagents and protocols comprises a novel and highly innovative assay system for spatial analysis of various biological molecules and activities. This allows the assays to measure numerous biological functions in a meaningful spatial environment, including functions such as gene expression and peptide localization. The systems provide the potential to open a new analytical window into the complex spatial patterns of cellular function and regulation in biological systems.

~~[00122]~~[0113] The biological molecules to be detected can be any biological molecules such as proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Further examples of subcellular objects include organelles, *e.g.,* mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplast, endocytic vesicle, exocytic vesicles, vacuole, lysosome, etc.

~~[00123]~~[0114] ~~Fig. 1~~ Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system ~~101~~ 401, two reagents ~~120~~ 420, ~~122~~ 422 that specifically bind to a biological molecule of interest are associated with coding identifiers ~~106~~ 406, ~~108~~ 408 that encode for a spatial location in the sample. These coding identifiers ~~106~~ 406,

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~~108~~ 408 are optionally associated with sites that assist in their identification in the assay format, *e.g.,* universal priming sites ~~104~~ 404, ~~110~~ 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section ~~116~~ 416 is encoded using the combination of the patterns ~~112~~ 412, ~~114~~ 414 created using the separate coding identifiers ~~106~~ 406, ~~108~~ 408 which provide a two dimensional code ~~118~~ 418 that shows the location of any positive detection of the biological molecule ~~102~~ 402 as well as quantifying the biological molecule ~~102~~ 402 at each location assayed in the tissue.

~~[00124]~~[0115] The assay systems of the invention are particularly advantageous in that they are compatible with numerous samples types, such as fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the binding agents are immobilized on a substrate surface in discrete, independently measureable areas. These discrete areas can be formed by spatially selective deposition of the binding agents on the substrate surface. Numerous methods can be used for the deposition of the agent and the coding identifiers associates with the agent. For example, the coding identifiers can be delivered together or separately from the agent. If delivered together they can be attached *(e.g.,* synthesized as a single molecule or attached through ligation or a chemical coupling mechanism) or simply mixed together to be attached after delivery to the substrate. In a preferred aspect, the agent and the coding identifier are made separately, mixed together for attachment, and delivered either attached or as a mixture to be attached on the surface. In a specific aspect the binding agents are delivered generally over the substrate surface and the coding identifiers are delivered in a pattern-specific manner.

~~[00125]~~[0116] Examples of methods that can be used for deposition of agents and/or coding identifiers onto the substrate surface include, but are not limited to, ink jet spotting, mechanical

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spotting by means of pin, pen or capillary, micro contact printing, fluidically contacting the measurement areas with the biological or biochemical or synthetic recognition elements upon their supply in parallel or crossed micro channels, upon exposure to pressure differences or to electric or electromagnetic potentials, and photochemical or photolithographic immobilization methods.

~~[00126]~~**[0117]** For several applications, it may be preferred to arrange the substrates into segments of one or more measurement areas for reagent distribution and agent determination. These regions may be physically separated using barriers or channels. They may still comprise several additional discrete measurement areas with agents that are different or in different combination from each other.

~~[00127]~~**[0118]** In certain aspects, the present invention provides a method, *e.g.,* a machine-based method, for evaluating changes in the presence and/or location of a biological molecule over time. The method includes providing a plurality of encoded array results representative of the biological molecule over time and evaluating the differences in detection and/or localization of the biological molecules.

Nucleic Acid Detection and Localization

~~[00128]~~**[0119]** In a particular aspect, the assay system is used to analyze nucleic acids, *e.g* genotyping, gene expression analysis, localization of particular transcripts within samples, and the like.

~~[00129]~~**[0120]** Genotyping may be performed using any technique known to those of skill in the art. Preferred techniques permit rapid, accurate determination of multiple variations with a minimum of sample handling. Some examples of suitable techniques involve but are not limited to direct DNA sequencing, capillary electrophoresis, hybridization, allele-specific probes or primers, single-strand conformation polymorphism analysis, nucleic acid arrays, bead arrays, restriction fragment length polymorphism analysis, cleavage fragment length polymorphism analysis, random amplified polymorphic DNA, ligase detection reaction, heteroduplex or fragment analysis, differential

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sequencing with mass spectrometry, atomic force microscopy, pyrosequencing, FRET *(e.g.,* TaqMan (Applied Biosystems, Inc., Foster City, Calif.) and Molecular Beacon (Stratagene, La Jolla, Calif.) assays), and other related techniques. Several methods for DNA sequencing are well known and generally available in the art. See, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, New York) (2001); Ausubel, et al., Current Protocols in Molecular Biology (John Wiley and Sons, New York) (1997), Twyman, et al. (2003) "Techniques Patents for SNP Genotyping", Pharmacogenomics 4(1):67-79; and Kristensen, et al. (2001) "High-Throughput Methods for Detection of Genetic Variation", BioTechniques 30(2):318-332. For details on the use of nucleic acid arrays (DNA chips) for the detection of, for example, SNPs, see U.S. Pat. No. 6,300,063 issued to Lipshultz, et al., and U.S. Pat. No. 5,837,832 to Chee, et al., HuSNP Mapping Assay, reagent kit and user manual, Affymetrix Part No. 90094 (Affymetrix, Santa Clara, Calif.). The molecular inversion probe (MIP) assay format (Hardenbol et al., 2003) is another example of a highly multiplexable assay that may be used with the assay systems of the invention.

~~[00130]~~**[0121]** In one exemplary and preferred method for analyzing nucleic acids using the assay system of the invention, the detection of nucleic acids uses two allele- specific oligonucleotides and a locus specific oligonucleotide. The assay methods are carried out according to the strategy outlined in Fig. 2 using next-generation sequencing or another highly parallel nucleic acid assay technology. In this assay, a set of two oligonucleotides is designed to hybridize to each target sequence, with a common oligonucleotide and two unique coding identifiers. The allele can be determined, *e,g,* by primer extension of the locus specific oligonucleotide. Following primer extension and ligation, an amplifiable template is formed with universal primer sequences at either end. Assay oligonucleotides are annealed to a template and enzymatic reactions are used to join the two oligonucleotides only when both are correctly annealed. The detection techniques and read out parameters used in this system of the invention include a much shorter tag than the oligonucleotides used in the assays that are based on capture by hybridization. These shorter tags are designed to be read out by sequencing or,

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preferably, used to ligate codes onto both ends of the fragment as illustrated in Fig. 2.

~~[00140]~~**[0122]** In ~~Figure 2~~ Figure 3, two target-specific assay oligonucleotides are ligated together ~~202~~ 302 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides containing tag sequence sets X and Y are ligated ~~204~~ 304 to the target specific oligonucleotides. Oligonucleotides containing X ligate specifically to one side of the targeting construct and oligonucleotides containing Y ligate to the other. The oligonucleotides contain universal primer sites Pl and P2. Following ligation, the constructs are eluted and, optionally, sequencing adapters can be attached ~~206~~ 306, *e.g.,* via PCR.

~~[00150]~~**[0123]** In one preferred aspect, the final construct created from the assay method is a substrate for next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of constructs. Such sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et al, U.S. patent publication 2005/0191656, and sequencing by synthesis methods, *e.g.,* Nyren et al, U.S. Pat. No. 6,210,891; Ronaghi, U.S. Pat. No. 6,828,100; Ronaghi et al (1998), Science, 281: 363-365; Balasubramanian, U.S. Pat. No. 6,833,246; Quake, U.S. Pat. No. 6,911,345; Li et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); Smith et al, PCT publication WO 2006/074351; use of reversible extension terminators, *e.g.,* Turner, U.S. Pat. No. 6,833,246 and Turner, U.S. Pat. No. 6,833,246 and ligation-based methods, *e.g.,* Shendure et al (2005), Science, 309: 1728-1739, Macevicz, U.S. Pat. No. 6,306,597; which references are incorporated by reference. Soddart et al., PNAS U S A. 2009 Apr 20; Xiao et al., Nat Methods. 2009 Mar;6(3):199-201. Epub 2009 Feb 8.

~~[00151]~~**[0124]** To maximize the efficiency of encoding, a combinatorial approach using pairs of oligonucleotides can be used. For example, with only two sets of 100 codes, a substrate can

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theoretically encode up to 10,000 locations. The number of assay oligonucleotides required is dramatically reduced, the cost decreased, and the robustness of the approach increased by decoupling the coding sequences from the genome-specific sequences. Alternative assay formats can also be used (e.g. ligation or primer extension followed by ligation).

~~[00152]~~[0125] By ligating the codes on separately, only 2n target-specific assay oligonucleotides are needed for n targets. For example, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 targeting oligonucleotides and 2 x 100 encoding oligonucleotides, using a combinatorial approach outlined in Fig. 2. The total count of assay oligonucleotides would be only 400 (200 target- specific and 200 encoding), not counting universal primers. In contrast, if the coding oligonucleotides were not decoupled, (n x X positional codes) + (n x Y positional codes) would be needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences.

~~[00153]~~[0126] Due to the matrix system of the invention, a large amount of information can be obtained even using five or more positions interrogated for five or more biological molecules. By varying one or the other of these, large amounts of information can be obtained, both in terms of locations and/or specific biological In specific aspects, multiple locations are interrogated for two or more biological molecules. As an example, for each datapoint ~1,000 reads may be sampled, for a total of -10E9 reads for 10E6 datapoints.

Peptide Detection Systems

~~[00154]~~[0127] The assay system of the invention can be used to analyze biological molecules using peptide agents that are associated with the substrate surface in a spatial pattern. Such peptides may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, etc.

~~[00155]~~[0128] The assay system of the invention allows the identification and spatial location of various forms of peptides, including isoforms and peptides that have undergone posttranslational

modification. Importantly, certain aspects of the invention allow the identification of active versus non-active forms of such peptides in a sample. This allows the identification of the presence or absence of specific peptide isoforms, and also acts as a proxy for identification of peptide activity in a sample.

~~[00156]~~**[0129]** In certain aspects of the invention, the binding agents associated with the substrate surfaces of the assay system include substrates for enzymes or proenzymes, *e.g.,* a kinase, a phosphatase, a zymogen, a protease, or a fragment thereof. In certain aspects, the binding agents associated with the substrate surfaces are phosphorylation substrates used to detect proteins involved with one or more signal transduction pathways, *e.g.,* a kinase or a phosphatase. In another specific aspect of the invention, the binding agents are specific protease substrates that associate only with individual or classes of proteases. In other aspects, the binding agents on the substrate surface are different processed forms, isoforms and/or domains of an enzyme.

Reagent Delivery

~~[00157]~~**[0130]** The reagent delivery system of the invention can be any system that allows the delivery of reagents to discrete portions of the array in order to keep the integrity of the defined spatial patterns of the encoding scheme. Such discrete delivery can be achieved in a number of different ways.

~~[00158]~~**[0131]** In one exemplary aspect, the reagent delivery system can be a flow-based system. The flow-based systems for reagent delivery in the present invention can include one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Such a reagent delivery system is configured to move fluid in contact with a discrete section of the substrate surface. Movement of the reagents can be driven through a fluid by a pump disposed, for example, downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, the reagents may be driven through the fluid by gravity.

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~~[00159]~~[0132] US Appln Nos. 20070166725 and 20050239192 disclose certain general- purpose fluidics tools that can be used with the assay systems of the invention. These allow the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

~~[00160]~~[0133] In a more specific example, one or more flow-cells can be attached to the substrate from above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump can be used to deliver the sample or reagents to the flow-cell and across the substrate. The flow cell is configured to deliver reagents only to certain portions of the array, restricting the amount and type of reagent delivered to any specific section of the array.

~~[00161]~~[0134] In another aspect, a microfluidic system can be integrated into the substrate or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid can be formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected according to selective opening and closing of valves disposed between reagent reservoirs.

~~[00162]~~[0135] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes (i.e., microfluidic structures). The pump can operate mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among others. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. The mechanical pumps may be micromachined, molded, etc. Exemplary electrical pumps can include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

~~[00163]~~[0136] Valves generally include any mechanism for regulating the passage of fluid

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through a channel. The valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into the channel to partially or completely block the channel, an electrocapillary structure, and/or the like.

~~[00164]~~**[0137]** In yet another aspect, an open gasket can be attached to the top of the substrate and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber formed by a gasket sandwiched between the substrate and a chemically inert, water resistant material such as, but not limited to, black-anodized aluminum, thermoplastics (e.g., polystyrene, polycarbonate, etc), glass, etc.

~~[00165]~~**[0138]** In a specific aspect of the present invention, the delivery system can compnse a microcircuit arrangement including an imager, such as a CCD or IGFET-based (e.g., CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Appln No. 20090197326, which is incorporated herein by reference.

~~[00166]~~**[0139]** In yet another aspect of the invention, the reagent delivery system controls the delivery of reagents to specific patterns on a substrate surface using semiconductor techniques such as masking and spraying. Specific areas of a substrate surface can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the substrate using conventional techniques such as spraying or fluid flow. The use of the masked substrate delivery results in a patterned delivery scheme on the substrate surface.

~~[00167]~~**[0140]** In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms *(e.g.,* thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions can be achieved.

Software for use in the Assay System

~~[00168]~~[0141] In order to target specific sites of interest, an informative image of the biological section to be analyzed can be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions can be identified using image processing *(e.g.,* images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with the other features of the assay system. In some aspects, software is used to automatically translate this information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample in a targeting system is thus a preferred component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and other very accurate physical positioning systems can be adapted to this purpose.

~~[00169]~~[0142] Additional software components will also be key components that will be part of a complete assay system. The invention thus preferably comprises a complete suite of software tailored to the assay system. Optionally, oligonucleotide design software will be customized for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for data analysis may be integrated to assist in determination of results of the assays. This can be especially useful, as the type of dataset that will be generated will be novel, particularly as a consequence of scale. The ability to provide algorithms and software tools that are specifically designed for analysis of spatially-associated data for significant patterns, including pattern-analysis software and visualization tools, is a novel feature that will enhance the value of the data generated by the assay systems.

~~[00170]~~[0143] In certain aspects, the assay system will comprise processes for making and carrying out quality control of reagents, *e.g.,* the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents will need to be formulated for compatibility with the reagent delivery

instrumentation. Factors such as volatility, stability at key temperatures, and chemical compatibility can be optimized by those skilled in the art upon reading the present disclosure.

Applications of Assay System

~~[00171]~~**[0144]** It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous very important areas of biological research, diagnostics, and drug development that will benefit from a high throughput means to simultaneously measure the presence or absence and spatial location of a biological molecule in a sample. For example, this technology combining the ability to analyze semi-quantitatively the expression of many different genes with the ability to image the spatial organization of expression across many cells in a tissue is enabling for many different areas of basic research. The following are exemplary uses and are by no means meant to be limiting in scope.

~~[00172]~~**[0145]** In one example, 3-dimensional patterns of expression can be determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning. This can be used to measure changes in gene expression in disease pathology, *e.g.,* in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues can be obtained. This may lead to new insights into the function and regulation both in normal and diseased states, and is likely to provide new hypotheses that can be tested. For example, a system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney et al., 2007) and modENCODE to be integrated at the tissue level. The assay systems will also aid in computational efforts to model interacting networks of gene expression in the field of systems biology.

~~[00173]~~**[0146]** The assay systems also provide a novel approach that enables the analysis of somatic variation, *e.g.,* somatic mutations in cancer or variability in response to infectious organisms. For

example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer related processes in both cancer and genetically normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers.

~~[00174]~~**[0147]** In another example, different people have varying susceptibility to infectious organisms, and much of this may be to underlying genetic differences in individuals and/or populations. Identifying these differences will aid in an understanding of the underlying disease pathologies and assist in the development of vaccines or therapeutics to prevent or ameliorate these disease states.

~~[00175]~~**[0148]** Importantly, in addition to providing spatially associated information, the technology of the invention will allow a great increase in the sensitivity of detecting rare mutations. The reason is that signal to noise can be dramatically increased because the approach of the invention assays a small location in any given reaction. In a typical assay for rare mutations in a mixed sample, the sample is treated in bulk, i.e. nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in 1 cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, the background can be reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed. This may be particularly important in detecting key mutations in tissue sections in cancer. Already, molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics (Choe et al., 2003). The technology of the invention promises to greatly increase the power of such approaches.

EXAMPLES

~~[00176]~~[0149] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventor regards as his invention, nor are they intended to represent or imply that the experiments below are all of or the only experiments performed. It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

~~[00177]~~[0150] Efforts have been made to ensure accuracy with respect to numbers used *(e.g.,* amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees centigrade, and pressure is at or near atmospheric.

EXAMPLE 1: Initial Proof of Concept of Encoding Scheme

~~[00178]~~[0151] As an initial proof of concept, a model system is developed using a microarray to demonstrate a working single-plex assay. The basic design validates the concept of the assay, and establishes a working assay prior to addressing issues related to the analysis of a more complicated biological sample. Conventional sequencing is used as a readout for this proof of concept.

~~[00179]~~[0152] A microarray is used as a proxy for a tissue section. The target sequences of the microarray are fully specified, so that the composition of the targets are known and can be varied systematically. Synthetic oligonucleotide templates are attached to a glass slide via a 5' amino modification. Each slide has a single oligonucleotide template sequence, and the assays that are carried out may employ either ligation, or extension followed by ligation as this may be useful in determining certain polymorphisms.

~~[00180]~~[0153] Once the *in situ* part of the assay is complete, the reaction products are eluted and analyzed by qPCR to determined presence or absence of a product and estimate yield, and by conventional sequencing to determine the structure of the assay products. The single plex assays that are tested include appropriate positive and negative controls, and a single nucleotide variant (SNV) to check ability to discriminate single base variants.

EXAMPLE 2: Scalability

~~[00181]~~[0154] The complexity of the assay system is increased to establish scalability of the assay for use in high throughput studies. Scalability of both the spatial encoding and assay systems is demonstrated by carrying out a 24-plex x 24-site assay using a microarray model system.

~~[00182]~~[0155] The amount of biological target, here a DNA target sequence, at each assay location is systematically varied on microarray substrate. For example, in a microarray with 50 micron spot size (center to center), a 1 mm^2 area contains ~400 spots. The region around each site is optionally occupied by a region that is devoid of these spots to allow individual resolvability of the target sequences. Alternatively, the spots may be clustered, with two or more directly adjacent spots surrounded by or adjacent to a region that is devoid of target sequences.

~~[00183]~~[0156] In order to demonstrate that spatial encoding is accurate, the sites comprise different target compositions to show that the assay readout matches the expected composition of each site. With 24 target sequences, a simple digital pattern is made with each site having a different set of 12 targets present and 12 targets absent, to make a binary code (0= absent, 1=present). The assay readout is then determined to show that the detected regions match the expected signal after spatial decoding. In this particular example, the code space is large enough (2^{24}) so that even a few errors would not result in different codes being mixed up. Moreover, this design allows identification of errors and allows an estimation not only of accuracy of spatial encoding but also of accuracy calling the presence or absence of target sequences.

In an exemplary aspect, a 4 x 4 arrangement of 16 sequences is used for the array configuration. A white square indicates that the sequence is absent and a black square that it is present, i.e. 8 of the 16 possible sequences are present in this sample. In a different sample, a different pattern of absent and present sequences can be constructed. In this way, unique patterns are associated with spatial locations so that the accuracy of spatial encoding can be measured.

~~[00184]~~[0157] The ability to detect quantitative differences is evaluated by generating dose-response curves for each of the 24 assays that are carried out at each site in a 24-site assay. This allows estimation of the limit of detection, dynamic range, and power to detect a given fold-change across the range.

~~[00185]~~[0158] In one aspect, a latin square design is used to represent individual targets at different ratios by varying the number of features for each target. In other words, with multiple spots in a site, the number of spots allocated to each of the 24 target sequences can be varied and each of the 24 sites can have a different composition. A 1 x 3 inch microarray is sufficiently large to permit multiple replicates. This larger set of 24 sequences will require deconvolution, and this is accomplished using high throughput techniques such as next-generation sequencing technologies *(e.g.,* SOLiD™ technology (Life Technologies, Inc., Carlsbad, CA) or Genome Analyzer (Illumina,

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Inc., San Diego, CA)). The use of the 24-plex assay demonstrates both the accuracy of spatial encoding and decoding, and the quantitative response of the assay system.

EXAMPLE 3: Adaptation of the assay to preserved samples.

~~[00186]~~[0159] Genomic DNA is assayed as a proof of concept for assaying RNA, as it provides a way to establish a single-copy reference signal. Once a working assay is developed for FFPE samples, it is adapted to an RNA assay. To this end, assay oligonucleotide concentrations are assayed to ensure compatibility with high multiplexing. Assuming a cell diameter of 10 microns, and delivery of a 10 micron diameter reagent droplet to an individual cell, the volume of the droplet will be ~500 µl and can contain ~3 x 10^{11} molecules at a l µM concentration. To assay 1,000 target sequences in 10,000 cells, ~2,000 targeting oligonucleotides would be required in a droplet. Therefore, each droplet could contain ~160 million copies of each assay oligo, a vast excess over the few thousand target sequences in a cell.

~~[00187]~~[0160] The handling of small absolute numbers of product molecules generated from very small or compromised samples are enhanced to counter the issue of low recovery efficiency; that is, elution is efficient and losses resulting from adsorption of molecules to surfaces are prevented. An approach to addressing the latter issue is to include a carrier material, such as glycogen or carrier nucleic acids.

Example 4: Adapting the Assay to a Biological Sample.

~~[00188]~~[0161] A control RNA template is immobilized to a solid support in order to create an artificial system. The assay is performed using T4 DNA ligase, which can repair nicks in DNA/RNA hybrids. Assays are carried out on matched slides, or different sections of the same slide, where in one case gDNA is assayed and in the other RNA is assayed. When assaying gDNA the slide can be pretreated with RNase, and when assaying RNA the slide is pretreated with DNase. Results of the assay are confirmed by extracting gDNA or RNA and quantitating the relative amounts by qPCR or RT-qPCR respectively.

~~[00189]~~[0162] In order make the tissue section RNA assays as informative as possible, pre-existing information on expression levels in specific tissues to target transcripts across a range of abundances are used in the assay design. Both high abundance transcripts, as well as some medium and low abundance transcripts, are targeted to enable an initial assessment of the quantitative performance characteristics of the assay.

~~[00190]~~[0163] The preceding merely illustrates the principles of the invention. It will be

appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims. In the claims that follow, unless the term "means" is used, none of the features or elements recited therein should be construed as means-plus-function limitations pursuant to 35 U.S.C. §112, ¶6.

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SPATIALLY ENCODED BIOLOGICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Patent Application No. 61/321,124, filed April 5, 2010 and is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] This invention relates to assays of biological molecules, and more particularly to assays for determining spatial distributions of a large number of biological molecules in a solid sample simultaneously.

BACKGROUND OF THE INVENTION

[0003] In the following discussion certain articles and methods will be described for background and introductory purposes. Nothing contained herein is to be construed as an "admission" of prior art. Applicant expressly reserves the right to demonstrate, where appropriate, that the articles and methods referenced herein do not constitute prior art under the applicable statutory provisions.

[0004] Comprehensive gene expression analysis and protein analysis have been useful tools in understanding mechanisms of biology. Use of these tools has allowed the identification of genes and proteins involved in development and in various diseases such as cancer and autoimmune disease. Conventional methods such as *in situ* hybridization and other multiplexed detection of different transcripts have revealed spatial patterns of gene expression and have helped shed light on the molecular basis of development and disease. Other technologies that have enabled the quantitative analysis of many RNA sequences per sample include microarrays (see Shi, et al., Nature Biotechnology, 24(9):1151-61 (2006); and Slonim and Yanai, Plos Computational Biology, 5(10):e1000543 (2009)); serial analysis of gene expression (SAGE) (see Velculescu, et al, Science, 270(5235):484-87 (1995)), high-throughput implementations of qPCR (see Spurgeon, et al., Plos ONE, 3(2):e1662 (2008)) and *in situ* PCR (see Nuovo, Genome Res., 4:151-67 (1995)). As useful as these methods are, however, they do not enable simultaneous measurement of the expression of many genes or the presence and/or activity of multiple proteins at many spatial locations in a sample. Laser capture microdissection has permitted the analysis of many genes at a small number of locations, but it is very expensive, laborious, and does not scale well. Certain PCR assays in a 2D format preserve spatial

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information (see Armani, et al., Lab on a Chip, 9(24): 3526-34 (2009)), but these methods have low spatial resolution because they rely on physical transference of tissue into wells, which also prevents random access to tissue samples and high levels of multiplexing.

[0005] At present, no practical method exists to analyze at high resolution the spatial expression patterns of large numbers of genes, proteins, or other biologically active molecules simultaneously. There is thus a need for reproducible, high-resolution spatial maps of biological molecules in tissues. The present invention addresses this need.

SUMMARY OF THE INVENTION

[0006] This Summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This Summary is not intended to identify key or essential features of the claimed subject matter, nor is it intended to be used to limit the scope of the claimed subject matter. Other features, details, utilities, and advantages of the claimed subject matter will be apparent from the following written Detailed Description including those aspects illustrated in the accompanying drawings and defined in the appended claims.

[0007] The invention encompasses assay systems that provide high-resolution spatial maps of biological activity in tissues. The assay system comprises an assay capable of high levels of multiplexing where encoded probes are provided to a biological sample in defined spatial patterns; instrumentation capable of controlled delivery of reagents according to the spatial patterns; and a decoding scheme providing a readout that is digital in nature. In short, the present invention provides the ability to look at many biological targets in many locations, providing the resolution of in situ hybridization with the highly- parallel data analysis of sequencing.

[0008] Thus, in some embodiments, the invention provides an assay system to determine spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered; allowing the encoded probes to interact with the biological targets; separating encoded probes that interact with the biological targets from encoded probes that do not interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[0009] In particular aspects of the invention the biological targets comprise nucleic acids and the

encoded probes are oligonucleotides, and in some aspects, there are two encoded probes for each of the multiple nucleic acid targets. In some aspects, the multiple biological targets comprise proteins, the probe regions of the encoding probes are proteins and the coding tags comprise oligonucleotides. In some aspects the multiple biological targets comprise enzymes. In some aspects the probe regions of the encoded probes comprise antibodies, aptamers or small molecules.

[0010] Some aspects of the assay system further comprise an amplification step between the separating step and the determining step. In some aspects, the determining step is performed by nucleic acid sequencing, and in preferred aspects, the sequencing is high- throughput digital nucleic acid sequencing.

[0011] In some aspects of the invention, the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 20, in some aspects product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 50, in some aspects the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 75, 100, 150, 500, 750, 1,000, 5,000, 10,000, 25,000, 50,000, 100,000, 500,000, or 1,000,000 or more. In other aspects, the sequence of at least fifty thousand encoding probes are determined in parallel, in other aspects the sequence of at least one hundred thousand encoding probes are determined in parallel, in some aspects the sequence of at least five hundred thousand encoding probes are determined in parallel, and in some aspects the sequence of at least one million, ten million, one hundred million, one billion, ten billion, one hundred billion or more encoding probes are determined in parallel.

[0012] In some aspects, the known spatial pattern is determined by histological features of the sample. Also in some aspects, software programmed hardware performs at least two steps of the delivering step, the separation step, the determining step and the associating step.

[0013] In some aspects, the probe regions of the encoded probes are proteins and the separating step is accomplished by encoded probes that interact with the biological targets being captured by an affinity capture agent. In some aspects the probe regions of the encoding probes are nucleic acids and the separating step is accomplished by a washing of the sample.

[0014] In other embodiments there is provided an assay system to determine spatial patterns of abundance or activity or both of multiple nucleic acid targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering oligonucleotide probes for multiple nucleic acid targets to the multiple sites in the sample in a known spatial pattern; allowing the oligonucleotide probes to hybridize with the nucleic acid targets; washing unhybridized encoded oligonucleotide probes from the sample; delivering one or more encoding agents to locations of the multiple sites in the sample according to a known spatial pattern, where the

combination of encoding agents delivered to each site is different; coupling the encoding agents and the oligonucleotide probes to form encoded probes; determining all or a portion of a sequence of the encoded probes using high-throughput sequencing, and associating the abundance or activity or both of multiple biological targets to the locations of multiple sites in the sample.

[0015] Other embodiments of the invention provide an assay system to determine spatial patterns of abundance or activity or both of multiple protein targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple protein targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a protein probe region that may interact with the protein targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and the protein probe region of the encoding probe of which the coding tag is part; allowing the encoded probes to interact with the protein targets; separating encoded probes that interact with the protein targets from encoded probes that do not interact with the protein targets; determining all or a portion of a sequence of the encoded probes by high throughput sequencing, and associating the abundance or activity or both of the multiple protein targets to the locations of the multiple sites in the sample.

[0016] Other embodiments provide an assay system to determine spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and identifies the biological target; allowing the encoded probes to interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[0017] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

DESCRIPTION OF THE FIGURES

[0018] Figure 1 provides a simplified overview of the assay system of the present invention.

[0019] Figure 2 provides a simplified overview of one embodiment of the assay system of the present invention for detecting nucleic acids.

[0020] Figure 3 is a representational depiction of one embodiment of the assay overviewed in Figure 2.

[0021] Figure 4 illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention.

[0022] Figure 5 provides a simplified, specific example of the embodiment of a combinatorial encoding scheme shown in Figure 4.

DEFINITIONS

[0023] The terms used herein are intended to have the plain and ordinary meaning as understood by those of ordinary skill in the art. The following definitions are intended to aid the reader in understanding the present invention, but are not intended to vary or otherwise limit the meaning of such terms unless specifically indicated.

[0024] The term "antibody" as used herein is intended to refer to an entire immunoglobulin or antibody or any functional fragment of an immunoglobulin molecule which is capable of specific binding to an antigen (antibodies and antigens are "binding partners" as defined herein). "Antibody" as used herein is meant to include the entire antibody as well as any antibody fragments capable of binding the antigen or antigenic fragment of interest. Examples of such peptides include complete antibody molecules, antibody fragments, such as Fab, F(ab')2, CDRS, VL, VH, and any other portion of an antibody which is capable of specifically binding to an antigen. Antibodies for assays of the invention are immunoreactive or immunospecific for, and therefore specifically and selectively bind to, proteins either detected *(i.e.,* biological targets) or used for detection *(i.e.,* probes) in the assays of the invention.

[0025] The term "binding agent" as used herein refers to any agent that specifically binds to a biological molecule of interest

[0026] "Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double-stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single-stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the other strand, usually at least about 90% to about 95%, and even about 98% to about 100%).

[0027] "Hybridization" refers to the process in which two single-stranded polynucleotides bind non-covalently to form a stable double-stranded polynucleotide, The resulting (usually) double-stranded polynucleotide is a "hybrid" or "duplex," "Hybridization conditions" will typically include salt concentrations of approximately less than 1M, often less than about 500 mM and may be less than about

200 mM. A "hybridization buffer" is a buffered salt solution such as 5% SSPE, or other such buffers known in the art. Hybridization temperatures can be as low as 5°C, but are typically greater than 22°C, and more typically greater than about 30°C, and typically in excess of 37°C. Hybridizations are often performed under stringent conditions, *i.e.,* conditions under which a primer will hybridize to its target subsequence but will not hybridize to the other, non-complementary sequences. Stringent conditions are sequence-dependent and are different in different circumstances. For example, longer fragments may require higher hybridization temperatures for specific hybridization than short fragments. As other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one parameter alone. Generally stringent conditions are selected to be about 5°C lower than the T_m for the specific sequence at a defined ionic strength and pH. Exemplary stringent conditions include a salt concentration of at least 0.01 M to no more than 1M sodium ion concentration (or other salt) at a pH of about 7.0 to about 8.3 and a temperature of at least 25°C. For example, conditions of 5xSSPE (750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA at pH 7.4) and a temperature of approximately 30°C are suitable for allele-specific hybridizations, though a suitable temperature depends on the length and/or GC content of the region hybridized.

[0028] "Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, *e.g.,* oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon terminal nucleotide of one oligonucleotide with a 3' carbon of another nucleotide.

[0029] "Nucleic acid", "oligonucleotide", "oligo" or grammatical equivalents used herein refers generally to at least two nucleotides covalently linked together. A nucleic acid generally will contain phosphodiester bonds, although in some cases nucleic acid analogs may be included that have alternative backbones such as phosphoramidite, phosphorodithioate, or methylphophoroamidite linkages; or peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids, positive backbones, non-ionic backbones and non-ribose backbones. Modifications of the ribose-phosphate backbone may be done to increase the stability of the molecules; for example, PNA:DNA hybrids can exhibit higher stability in some environments.

[0030] "Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed.

The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Primers usually are extended by a DNA polymerase.

[0031] The term "SNP" or "single nucleotide polymorphism" refers to a genetic variation between individuals; *e.g.,* a single nitrogenous base position in the DNA of organisms that is variable. SNPs are found across the genome; much of the genetic variation between individuals is due to variation at SNP loci, and often this genetic variation results in phenotypic variation between individuals. SNPs for use in the present invention and their respective alleles may be derived from any number of sources, such as public databases (U.C. Santa Cruz Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway) or the NCBI dbSNP website (http://www.ncbi.nlm.nih.gov/SNP/), or may be experimentally determined as described in U.S. Pat No. 6,969,589; and US Pub. No. 2006/0188875 entitled "Human Genomic Polymorphisms." Although the use of SNPs is described in some of the embodiments presented herein, it will be understood that other biallelic or multi-allelic genetic markers may also be used. A biallelic genetic marker is one that has two polymorphic forms, or alleles. As mentioned above, for a biallelic genetic marker that is associated with a trait, the allele that is more abundant in the genetic composition of a case group as compared to a control group is termed the "associated allele," and the other allele may be referred to as the "unassociated allele." Thus, for each biallelic polymorphism that is associated with a given trait *(e.g.,* a disease or drug response), there is a corresponding associated allele. Other biallelic polymorphisms that may be used with the methods presented herein include, but are not limited to multinucleotide changes, insertions, deletions, and translocations. It will be further appreciated that references to DNA herein may include genomic DNA, mitochondrial DNA, episomal DNA, and/or derivatives of DNA such as amplicons, RNA transcripts, cDNA, DNA analogs, etc. The polymorphic loci that are screened in an association study may be in a diploid or a haploid state and, ideally, would be from sites across the genome.

[0032] The term "selectively binds", "selective binding" and the like as used herein, when referring to a binding partner *(e.g.*. protein, nucleic acid, antibody or other affinity capture agent, etc.), refers to a binding reaction of two or more binding partners with high affinity and/or complementarity to ensure selective hybridization under designated assay conditions. Typically, specific binding will be at least three times the standard deviation of the background signal. Thus, under designated conditions the binding partner binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

[0033] "Sequencing", "sequence determination" and the like means determination of information relating to the nucleotide base sequence of a nucleic acid. Such information may include the identification or determination of partial as well as full sequence information of the nucleic acid.

Sequence information may be determined "with varying degrees of statistical reliability or confidence. In one aspect, the term includes the determination of the identity and ordering of a plurality of contiguous nucleotides in a nucleic acid, "High throughput digital sequencing" or "next generation sequencing" means sequence determination using methods that determine many (typically thousands to billions) of nucleic acid sequences in an intrinsically parallel manner, *i.e.* where DNA templates are prepared for sequencing not one at a time, but in a bulk process, and where many sequences are read out preferably in parallel, or alternatively using an ultra-high throughput serial process that itself may be parallelized. Such methods include but are not limited to pyrosequencing (for example, as commercialized by 454 Life Sciences, Inc., Branford, CT); sequencing by ligation (for example, as commercialized in the SOLiD™ technology, Life Technology, Inc., Carlsbad, CA); sequencing by synthesis using modified nucleotides (such as commercialized in TruSeq™ and HiSeq™ technology by Illumina, Inc., San Diego, CA, HeliScope™ by Helicos Biosciences Corporation, Cambridge, MA, and PacBio RS by Pacific Biosciences of California, Inc., Menlo Park, CA), sequencing by ion detection technologies (Ion Torrent, Inc., South San Francisco, CA); sequencing of DNA nanoballs (Complete Genomics, Inc., Mountain View, CA); nanopore-based sequencing technologies (for example, as developed by Oxford Nanopore Technologies, LTD, Oxford, UK), and like highly parallelized sequencing methods.

[0034] The term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation, T_m=81.5+0.41 (% G+C), when a nucleic acid is in aqueous solution at 1M NaCl (see *e.g.,* Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985)). Other references *(e.g.,* Allawi and SantaLucia, Jr., Biochemistry, 36:10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

DETAILED DESCRIPTION OF THE INVENTION

[0035] The practice of the techniques described herein may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and sequencing technology, which are within the skill of those who practice in the art. Such conventional techniques include polymer array synthesis, hybridization and ligation of polynucleotides, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the examples

herein. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Green, et al., Eds., *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (1999); Weiner, Gabriel, Stephens, Eds., *Genetic Variation: A Laboratory Manual* (2007); Dieffenbach, Dveksler, Eds., *PCR Primer: A Laboratory Manual* (2003); Bowtell and Sambrook, *DNA Microarrays: A Molecular Cloning Manual* (2003); Mount, *Bioinformatics: Sequence and Genome Analysis* (2004); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); Stryer, *Biochemistry* (4th Ed.) (1995) W.H, Freeman, New York N.Y.; Gait, *"Oligonucleotide Synthesis: A Practical Approach"* (2002) IRL Press, London; Nelson and Cox, *Lehninger, Principles of Biochemistry* (2000) 3rd Ed., W. H. Freeman Pub., New York, N.Y,; and Berg, et al., *Biochemistry* (2002) 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

[0036] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" refers to one or more nucleic acids, and reference to "the assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0037] 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 this invention belongs. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing devices, formulations and methodologies that may be used in connection with the presently described invention.

[0038] Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention,

[0039] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been

described in order to avoid obscuring the invention.

The Invention in General

[0040] The assay systems of the invention provide spatially-encoded, multiplexed assays comprising 1) an assay capable of high levels of multiplexing with an efficient spatial encoding scheme; 2) instrumentation capable of delivering reagents according to a spatial pattern; and 3) decoding determined by a readout that is digital in nature. The assay systems of the invention detect the presence or absence and relative amount of a biological target or biological activity indicative of a biological target, as well as the location of the biological target or activity in a biological sample, *e.g.*, a tissue section or other biological structure disposed upon a support such as a microscope slide or culture dish.

[0041] The assay system further provides instrumentation with an ability to deliver reagents in a spatially-defined pattern. This instrumentation, together "with software, reagents and protocols, provides a key component of the highly innovative assay system of the invention, allowing for measurement of numerous biological targets or activities in a meaningful spatial environment, including gene expression and peptide localization, An encoding scheme used in these assay systems allows one to determine the location of biological targets or activity (or lack thereof) in the biological samples after the products of the multiplexed assay are removed from the biological sample and pooled for analysis. Decoding of the encoding scheme can be performed by, *e.g.*, next-generation sequencing, which easily provides millions to trillions of data points at low cost. The assay results such as the amount or activity of biological targets can then be mapped back to specific location in the biological sample. The assay systems open a new analytical window into the complex spatial patterns of cellular function and regulation in biological samples.

[0042] A simplified overview of the assay system 100 of the present invention is provided at Figure 1, At step 110, a biological sample affixed to a support is provided. The biological sample contains biological targets of interest. Biological targets can include any molecule of interest, such as nucleic acids (including, *e.g.* RNA transcripts, genomic DNA sequences, cDNAs, amplicons, or other nucleic acid sequences) and proteins, enzymes and the like. At step 120, encoded probes are delivered to the biological sample according to a known spatial pattern. Encoded probes comprise probes, which can interact "with biological targets of interest, and coding tags, which identify the positions in the sample of the biological targets being assayed, and thus can be used to link assay results back to locations in the sample. Coding tags in most embodiments are oligonucleotides. However, coding tags may also be mass tags, fluorescent labels, or other moieties.

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[0043] In some embodiments, the probe and coding tag portions of the encoded probe are pre-coupled before being delivered to the biological sample, For example, in the case where the encoded probes are oligonucleotides, both the probe and coding tag sequence can be synthesized as a single oligonucleotide. Alternatively, the probe and coding tag portions of the encoding probes can be synthesized or obtained separately and combined before delivery to the biological sample *(e.g.,* two separate oligonucleotides can be synthesized and coupled by, *e.g.,* ligation; or an antibody and an oligonucleotide can be prepared separately and conjugated before delivery to the biological sample). Also, as is described in Figures 2-5, the probes and the coding tags (in encoding oligonucleotides) are synthesized separately, and are delivered to the biological sample at different steps (*e.g.,* probes first and coding tags thereafter, or vice versa) in the assay.

[0044] At step 130, the encoded probes are allowed to react or interact with the biological targets, *i.e.,* conditions are provided to allow *e.g.,* oligonucleotides to hybridize to nucleic acid targets, enzymes to catalyze reactions with protein targets, antibodies to bind epitopes, etc. In the case where the biological targets are nucleic acids, the encoded probes are typically oligonucleotides and hybridize to the target nucleic acids. In the case that the biological targets are proteins, the encoded probes typically are aptamers, small molecules, or oligonucleotide-conjugated proteins that interact with target proteins by binding to them or by reacting with them (that is, one of the proteins is a substrate for the other). Encoding oligonucleotides may be coupled to the probes (proteins) by conjugation, chemical or photo-crosslinking via suitable groups and the like.

[0045] Once encoded probes interact with the biological targets, the encoded probes that interacted with the biological targets must be separated from the encoded probes that did not interact with the biological targets at step 140. In the case where the biological targets are nucleic acids and the encoded probes are oligonucleotides, the separation can be accomplished by, *e.g.,* washing the unhybridized encoded probes from the sample. Similarly, for other assays that are based on affinity binding, including those using aptamer, small molecule, and protein probes, washing steps can be used to remove low affinity binders. In the case where the probe is transformed via interaction with the target, *e.g.,* in the case of a peptide, *e.g.,* via cleavage by a protease or phosphorylation by a kinase, it is convenient to collect, all encoded probes--both encoded probes that interacted with the biological targets and were transformed and encoded probes that were not transformed. After collection or pooling, an antibody or other affinity capture agent can be used to capture probes that were transformed by addition of a moiety (*e.g.,* a phosphate group). In cases where probes have been transformed via cleavage, the transformed probes can be separated, *e.g.,* by capturing the non-transformed probes via a tag that is removed from the transformed probes during the transformation *(e.g.,* by cleavage), or by adding a new tag at the site of cleavage.

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[0046] Once the reacted (transformed) or interacted encoded probes are separated from the unreacted or un-interacted encoded probes, the sequence of the reacted and/or interacted encoded probes is determined by, preferably, sequencing. The sequence of the encoded probes allows the mapping of the assay results back to locations in the biological sample.

[0047] Figure 2 provides a simplified overview of an assay system of the present invention embodying an efficient implementation of a combinatorial coding scheme for the encoding of spatial information. For purposes of this overview, the probes are oligonucleotides, but as explained elsewhere, other types of probes can also be used. In step 210, a biological sample affixed to a support, *e.g.,* a tissue sample or other biological structure, is provided. In step 220, one or more oligonucleotide probes are delivered to the biological sample, where the oligonucleotide probes are capable of hybridizing with biological targets in the biological sample. In step 230, the oligonucleotide probes are allowed to interact with (hybridize to) the nucleic acid targets; that is, appropriate conditions are provided where oligonucleotide probes can hybridize to the target nucleic acids.

[0048] In step 240, the oligonucleotide probes that did not hybridize to target nucleic acids are removed, and thereby separated from oligonucleotide probes that did hybridize to target nucleic acids. In this embodiment, separation can be accomplished by, *e.g.,* washing the sample to remove unhybridized oligonucleotide probes. Next, in step 250, encoding oligonucleotides (the encoding agents) are delivered to the biological sample according to a chosen spatial pattern, where the encoding oligonucleotides comprise coding tags that are used to encode the location of biological targets in the biological sample. Note that in contrast to the assay system of Figure 1, here the probes and encoding agents (encoding oligonucleotides) are delivered in separate steps. In step 260, the encoding oligonucleotides are coupled to the oligonucleotide probes to create encoded probes. In this case where the probes are oligonucleotides, the encoding oligonucleotides may be coupled to the oligonucleotides probes by, *e.g.,* ligation. Alternatively, the information in the encoding oligonucleotides can be transferred by using a DNA polymerase to extend a probe oligonucleotide that acts as a primer, and thereby copy and incorporate the sequence of the encoding oligonucleotides.

[0049] In step 270, the sequence of the coding tags in the encoded probes as well as the sequence or a portion of the sequence of the probe itself is determined, and in step 280, the target nucleic acids are mapped back to the biological sample. In some embodiments, the abundance of sequences reveals the relative quantity of biological targets at the location. Although this embodiment shows the individual steps in a particular order, so as to better explain the invention,

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the precise order of the steps can be varied. For example, steps 220 and 250 can be combined, so that a mixture of the probes and encoding oligonucleotides is delivered according to a chosen spatial pattern. Coupling step 260 can then be carried out immediately after the combined steps 220 and 250, or concomitantly with them. In this case, step 240 would then occur after step 260. It can therefore be appreciated that the two key results of this series of steps, *i.e.,* the location-specific encoding of probe molecules and the separation of probe molecules based on their ability to interact with corresponding target molecules, can be accomplished with some flexibility in the implementation of the particular steps. Similarly, there is considerable flexibility in the design of the coding scheme. As described *infra,* the assays of the invention are particularly amenable to combinatorial methods.

[0050] Thus, the present invention provides an ability to look at many different biological targets in many locations, providing the resolution of in situ hybridization with the highly-parallel data analysis of sequencing. In some embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 20, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 50, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 100, greater than 500, 1,000, 10,000, 25,000, 100,000, 500,000, 1,000,000. It will be appreciated that, due to the spatial encoding dimension of the invention, even much larger numbers can be contemplated. For example, assaying l 0,000 targets per location x 10,000 locations would generate $1O^{8}$ different assays, and even larger numbers than these can easily be contemplated, particularly if spatial locations with resolution on the order of that of single cells are utilized. Further, in embodiments where high-throughput digital sequencing is employed, the sequences of at least 1,000 encoding probes are typically determined in parallel. More typically, using a digital readout, it is desirable to obtain multiple sequence reads for each assay (defined by a probe and a spatial location code). It is desirable to obtain an average of at least 3 copies per assay, and more typically at least 10 or at least 30 copies per assay, depending on the design of the experiment and requirements of the assay. For a quantitative readout with suitable dynamic range, it may be desirable to obtain at least 1,000 reads per assay. Therefore, if 1,000,000 assays are carried out, the number of sequence reads may be 1 billion or more. With high-throughput digital sequencing, and allowing for redundancy, the sequence of at least 10,000 encoding probes are determined in parallel, or the sequence of at least 100,000, 500,000, 1,000,000, 10,000,000, 100,000,000, 1,000,000,000 or more encoding probes are determined in parallel.

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Assays

[0051] The assay portion of the assay systems of the present invention comprise the following general steps: delivering probes and encoding agents where the encoding agents (in some embodiments pre-coupled to the probes) are delivered to the sample according to a known spatial pattern, allowing the probes to interact or react with biological targets in the sample, and, if the probes and encoding agents have not been pre- coupled, coupling the encoding agents to probes.

[0052] The samples of the present invention include virtually any biological sample or samples that can be affixed to a support or provided essentially in a two-dimensional manner, where the ability to tie an assayed biological target or activity back to the location within the biological sample is important. Exemplary biological samples include tissue sections (e.g., including whole animal sectioning and tissue biopsies), cell populations on slides or culture dishes, and the like. The assay systems of the invention are particularly advantageous in that they are compatible with numerous biological sample types, including fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the biological samples are immobilized on a substrate surface having discrete, independently measurable areas.

[0053] The biological targets to be detected can be any biological molecules including but not limited to proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Examples of subcellular targets include organelles, e.g., mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplasts, endocytic vesicles, exocytic vesicles, vacuoles, lysosomes, etc.

[0054] In some particular embodiments, the assay system is used to analyze nucleic acids, *e.g.,* by genotyping, quantitation of DNA copy number or RNA transcripts, localization of particular transcripts within samples, and the like. Figure 3 illustrates an overall scheme for an exemplary assay for, *e.g.,* detecting single nucleotide polymorphisms (SNPs) that can be used with the assay system of the invention. In Figure 3, two oligonucleotide probes are provided. Each oligonucleotide probe comprises a target-specific region (located on either side of the SNP to be analyzed) seen at 305 and 307, and ligation regions, seen at 301 and 303, The oligonucleotide probes are allowed to hybridize to a target nucleic acid (not shown) in the biological sample. At step 302, one of the oligonucleotide probes is extended to incorporate the SNP sequence and ligated to the other probe to form an extended probe comprising target nucleic acid region 309 and ligation regions 301 and 303.

[0055] Two encoding agents, both comprising a coding tag (seen at 315 and 317), a ligation region

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(seen at 311 and 313), and a primer region (seen at 319 and 321) are combined with and ligated to the extended probe at step 304 to form an encoded target- specific oligonucleotide. Again, in contrast with Figure 1, the probes and encoding agents are delivered at separate steps. Doing so allows use of the combinatorial embodiments described *infra.* In preferred embodiments, the encoding oligonucleotides within a pair of encoding oligonucleotides ligate specifically to one side of the target sequence or the other *(i.e., 5'* or 3' of the target sequence). Also, typically, the ligation and primer regions of the encoding oligonucleotides and probes are universal; that is, the set of ligation and primer regions used in constructing the probes and encoding oligonucleotides are constant, and only the target-specific regions of the probes and the coding tags of the encoding oligonucleotides differ. However, again in alternative embodiments, the ligation and primer regions are not universal and differ between probes and encoding agents.

[0056] Following ligation, the encoded probes are eluted, pooled, and, optionally, sequencing adapters are added to the encoded probes via PCR. In alternative embodiments, sequencing primers may be ligated to the encoding oligonucleotides, or sequencing primer sequences can be included as part of the encoding oligonucleotide. As seen in Figure 3, each sequencing adapter comprises primer region 319 or 321, compatible with the primer regions 319 and 321 on the encoded probes. The final construct comprising first adapter 327, first primer region 319, first coding tag 315, ligation regions 311 and 301, target region 309, ligation regions 313 and 303, second coding tag 317, second primer region 325 and second adapter 329 is now ready for input into a digital high-throughput sequencing process.

[0057] A combination of extension and ligation reactions are exemplified in Figure 3, but it should be appreciated that a variety of reactions may be used to couple the encoding oligonucleotides to the target-specific oligonucleotides, including ligation only (e.g., for oligonucleotides that hybridize to contiguous portions of the target nucleic acid sequence). Alternatively, an assay utilizing an additional oligonucleotide, such as in the GOLDENGATE® assay (see Fan, et al., Cold Spring Symp. Quant. Biol., 68:69-78 (2003); (Ilumina, Inc., San Diego, CA)), may be employed.

[0058] In other embodiments, the assay system of the invention also can be used to analyze peptides or proteins, the presence of antibodies, enzymatic and other protein activities, posttranslational modifications, active and non-active forms of peptides, as well as peptide isoforms in a biological sample. Accordingly, the probes may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, aptamers and the like.

[0059] In certain aspects, the probes are substrates for enzymes or proenzymes, *e.g.,* kinases, phosphatases, zymogens, proteases, or fragments thereof. In certain aspects, the probes are

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phosphorylation substrates used to detect proteins involved in one or more signal transduction pathways, *e.g.,* a kinase or a phosphatase. In another specific aspect of the invention, the probes are specific protease substrates that associate only with individual proteases or classes of proteases. In other aspects, the probes are different processed forms, isoforms and/or domains of an enzyme. Protein-based probes are typically conjugated or otherwise linked to oligonucleotide encoding agents. The oligonucleotide encoding agents in this case would also include a nucleotide sequence component that allows for identification of the protein probe.

[0060] In certain aspects, the present invention provides assays for evaluating differences in the amount and/or activity of biological targets between different locations in a sample and/or between samples. The method includes determining a plurality of encoded results from the biological sample and evaluating the differences in quantity of the biological targets at each location in the biological sample.

Combinatorial Embodiments

[0061] To maximize the efficiency of encoding, a combinatorial approach using pairs of coding tags in the encoding oligonucleotides can be used. By de-coupling the target-specific information and the coding tags, the number of oligonucleotides required is dramatically reduced, with a concomitant decrease in cost.

[0062] Figure 4 illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention, where nucleic acids in a representative tissue section (shown at 416) are assayed. Figure 4 at A shows two target-specific/encoding oligonucleotide constructs 420 and 422 *(e.g.,* formed between steps 302 and 304 of Figure 3) specifically bound to a target nucleic acid 402 of interest, The first encoded probe 420 comprises coding tag 408, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 404. The second encoded probe 422 comprises coding tag 406, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 410.

[0063] Figure 4 at B shows the spatial pattern that may be used for twenty different coding tags, al through a10 (coding tag 406 on encoded probe 420) and b1 through b10 (coding tag 408 encoded probe 422). Coding tag al., for example, is deposited on the biological sample in ten discrete areas or spots (shown as the first horizontal line of spots in 412). Coding tag a2 is deposited on the biological sample in ten spots on the second horizontal line in 412. Coding tag a3 is deposited on the biological sample in ten spots on the third horizontal line in 412, and so on. Whereas the "a" tags are deposited

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in ten horizontal rows, the "b" tags are deposited in ten vertical rows as shown in 414. For example, coding tag b1 is deposited on the biological sample in ten discrete spots in the first vertical row of 414, coding tag b2 is deposited on the biological sample in ten discrete spots in the second vertical row of 414, and so on. Using such a configuration allows for twenty coding tags to uniquely define 100 different locations on the biological sample,

[0064] Figure 4 at C shows a representative tissue section 416 coincident with coding tag grid 418. The arrows show how the "a" coding tags and the "b" coding tags are deposited on grid 418 that is coincident with tissue section 416. If, once sequenced, coding tags al and b4, *e.g.,* are associated with a target nucleic acid sequence, then that target nucleic acid sequence *(i.e.,* biological target) was present in the tissue section at location al, b4.

[0065] Figure 5 provides a simplified, specific example of the encoding scheme of the assay systems of the invention. Figure 5 shows encoding oligonucleotides 510, comprising al, a2, a3, a4 and b1, b3, b3 and b4. Target-specific oligonucleotides (TSOs) (probes) 1 and 2 are shown at 520. A deposit or dispensing scheme is shown at 530. Like the grid exemplified in Figure 4, encoding oligonucleotides al through a4 are deposited in spots in a pattern (here, in a vertical pattern), and encoding oligonucleotides bl through b4 are deposited in spots in a pattern (here, a horizontal pattern). The grid though shown as a square with spots is actually a deposition pattern on a biological sample (not shown) such as tissue section 416 shown in Figure 4.

[0066] The target-specific oligonucleotides are delivered to the biological sample, where the target-specific oligonucleotides hybridize to target nucleic acids in the biological sample if target nucleic acids are present. Unhybridized target-specific oligonucleotides are then removed, *e.g.,* by washing. The encoding oligonucleotides are then delivered to the biological sample according to the spatial pattern shown at 530. The encoding oligonucleotides are ligated (or, *e.g.,* extended and ligated) to any target-specific oligonucleotides that hybridized to the target nucleic acid in the biological sample, the ligated constructs are then eluted from the biological sample, pooled, and sequencing adapters are added through, *e.g.,* PCR or ligation, if the sequences were not previously included in the encoding oligonucleotides. The ligated constructs are sequenced by, *e.g.,* high throughput or "next generation" sequencing.

[0067] The pool of resulting sequences is shown at 540. A sequence readout was obtained for target-specific oligonucleotide 1 only at a4bl, a4b2, alb3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at a1b1 (position shown with vertical lines). A sequence readout was obtained for both target-specific oligonucleotides 1 and 2 at positions a2b1, a3b1, a1b2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at alb4, a2b4

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or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample, and neither target nucleic acid was detected in the upper right portion of the biological sample. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample.

[0068] In addition to location information, information relating to relative abundance of the encoded tags can be obtained. For example, if it is found that there are ten times as many a4T1b1 sequences occurring in the data set as compared to a4T1b2 sequences, this would indicate that target nucleic acid sequence 1 is ten times more abundant at the a4T1b1 location than at the a4T1b2 location.

[0069] In the case of nucleotide analysis as shown in Figure 3, by ligating the coding tags directly to target-specific oligonucleotides, only 2n target-specific oligonucleotides are needed for n targets. For example, using the combinatorial approach outlined in Figure 2, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 target-specific oligonucleotides and 2 x 100 encoding oligonucleotides. The total count of assay oligonucleotides would be only 400 (200 target-specific and 200 encoding), not counting universal primers. In contrast, if the coding oligonucleotides were not decoupled from the target-specific oligonucleotides, (n x X positional codes) + (n x Y positional codes) would be needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences. Moreover, though the embodiments shown in Figures 2- 5 depict a combinatorial scheme using two encoding agents (coding tags), three, four or more encoding agents and coding tags may be used, and attached to the probe or one another by varying means and in varying combinations of steps.

[0070] Due to the spatial encoding aspect of the assay system of the invention, a large amount of information can be generated with even a modest number of assays. For example, five or more biological targets assayed at five or more positions in the sample generates 25 or more combinations. Using digital sequencing as a readout, the optimum number of sequence reads per combination depends on the sensitivity and dynamic range required, and can be adjusted, For example, if for each combination on average 100 reads are sampled, the total for 25 combination is 25,000 reads. If 1,000 targets are assayed at 1,000 locations with an average sampling depth of 1,000, then 10^9 reads are required. These numbers, although large, are within the capacity of intrinsically parallel digital sequencing methods, which can generate datasets of billions or even trillions of reads in a reasonable timeframe and at a very low cost per read. Therefore, by varying the numbers of positions interrogated or biological targets assayed, or both, and using digital sequencing, large

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amounts of information can be obtained. In specific aspects, multiple locations are interrogated for two or more biological molecules.

Reagent Delivery Systems

[0071] The reagent delivery system of the invention includes instrumentation that allows the delivery of reagents to discrete portions of the biological sample, maintaining the integrity of the spatial patterns of the encoding scheme. Reagent delivery systems of the assay systems of the invention comprise optional imaging means, reagent delivery hardware and control software. Reagent delivery can be achieved in a number of different ways. It should be noted that reagent delivery may be to many different biological samples at one time. A single tissue section has been exemplified herein; however, multiple biological samples may be affixed and analyzed simultaneously. For example, pions of a tissue sample can be analyzed in parallel and the data combined to build a 3D map.

[0072] Integral to the assay system of the invention is instrumentation that allows for spatial patterning of reagents onto the biological sample. Technologies for formulating and delivering both biological molecules *(e.g.* oligonucleotides or antibodies) and chemical reagents (e.g., small molecules or dNTPs) are known in the art, and uses of these instrument systems are known to one skilled in the art and easily adaptable to the assay systems of the invention. One example of a suitable reagent delivery system is the Labcyte™ Echo acoustic liquid handier, which can he used to deliver nanoliter scale droplets containing biological molecules with high precision and reproducibility. One skilled in the art could incorporate this reagent delivery device into the overall system, using software to specify the locations to which reagents should be delivered.

[0073] Other instruments that can be used for the deposition of agents and/or coding identifiers onto biological samples include, but are not limited to, ink jet spotting; mechanical spotting by means of pin, pen or capillary; micro contact printing; photochemical or photolithographic methods; and the like. For several applications, it may be preferred to segment or sequester certain areas of the biological samples into one or more assay areas for different reagent distributions and/or biological target determination. The assay areas may be physically separated using barriers or channels.

[0074] In one exemplary aspect, the reagent delivery system may be a flow-based system. The flow-based systems for reagent delivery in the present invention can include instrumentation such as one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Reagent delivery systems are configured to move fluid to contact a discrete section of the biological sample. Movement

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of the reagents can be driven by a pump disposed, for example, downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, reagents may be driven through the fluid by gravity. US Pub. Nos. 20070166725 and 20050239192 disclose certain general-purpose fluidics tools that can be used with the assay systems of the invention, allowing for the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

[0075] In a more specific example, one or more flow-cells can be attached to the substrate-affixed biological sample from above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump is used to deliver reagents to the flow-cell and across the biological sample. The flow cells are configured to deliver reagents only to certain portions of the biological sample, restricting the amount and type of reagent delivered to any specific section of the biological sample.

[0076] In another aspect, a microfluidic system can be integrated into the substrate upon which the biological sample is disposed or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid may be formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected and delivered according to selective opening and closing of valves disposed between reagent reservoirs.

[0077] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes *(i.e.,* microfluidic structures). The pump can operate mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among other means. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. Mechanical pumps may be micromachined, molded, etc. Exemplary electrical pumps may include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[0078] Valves generally include any mechanism for regulating the passage of fluid through a channel. Valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into a channel to partially or completely block a channel, an electrocapillary structure, and/or the like.

[0079] An open gasket can be attached to the top of the biological sample and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber may be formed by a gasket sandwiched between the biological sample on the substrate and a chemically inert, water

resistant material such as, but not limited to, black-anodized aluminum, thermoplastics *(e.g.,* polystyrene, polycarbonate, etc), glass, etc.

[0080] In an optional embodiment, the assay system comprises imaging means to determine features and organization of the biological sample of interest. The images obtained, *e.g.,* may be used to design the deposition pattern of the reagents, Imaging means are optional, as an individual can instead view the biological sample using, *e.g.,* a microscope, analyze the organization of the biological sample, and specify a spatial pattern for delivery assay reagents. If included, the delivery system can comprise a microcircuit arrangement including an imager, such as a CCD or IGFET-based *(e.g.,* CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Pub. No. 20090197326, which is incorporated herein by reference. Also, it should be noted that although Figures 4 and 5 illustrate using a x,y grid configuration, other configurations can be used, such as, *e.g.,* following the topology of a tissue sample; targeting certain groups of cells, cell layers and/or cell types in a tissue, and the like.

[0081] In yet another alternative, the reagent delivery system controls the delivery of reagents to specific patterns on a biological sample surface using semiconductor techniques such as masking and spraying. Specific areas of a biological sample can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the biological sample using conventional techniques such as spraying or fluid flow. The use of masked delivery results in a patterned delivery scheme on the substrate surface.

[0082] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms (*e.g.,* thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions are be achieved.

[0083] In order to target specific sites of interest, an informative image of the biological sample to be assayed may be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions of the biological sample can be identified using image processing (e.g., images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with other features of the assay system. In some aspects, software is used to automatically translate image information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample for reagent delivery is thus an important component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and/or other very accurate physical positioning systems can be adapted to this purpose.

[0084] The invention preferably comprises a complete suite of software tailored to the assay

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system. Optionally, oligonucleotide design software is used to design the encoding nucleotides (and in embodiments where nucleic acids are assayed, the target-specific oligonucleotides) for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for reagent delivery and data analysis (i.e., sequence analysis) may be integrated to determine assay results. Integrated data analysis is particularly useful, as the type of dataset that is generated may be massive as a consequence of scale. Algorithms and software tools that are specifically designed for analysis of the spatially-associated data generated by the assay systems, including pattern-analysis software and visualization tools, enhance the value of the data generated by the assay systems.

[0085] In certain aspects, the assay system comprises processes for making and carrying out the quality control of reagents, *e.g.,* the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents are formulated according to factors such as volatility, stability at key temperatures, and chemical compatibility for compatibility with the reagent delivery instrumentation and may be analyzed by instrumentation integrated within the assay system.

Sequencing

[0086] Numerous methods can be used to identify the coding tags and probe sequences in the encoded probes of the assay systems of the invention. The coding tags can be detected using techniques such as mass spectroscopy *(e.g.,* Maldi-T of, LC-MS/MS), nuclear magnetic resonance imaging, or, preferably, nucleic acid sequencing. Examples of techniques for decoding the coding tags of the present invention can be found, for example, in US Pub. No. 20080220434, which is incorporated herein by reference. For example, the coding tags may be oligonucleotide mass tags (OMTs or massTags). Such tags are described, *e.g.,* in US Pub. No. 20090305237, which is incorporated by reference in its entirety. In yet another alternative, the encoded probes can be amplified and hybridized to a microarray. This would require separate amplification reactions to be carried out, in which each amplification is specific to a particular spatial code or subset of codes, accomplished by using code-specific primers. Each amplification would also incorporate a different resolvable label (e.g. fluorophor). Following hybridization, the relative amounts of a particular target mapping to different spatial locations in the sample can he determined by the relative abundances of the resolvable labels.

[0087] In one particularly preferred aspect, the resulting coding tags according to the assay system are substrates for high-throughput, next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of the coding tags, for example, with SOLiD™ technology (Life Technologies, Inc.) or Genome Ananlyzer (Illumina, Inc.). Such next-

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generation sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in *e.g.,* Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et al, U.S. patent publication 2005/0191656; sequencing-by-synthesis methods, *e.g.,* U.S. Pat Nos. 6,210,891; 6,828,100; 6,969,488; 6,897,023; 6,833,246; 6,911,345; 6,787,308; 7,297,518; 7,462,449 and 7,501,245; US Publication Application Nos. 20110059436; 20040106110; 20030064398; and 20030022207; Ronaghi, et al, Science, 281: 363-365 (1998); and Li, et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); ligation-based methods, *e.g.,* U.S, Patent Nos. 5,912,148 and 6,130,073; and U.S. Pat. Appln Nos. 20100105052, 20070207482 and 20090018024; nanopore sequencing *e.g.,* U.S. Pat. Appln Nos. 20070036511; 20080032301; 20080128627; 20090082212; and Soni and Meller, Clin Chem 53: 1996-2001 (2007)), as well as other methods, *e.g.,* U.S. Pat. Appln Nos. 20110033854; 20090264299; 20090155781; and 20090005252; also, see, McKernan, et al., Genome Res., 19:1527-41 (2009) and Bentley, et al., Nature 456:53-59 (2008), all of which are incorporated herein in their entirety for all purposes.

Applications of Assay System

[0088] It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous important areas of biological research, diagnostics, and drug development that will benefit from a high throughput multiplexed assay system that can measure simultaneously the amount and spatial location of a biological target in a biological sample. For example, combining the ability to estimate the relative abundance of different RNA transcripts with the ability to reconstruct an image of spatial patterns of abundance across many locations, which may be as small as or even smaller than individual cells, in a tissue enables many different areas of basic research. The following are exemplary uses and are by no means meant to be limiting in scope.

[0089] In one example, 3-dimensional patterns of gene expression are determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning, Such a method can be used to measure changes in gene expression in disease pathology, *e.g.,* in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues is obtained, leading to new insights into the function and regulation both in normal and diseased states, and provides new hypotheses that can be tested. For example, an assay system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney, et al., Nature, 447:799-816 (2007)) and modENCODE to be integrated at the tissue level. The assay

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systems also aid computational efforts to model interacting networks of gene expression in the field of systems biology.

[0090] The assay systems also provide a novel approach to analysis of somatic variation, *e.g.*, somatic mutations in cancer or variability in response to infectious organisms. For example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer-related processes in both cancer and genetically normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers. In another example, individuals have varying susceptibility to infectious organisms, and the assay systems of the invention can be used to study the interaction between microbes and tissues or the various cell types within the tissue.

[0091] Importantly, in addition to providing spatially-associated information, the invention allows a great increase in the sensitivity of detecting rare mutations, as signal to noise can he dramatically increased since only a small location is assayed in any given reaction. In a typical assay for rare mutations in a mixed sample, the sample is treated in bulk, *i.e.*, nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in one cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the assay systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, in the assay systems of the present invention, background is reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed, which may be particularly important in detecting key mutations in tissue sections in cancer. Already molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics. The technology of the invention promises to greatly increase the power of such approaches.

[0092] The present invention provides assays, assay systems, and methods of using such assays in spatially encoded biological assays. The invention provides an assay system comprising one or more agents provided in defined spatial patterns on a substrate surface, and a detection system for identifying the presence or absence, relative amount, and location of a biological molecule. Such biological molecules include, but are not limited to, nucleic acids, peptides, carbohydrates, cellular components, and the like. The assay system is a novel multiplexing approach, as it allows multiple molecules and their respective multiple locations to be identified in a single system using a unique

encoding scheme. This encoding scheme uses both molecule-specific binding agents and coding identifiers to provide a practical and cost-effective determination of information on multiple biological molecules, including specific positional information of such molecules in a biological sample, e.g., a tissue section. The single molecule detection analysis using the encoding system also allows relative amounts of biological molecules to be detected, thus providing information on expression levels, sequestering in specific locales, and the like.

[0093] The assay systems detect the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. In addition, the assays provide methods for doing this for multiple biological molecules simultaneously. The assay systems utilize one or more binding agents that specifically bind to the biological molecule of interest and unique coding identifiers associated with specific binding agents. The detection system utilizes a method for identifying the presence and spatial address of the agent binding based on the positive and/or negative results that are obtained using detection of the agent and identifier and the encoding scheme of the spatial patterns on the substrate surface. In a specific aspect, the encoding scheme employs limited reagent delivery to the spatial patterns on the substrate surface, and access of the coding identifiers and/or binding agents to portions of the sample is controlled through such limited delivery.

[0094] In one aspect, the assay system detects the presence or absence and spatial location of a biological molecule based on the positive and/or negative results that are obtained using limited reagent delivery and the encoding scheme of the spatial patterns on the substrate surface.

[0095] The assay system and methods of the invention are based on relational, solid-state substrates with positions that represent specific spatial locations within a biological sample, e.g., a cell, organelle or tissue. The ability to use encoding features to represent locations allows high-throughput analysis of the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. The encoding features also allow provide assaying of multiple biological molecules at these multiple locations simultaneously.

[0096] A primary feature of the invention is the preservation of the spatial organization of elements in a sample of interest through the use of an encoding scheme. For example, the assay may be designed to preserve the relative position of cells in a tissue, and the assay may interrogate the individual cells for genomic DNA variation (including epigenetic modifications), and RNA and protein expression.

[0097] In one specific aspect, the encoding scheme of the assay system comprises the use of two or more coding patterns, each comprising regions defined by spatial patterns on the substrate surface. For example, the assay system can utilize an encoding scheme that comprises a 2-dimensional grid format based on the discrete positioning of the binding agents in the substrate surfaces. In another example, the spatial patterns may be based on more randomized cell locations, e.g., the patterns on the substrate

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surface follow an underlying biological structure rather than a strict, x,y grid pattern. This aspect includes systems with two or more substantially identical spatial patterns using different binding agents and/or coding identifiers, as well as systems having different patterns for different agents and/or coding identifiers. The encoding scheme of the systems can be controlled by delivery of different reagents to discrete regions on the substrate surfaces, which allows different reactions to take place on substantially similar agents of known location on the substrate surfaces.

[0098] In one specific aspect, the invention provides high resolution, high- throughput analysis of nucleic acids and/or expression levels that provides both detection and spatial identification of large numbers of nucleic acids, e.g., DNA or RNA.

[0099] In another specific aspect, the invention provides high resolution, high- throughput analysis of proteins that provides both detection and spatial identification of large numbers of such proteins, e.g., kinases or proteases.

[0100] Numerous reagent delivery systems can be used with the assay system of the invention. The primary criteria of such reagent delivery systems is the ability to direct delivery of specific agents based on spatial patterns on the substrate surface.

[0101] In one preferred aspect, the encoding scheme utilizes a reagent delivery system based on printing and informatics technologies to implement the spatial patterns used for identification and localization of the biological materials. For example, the patterns found in the encoding scheme may be created using ink jet printing technology to provide reagents at specific locations on one or more substrate surfaces. The desired patterns are set out in specific coding patterns on the substrate surface.

[0102] In certain aspects of the invention, the binding agents are immobilized directly to the substrate surface, and the location of the binding agents is known or determined prior to use of the substrate surface in the assay system. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, e.g., provided in wells or channels.

[0103] In specific aspects of the invention, the binding agents are nucleic acids immobilized directly or indirectly to the substrate surface, e.g., directly through the use of amino groups on the substrate surface or indirectly through the use of a linker. The location of the nucleic acid sequences is known or determined prior to use of the substrate surface in the assay system. In another specific aspect, the nucleic acids may be immobilized directly or indirectly onto beads that are then provided in known locations on the substrate surface. In yet another aspect, the nucleic acids may be provided in or on features of the substrate surface, e.g., provided in wells.

[0104] In these aspects involving nucleic acid agents, any methods of sequence determination can be

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used, e.g., sequencing, hybridization and the like. In a preferred aspect, nucleic acid sequencing, and preferably next-generation sequencing, is used to decode the spatial encoding scheme in the assay system of the invention. This provides a very wide dynamic range for very large numbers of assays, allowing for efficient multiplexing.

[0105] In some aspects, the assay utilizes two or more oligonucleotides, the oligonucleotides comprising a universal primer region and a region that correlates specifically to a single spatial pattern within the spatial encoding scheme. In a specific aspect, the assay comprises two allele specific oligonucleotides and one locus specific oligonucleotides. These oligonucleotides allow the identification of specific SNPs, indels or mutations within an allele. This is useful in the identification of genetic changes in somatic cells, genotyping of tissues, and the like.

[0106] In other specific aspects of the invention, the binding agents are peptides. In one aspect, these peptides are associated directly or indirectly to known locations on a substrate surface, e.g., using binding protein pairs or through oligonucleotide linkers complementary to oligonucleotides on the substrate surface. In another aspect, the binding agents are peptides are immobilized directly or indirectly onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the peptides may be provided in or on features of the substrate surface, e.g., provided in wells.

[0107] In yet other specific aspects of the invention, the binding agents are chemical entities (e.g., small molecules) that are coded, e.g. using sequence tags or mass spectroscopy tags as coding identifiers. In one aspect, these chemical entities can be are immobilized directly to the substrate surface. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, e.g., provided in wells.

[0108] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

[0109] The Invention in General

[0110] The assay system and methods of the invention are based on relational methods that allow extraction of data to detect the presence or absence and relative amount of a biological molecule, and the location of this molecule in a sample having a distinct structure, *e.g.,* a tissue section or other biological structure with distinct locations of specific biological molecules. The encoding scheme used in these systems corresponds to the structural elements of the sample, and the information

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obtained using a two-dimensional coding system is indicative of the spatial addresses of these molecules in a sample of interest.

[0111] Integral to the assay system of the invention is a method for spatial patterning of reagents. Technologies for formulating and delivering both biological molecules *(e.g.* DNA or antibodies) and chemical reagents *(e.g.,* small molecules or dNTPs) have already been demonstrated, and use of these systems will be available to one skilled in the art and easily adaptable upon reading this specification.

[0112] The assay design of the invention provides an accurate and easily scalable spatial encoding system. The ability to deliver reagents in a spatially defined pattern together with software, reagents and protocols comprises a novel and highly innovative assay system for spatial analysis of various biological molecules and activities. This allows the assays to measure numerous biological functions in a meaningful spatial environment, including functions such as gene expression and peptide localization. The systems provide the potential to open a new analytical window into the complex spatial patterns of cellular function and regulation in biological systems.

[0113] The biological molecules to be detected can be any biological molecules such as proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Further examples of subcellular objects include organelles, *e.g.,* mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplast, endocytic vesicle, exocytic vesicles, vacuole, lysosome, etc.

[0114] Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system 401, two reagents 420, 422 that specifically bind to a biological molecule of interest are associated with coding identifiers 406, 408 that encode for a spatial location in the sample. These coding identifiers 406, 408 are optionally associated with sites that assist in their identification in the assay format, *e.g.,* universal priming sites 404, 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a

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tissue section 416 is encoded using the combination of the patterns 412, 414 created using the separate coding identifiers 406, 408 which provide a two dimensional code 418 that shows the location of any positive detection of the biological molecule 402 as well as quantifying the biological molecule 402 at each location assayed in the tissue.

[0115] The assay systems of the invention are particularly advantageous in that they are compatible with numerous samples types, such as fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the binding agents are immobilized on a substrate surface in discrete, independently measureable areas. These discrete areas can be formed by spatially selective deposition of the binding agents on the substrate surface. Numerous methods can be used for the deposition of the agent and the coding identifiers associates with the agent. For example, the coding identifiers can be delivered together or separately from the agent. If delivered together they can be attached *(e.g.,* synthesized as a single molecule or attached through ligation or a chemical coupling mechanism) or simply mixed together to be attached after delivery to the substrate. In a preferred aspect, the agent and the coding identifier are made separately, mixed together for attachment, and delivered either attached or as a mixture to be attached on the surface. In a specific aspect the binding agents are delivered generally over the substrate surface and the coding identifiers are delivered in a pattern-specific manner.

[0116] Examples of methods that can be used for deposition of agents and/or coding identifiers onto the substrate surface include, but are not limited to, ink jet spotting, mechanical spotting by means of pin, pen or capillary, micro contact printing, fluidically contacting the measurement areas with the biological or biochemical or synthetic recognition elements upon their supply in parallel or crossed micro channels, upon exposure to pressure differences or to electric or electromagnetic potentials, and photochemical or photolithographic immobilization methods.

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[0117] For several applications, it may be preferred to arrange the substrates into segments of one or more measurement areas for reagent distribution and agent determination. These regions may be physically separated using barriers or channels. They may still comprise several additional discrete measurement areas with agents that are different or in different combination from each other.

[0118] In certain aspects, the present invention provides a method, *e.g.,* a machine-based method, for evaluating changes in the presence and/or location of a biological molecule over time. The method includes providing a plurality of encoded array results representative of the biological molecule over time and evaluating the differences in detection and/or localization of the biological molecules.

(Original) Nucleic Acid Detection and Localization

[0119] In a particular aspect, the assay system is used to analyze nucleic acids, *e.g* genotyping, gene expression analysis, localization of particular transcripts within samples, and the like.

[0120] Genotyping may be performed using any technique known to those of skill in the art. Preferred techniques permit rapid, accurate determination of multiple variations with a minimum of sample handling. Some examples of suitable techniques involve but are not limited to direct DNA sequencing, capillary electrophoresis, hybridization, allele-specific probes or primers, single-strand conformation polymorphism analysis, nucleic acid arrays, bead arrays, restriction fragment length polymorphism analysis, cleavage fragment length polymorphism analysis, random amplified polymorphic DNA, ligase detection reaction, heteroduplex or fragment analysis, differential sequencing with mass spectrometry, atomic force microscopy, pyrosequencing, FRET *(e.g.,* TaqMan (Applied Biosystems, Inc., Foster City, Calif.) and Molecular Beacon (Stratagene, La Jolla, Calif.) assays), and other related techniques. Several methods for DNA sequencing are well known and generally available in the art. See, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, New York) (2001); Ausubel, et al., Current Protocols in

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Molecular Biology (John Wiley and Sons, New York) (1997), Twyman, et al. (2003) "Techniques Patents for SNP Genotyping", Pharmacogenomics 4(1):67-79; and Kristensen, et al. (2001) "High-Throughput Methods for Detection of Genetic Variation", BioTechniques 30(2):318-332. For details on the use of nucleic acid arrays (DNA chips) for the detection of, for example, SNPs, see U.S. Pat. No. 6,300,063 issued to Lipshultz, et al., and U.S. Pat. No. 5,837,832 to Chee, et al., HuSNP Mapping Assay, reagent kit and user manual, Affymetrix Part No. 90094 (Affymetrix, Santa Clara, Calif.). The molecular inversion probe (MIP) assay format (Hardenbol et al., 2003) is another example of a highly multiplexable assay that may be used with the assay systems of the invention.

[0121] In one exemplary and preferred method for analyzing nucleic acids using the assay system of the invention, the detection of nucleic acids uses two allele- specific oligonucleotides and a locus specific oligonucleotide. The assay methods are carried out according to the strategy outlined in Fig. 2 using next-generation sequencing or another highly parallel nucleic acid assay technology. In this assay, a set of two oligonucleotides is designed to hybridize to each target sequence, with a common oligonucleotide and two unique coding identifiers. The allele can be determined, *e,g,* by primer extension of the locus specific oligonucleotide. Following primer extension and ligation, an amplifiable template is formed with universal primer sequences at either end. Assay oligonucleotides are annealed to a template and enzymatic reactions are used to join the two oligonucleotides only when both are correctly annealed. The detection techniques and read out parameters used in this system of the invention include a much shorter tag than the oligonucleotides used in the assays that are based on capture by hybridization. These shorter tags are designed to be read out by sequencing or, preferably, used to ligate codes onto both ends of the fragment as illustrated in Fig. 2.

[0122] In Figure 3, two target-specific assay oligonucleotides are ligated together 302 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides containing tag sequence sets X and Y are ligated 304 to the target specific oligonucleotides.

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Oligonucleotides containing X ligate specifically to one side of the targeting construct and oligonucleotides containing Y ligate to the other. The oligonucleotides contain universal primer sites Pl and P2. Following ligation, the constructs are eluted and, optionally, sequencing adapters can be attached 306, *e.g.,* via PCR.

[0123] In one preferred aspect, the final construct created from the assay method is a substrate for next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of constructs. Such sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et al, U.S. patent publication 2005/0191656, and sequencing by synthesis methods, *e.g.,* Nyren et al, U.S. Pat. No. 6,210,891; Ronaghi, U.S. Pat. No. 6,828,100; Ronaghi et al (1998), Science, 281: 363-365; Balasubramanian, U.S. Pat. No. 6,833,246; Quake, U.S. Pat. No. 6,911,345; Li et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); Smith et al, PCT publication WO 2006/074351; use of reversible extension terminators, *e.g.,* Turner, U.S. Pat. No. 6,833,246 and Turner, U.S. Pat. No. 6,833,246 and ligation-based methods, *e.g.,* Shendure et al (2005), Science, 309: 1728-1739, Macevicz, U.S. Pat. No. 6,306,597; which references are incorporated by reference. Soddart et al., PNAS U S A. 2009 Apr 20; Xiao et al., Nat Methods. 2009 Mar;6(3):199-201. Epub 2009 Feb 8.

[0124] To maximize the efficiency of encoding, a combinatorial approach using pairs of oligonucleotides can be used. For example, with only two sets of 100 codes, a substrate can theoretically encode up to 10,000 locations. The number of assay oligonucleotides required is dramatically reduced, the cost decreased, and the robustness of the approach increased by decoupling the coding sequences from the genome-specific sequences. Alternative assay formats can also be used (e.g. ligation or primer extension followed by ligation).

[0125] By ligating the codes on separately, only 2n target-specific assay oligonucleotides are needed for n targets. For example, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 targeting oligonucleotides and 2 x 100 encoding oligonucleotides, using a combinatorial approach outlined in Fig. 2. The total count of assay oligonucleotides would be only 400 (200 target- specific and 200 encoding), not counting universal primers. In contrast, if the coding oligonucleotides were not decoupled, (n x X positional codes) + (n x Y positional codes) would be needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences.

[0126] Due to the matrix system of the invention, a large amount of information can be obtained even using five or more positions interrogated for five or more biological molecules. By varying one or the other of these, large amounts of information can be obtained, both in terms of locations and/or specific biological In specific aspects, multiple locations are interrogated for two or more biological molecules. As an example, for each datapoint ~1,000 reads may be sampled, for a total of -10E9 reads for 10E6 datapoints.

Peptide Detection Systems

[0127] The assay system of the invention can be used to analyze biological molecules using peptide agents that are associated with the substrate surface in a spatial pattern. Such peptides may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, etc.

[0128] The assay system of the invention allows the identification and spatial location of various forms of peptides, including isoforms and peptides that have undergone posttranslational modification. Importantly, certain aspects of the invention allow the identification of active versus non-active forms of such peptides in a sample. This allows the identification of the presence or absence of specific peptide isoforms, and also acts as a proxy for identification of peptide activity in a sample.

[0129] In certain aspects of the invention, the binding agents associated with the substrate surfaces of the assay system include substrates for enzymes or proenzymes, *e.g.,* a kinase, a phosphatase, a zymogen, a protease, or a fragment thereof. In certain aspects, the binding agents associated with the substrate surfaces are phosphorylation substrates used to detect proteins involved with one or more signal transduction pathways, *e.g.,* a kinase or a phosphatase. In another specific aspect of the invention, the binding agents are specific protease substrates that associate only with individual or classes of proteases. In other aspects, the binding agents on the substrate surface are different processed forms, isoforms and/or domains of an enzyme.

Reagent Delivery

[0130] The reagent delivery system of the invention can be any system that allows the delivery of reagents to discrete portions of the array in order to keep the integrity of the defined spatial patterns of the encoding scheme. Such discrete delivery can be achieved in a number of different ways.

[0131] In one exemplary aspect, the reagent delivery system can be a flow-based system. The flow-based systems for reagent delivery in the present invention can include one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Such a reagent delivery system is configured to move fluid in contact with a discrete section of the substrate surface. Movement of the reagents can be driven through a fluid by a pump disposed, for example, downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, the reagents may be driven through the fluid by gravity.

[0132] US Appln Nos. 20070166725 and 20050239192 disclose certain general- purpose fluidics tools that can be used with the assay systems of the invention. These allow the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

[0133] In a more specific example, one or more flow-cells can be attached to the substrate from

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above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump can be used to deliver the sample or reagents to the flow-cell and across the substrate. The flow cell is configured to deliver reagents only to certain portions of the array, restricting the amount and type of reagent delivered to any specific section of the array.

[0134] In another aspect, a microfluidic system can be integrated into the substrate or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid can be formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected according to selective opening and closing of valves disposed between reagent reservoirs.

[0135] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes (i.e., microfluidic structures). The pump can operate mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among others. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. The mechanical pumps may be micromachined, molded, etc. Exemplary electrical pumps can include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[0136] Valves generally include any mechanism for regulating the passage of fluid through a channel. The valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into the channel to partially or completely block the channel, an electrocapillary structure, and/or the like.

[0137] In yet another aspect, an open gasket can be attached to the top of the substrate and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber formed by a

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gasket sandwiched between the substrate and a chemically inert, water resistant material such as, but not limited to, black-anodized aluminum, thermoplastics (e.g., polystyrene, polycarbonate, etc), glass, etc.

[0138] In a specific aspect of the present invention, the delivery system can compnse a microcircuit arrangement including an imager, such as a CCD or IGFET-based (e.g., CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Appln No. 20090197326, which is incorporated herein by reference.

[0139] In yet another aspect of the invention, the reagent delivery system controls the delivery of reagents to specific patterns on a substrate surface using semiconductor techniques such as masking and spraying. Specific areas of a substrate surface can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the substrate using conventional techniques such as spraying or fluid flow. The use of the masked substrate delivery results in a patterned delivery scheme on the substrate surface.

[0140] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms *(e.g.,* thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions can be achieved.

Software for use in the Assay System

[0141] In order to target specific sites of interest, an informative image of the biological section to be analyzed can be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions can be identified using image processing *(e.g.,* images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with the other features of the assay

system. In some aspects, software is used to automatically translate this information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample in a targeting system is thus a preferred component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and other very accurate physical positioning systems can be adapted to this purpose.

[0142] Additional software components will also be key components that will be part of a complete assay system. The invention thus preferably comprises a complete suite of software tailored to the assay system. Optionally, oligonucleotide design software will be customized for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for data analysis may be integrated to assist in determination of results of the assays. This can be especially useful, as the type of dataset that will be generated will be novel, particularly as a consequence of scale. The ability to provide algorithms and software tools that are specifically designed for analysis of spatially-associated data for significant patterns, including pattern-analysis software and visualization tools, is a novel feature that will enhance the value of the data generated by the assay systems.

[0143] In certain aspects, the assay system will comprise processes for making and carrying out quality control of reagents, *e.g.,* the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents will need to be formulated for compatibility with the reagent delivery instrumentation. Factors such as volatility, stability at key temperatures, and chemical compatibility can be optimized by those skilled in the art upon reading the present disclosure.

(Original) Applications of Assay System

[0144] It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous very important areas of biological research, diagnostics, and drug development that will

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benefit from a high throughput means to simultaneously measure the presence or absence and spatial location of a biological molecule in a sample. For example, this technology combining the ability to analyze semi-quantitatively the expression of many different genes with the ability to image the spatial organization of expression across many cells in a tissue is enabling for many different areas of basic research. The following are exemplary uses and are by no means meant to be limiting in scope.

[0145] In one example, 3-dimensional patterns of expression can be determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning. This can be used to measure changes in gene expression in disease pathology, *e.g.,* in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues can be obtained. This may lead to new insights into the function and regulation both in normal and diseased states, and is likely to provide new hypotheses that can be tested. For example, a system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney et al., 2007) and modENCODE to be integrated at the tissue level. The assay systems will also aid in computational efforts to model interacting networks of gene expression in the field of systems biology.

[0146] The assay systems also provide a novel approach that enables the analysis of somatic variation, *e.g.,* somatic mutations in cancer or variability in response to infectious organisms. For example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer related processes in both cancer and genetically normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers.

[0147] In another example, different people have varying susceptibility to infectious organisms,

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and much of this may be to underlying genetic differences in individuals and/or populations. Identifying these differences will aid in an understanding of the underlying disease pathologies and assist in the development of vaccines or therapeutics to prevent or ameliorate these disease states.

[0148] Importantly, in addition to providing spatially associated information, the technology of the invention will allow a great increase in the sensitivity of detecting rare mutations. The reason is that signal to noise can be dramatically increased because the approach of the invention assays a small location in any given reaction. In a typical assay for rare mutations in a mixed sample, the sample is treated in bulk, i.e. nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in 1 cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, the background can be reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed. This may be particularly important in detecting key mutations in tissue sections in cancer. Already, molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics (Choe et al., 2003). The technology of the invention promises to greatly increase the power of such approaches.

(Original) EXAMPLES

[0149] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventor regards as his invention, nor are they intended to represent or imply that the experiments below are all of or the only experiments performed. It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

[0150] Efforts have been made to ensure accuracy with respect to numbers used *(e.g.,* amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees centigrade, and pressure is at or near atmospheric.

EXAMPLE 1: Initial Proof of Concept of Encoding Scheme

[0151] As an initial proof of concept, a model system is developed using a microarray to demonstrate a working single-plex assay. The basic design validates the concept of the assay, and establishes a working assay prior to addressing issues related to the analysis of a more complicated biological sample. Conventional sequencing is used as a readout for this proof of concept.

[0152] A microarray is used as a proxy for a tissue section. The target sequences of the microarray are fully specified, so that the composition of the targets are known and can be varied systematically. Synthetic oligonucleotide templates are attached to a glass slide via a 5' amino modification. Each slide has a single oligonucleotide template sequence, and the assays that are carried out may employ either ligation, or extension followed by ligation as this may be useful in determining certain polymorphisms.

[0153] Once the *in situ* part of the assay is complete, the reaction products are eluted and analyzed by qPCR to determined presence or absence of a product and estimate yield, and by conventional sequencing to determine the structure of the assay products. The single plex assays that are tested include appropriate positive and negative controls, and a single nucleotide variant (SNV) to check ability to discriminate single base variants.

EXAMPLE 2: Scalability

[0154] The complexity of the assay system is increased to establish scalability of the assay for use in high throughput studies. Scalability of both the spatial encoding and assay systems is demonstrated by carrying out a 24-plex x 24-site assay using a microarray model system.

[0155] The amount of biological target, here a DNA target sequence, at each assay location is systematically varied on microarray substrate. For example, in a microarray with 50 micron spot size (center to center), a 1 mm^2 area contains ~400 spots. The region around each site is optionally occupied by a region that is devoid of these spots to allow individual resolvability of the target sequences. Alternatively, the spots may be clustered, with two or more directly adjacent spots surrounded by or adjacent to a region that is devoid of target sequences.

[0156] In order to demonstrate that spatial encoding is accurate, the sites comprise different target compositions to show that the assay readout matches the expected composition of each site. With 24 target sequences, a simple digital pattern is made with each site having a different set of 12 targets

present and 12 targets absent, to make a binary code (0= absent, 1=present). The assay readout is then determined to show that the detected regions match the expected signal after spatial decoding. In this particular example, the code space is large enough (2^{24}) so that even a few errors would not result in different codes being mixed up. Moreover, this design allows identification of errors and allows an estimation not only of accuracy of spatial encoding but also of accuracy calling the presence or absence of target sequences.

(Original) In an exemplary aspect, a 4 x 4 arrangement of 16 sequences is used for the array configuration. A white square indicates that the sequence is absent and a black square that it is present, i.e. 8 of the 16 possible sequences are present in this sample. In a different sample, a different pattern of absent and present sequences can be constructed. In this way, unique patterns are associated with spatial locations so that the accuracy of spatial encoding can be measured.

[0157] The ability to detect quantitative differences is evaluated by generating dose-response curves for each of the 24 assays that are carried out at each site in a 24-site assay. This allows estimation of the limit of detection, dynamic range, and power to detect a given fold-change across the range.

[0158] In one aspect, a latin square design is used to represent individual targets at different ratios by varying the number of features for each target. In other words, with multiple spots in a site, the number of spots allocated to each of the 24 target sequences can be varied and each of the 24 sites can have a different composition. A 1 x 3 inch microarray is sufficiently large to permit multiple replicates. This larger set of 24 sequences will require deconvolution, and this is accomplished using high throughput techniques such as next-generation sequencing technologies *(e.g.,* SOLiD™ technology (Life Technologies, Inc., Carlsbad, CA) or Genome Analyzer (Illumina, Inc., San Diego, CA)). The use of the 24-plex assay demonstrates both the accuracy of spatial encoding and decoding, and the quantitative response of the assay system.

EXAMPLE 3: Adaptation of the assay to preserved samples.

[0159] Genomic DNA is assayed as a proof of concept for assaying RNA, as it provides a way to establish a single-copy reference signal. Once a working assay is developed for FFPE samples, it is adapted to an RNA assay. To this end, assay oligonucleotide concentrations are assayed to ensure compatibility with high multiplexing. Assuming a cell diameter of 10 microns, and delivery of a 10 micron diameter reagent droplet to an individual cell, the volume of the droplet will be ~500 µl and can contain ~3 x 10^{11} molecules at a 1 µM concentration. To assay 1,000 target sequences in 10,000 cells, ~2,000 targeting oligonucleotides would be required in a droplet. Therefore, each droplet could contain ~160 million copies of each assay oligo, a vast excess over the few thousand target sequences in a cell.

Curio EX1010-p. 846
Curio v Prognosys

[0160] The handling of small absolute numbers of product molecules generated from very small or compromised samples are enhanced to counter the issue of low recovery efficiency; that is, elution is efficient and losses resulting from adsorption of molecules to surfaces are prevented. An approach to addressing the latter issue is to include a carrier material, such as glycogen or carrier nucleic acids.

Example 4: Adapting the Assay to a Biological Sample.

[0161] A control RNA template is immobilized to a solid support in order to create an artificial system. The assay is performed using T4 DNA ligase, which can repair nicks in DNA/RNA hybrids. Assays are carried out on matched slides, or different sections of the same slide, where in one case gDNA is assayed and in the other RNA is assayed. When assaying gDNA the slide can be pretreated with RNase, and when assaying RNA the slide is pretreated with DNase. Results of the assay are confirmed by extracting gDNA or RNA and quantitating the relative amounts by qPCR or RT-qPCR respectively.

[0162] In order make the tissue section RNA assays as informative as possible, pre-existing information on expression levels in specific tissues to target transcripts across a range of abundances are used in the assay design. Both high abundance transcripts, as well as some medium and low abundance transcripts, are targeted to enable an initial assessment of the quantitative performance characteristics of the assay.

[0163] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims. In the claims that follow, unless the term "means" is used, none of the features or elements

Curio EX1010-p. 847
Curio v Prognosys

recited therein should be construed as means-plus-function limitations pursuant to 35 U.S.C. §112, ¶6.

Curio EX1010-p. 848
Curio v Prognosys

Electronic Acknowledgement Receipt

EFS ID:	36271481
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Lisa Gray
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	11-JUN-2019
Filing Date:	14-FEB-2019
Time Stamp:	20:26:53
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		47706-0004003suppresponse.pdf	948610 64603b86352492198d73ae21ffb24a454a0e24b0	yes	47

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Supplemental Response or Supplemental Amendment	1	1
	Specification	2	3
	Applicant Arguments/Remarks Made in an Amendment	4	47

Warnings:					
Information:					
2	Specification	47706-0004003subspec.pdf	930752 031571d896694b32cc750b7407a28a60400fadb3	no	43
Warnings:					
Information:					
		Total Files Size (in bytes):	1879362		

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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.

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003

CONFIRMATION NO. 7944

159367
FISH & RICHARDSON P.C.
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P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

PUBLICATION NOTICE

OC00000108894916

Title: Spatially Encoded Biological Assays

Publication No.US-2019-0177777-A1
Publication Date:06/13/2019

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

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	16276235
Filing Date	14-Feb-2019
First Named Inventor	Mark CHEE
Attorney Docket Number	47706-0004003
Title of Invention	Spatially Encoded Biological Assays

☒ 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
Prognosys Biosciences, 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)

16430109 filed on 06/03/2019

16430015 filed on 06/03/2019

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 58674

○ 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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*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:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Attorney Docket Number:	47706-0004003

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	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16276235

Filing Date: 14-Feb-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on June 20, 2019

☒ 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:	36360872
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	20-JUN-2019
Filing Date:	14-FEB-2019
Time Stamp:	14:45:45
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$160
RAM confirmation Number	062119INTEFSW00001158061050
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	34691 409380dfa9e73900d6b1f0c5a1efb224015c96dc	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30653 2510d509b3de9f37ad9c9ac1c18a9edaa7616ab1	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	65344		

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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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

SUPPLEMENTAL RESPONSE TO AMENDMENT(S) FILED ON MAY 15, 2019

Please consider the following reply.

FIG. 4A

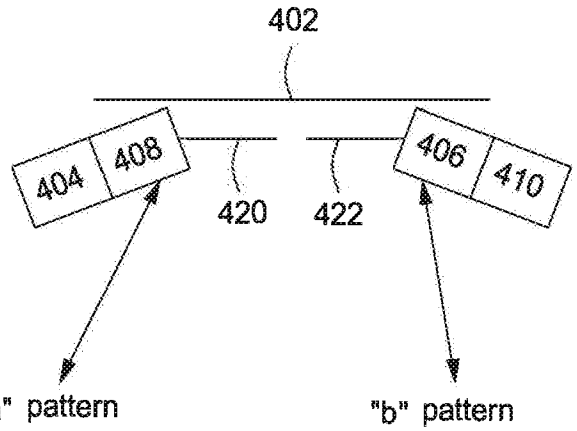


FIG. 4B

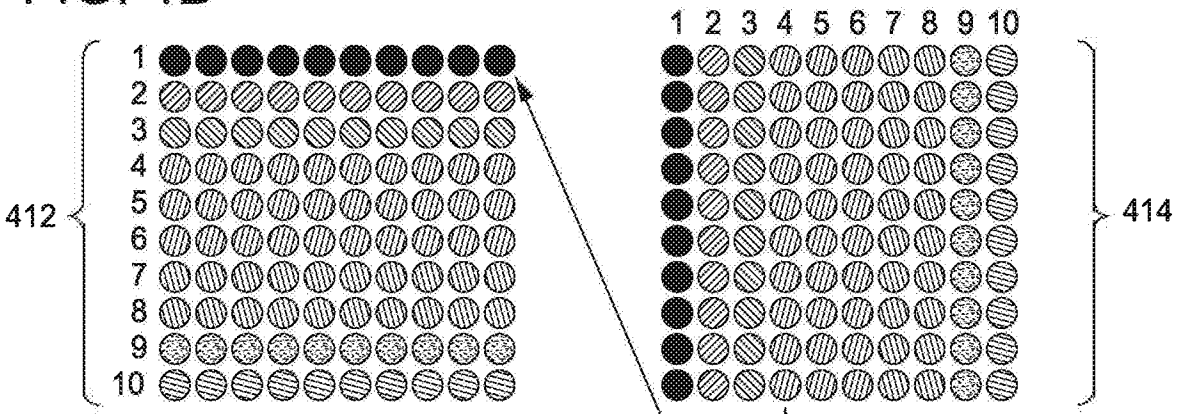
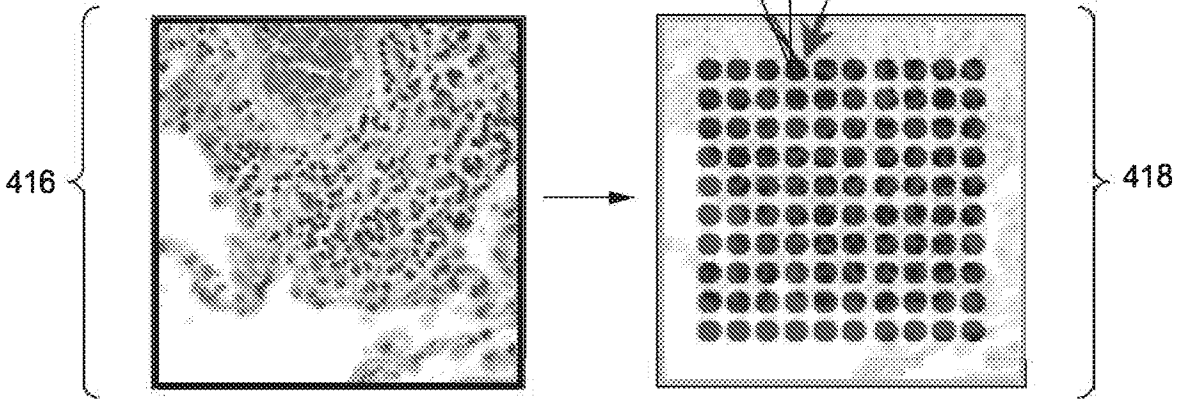


FIG. 4C



Electronic Acknowledgement Receipt

EFS ID:	36379194
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Lisa Gray
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	21-JUN-2019
Filing Date:	14-FEB-2019
Time Stamp:	17:40:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		47706-0004003response.pdf	77249 9997c84220914c909ebba9db3b33c6a0890648d2	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Supplemental Response or Supplemental Amendment	1	1
	Drawings-only black and white line drawings	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:					
Information:					
2	Drawings-only black and white line drawings	47706-0004003replacementfig.pdf	1688578 f918e52fcbbb38daafdee0c6e8ab2efff2a4a6f4	no	1
Warnings:					
Information:					
			Total Files Size (in bytes):	1765827	

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

REMARKS

A replacement drawing sheet for Figure 4 is submitted herewith.

No new matter has been added.

Apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: June 21, 2019

/Cameron Luitjens/

Cameron M. Luitjens, Ph.D., J.D.
Reg. No. 58,674

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61959512.doc

Amendments to the Drawings:

The attached replacement sheet of drawings includes changes to Figs. 4A-4C and replaces the original sheet including Figs. 4A-C.

In Figures 4A, 4B, and 4C, each of the figures have been labeled as "FIG. 4A," "FIG. 4B," and "FIG. 4C," respectively.

Attachments following last page of this Amendment:

Replacement Sheet (1 page)

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	16276235
Filing Date	14-Feb-2019
First Named Inventor	Mark CHEE
Attorney Docket Number	47706-0004003
Title of Invention	Spatially Encoded Biological Assays

☒ 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
Prognosys Biosciences, 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)

16435176 filed on 06/07/2019

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 58674

○ 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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*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:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Attorney Docket Number:	47706-0004003

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	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16276235

Filing Date: 14-Feb-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on June 25, 2019

☒ 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:	36397743
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	25-JUN-2019
Filing Date:	14-FEB-2019
Time Stamp:	11:26:08
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$160
RAM confirmation Number	062519INTEFSW00012149061050
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	33896 eba4dd30d19c55afa13b4c935aada3365c679bf3	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30653 9ed54c4c162d1545a9b9050e7fa555c8ea992dc2	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	64549		

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

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

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New International Application Filed with the USPTO as a Receiving Office

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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	16276235
Filing Date	14-Feb-2019
First Named Inventor	Mark CHEE
Attorney Docket Number	47706-0004003
Title of Invention	Spatially Encoded Biological Assays

☒ 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
Prognosys Biosciences, 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)

16437726 filed on 06/11/2019

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

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◉ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application

Registration Number 58674

○ 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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*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:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Attorney Docket Number:	47706-0004003

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	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16276235

Filing Date: 14-Feb-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on June 27, 2019

☒ 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:	36425720
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	27-JUN-2019
Filing Date:	14-FEB-2019
Time Stamp:	12:10:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$160
RAM confirmation Number	062719INTEFSW00013551061050
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	33896 0a600cc5061bd624401aed18b5a7686f82e1b30c	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30654 8112bab15fe4654d0b2d802be65cc694cc963cfa	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	64550		

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

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

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New International Application Filed with the USPTO as a Receiving Office

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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	16276235
Filing Date	14-Feb-2019
First Named Inventor	Mark CHEE
Attorney Docket Number	47706-0004003
Title of Invention	Spatially Encoded Biological Assays

☒ 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
Prognosys Biosciences, 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)

16435295 filed on 06/07/2019

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.

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◉ 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.

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I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

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Registration Number 58674

○ 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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*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:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Attorney Docket Number:	47706-0004003

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	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16276235

Filing Date: 14-Feb-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on July 2, 2019

☒ 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:	36464182
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	02-JUL-2019
Filing Date:	14-FEB-2019
Time Stamp:	13:45:45
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$160
RAM confirmation Number	070319INTEFSW00000008061050
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	33897 f045e118a867e9a7c6805ad90c2e42e2f4d26dba	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30652 0c365034f3745372d42b3e7253b1a8ed95c6992a	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	64549		

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

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

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New International Application Filed with the USPTO as a Receiving Office

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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	16276235
Filing Date	14-Feb-2019
First Named Inventor	Mark CHEE
Attorney Docket Number	47706-0004003
Title of Invention	Spatially Encoded Biological Assays

☒ 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
Prognosys Biosciences, Inc.	100%

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16437637 filed on 06/11/2019

16443771 filed on 06/17/2019

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○ Micro Entity

◉ Regular Undiscounted

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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 58674

○ 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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

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Electronic Patent Application Fee Transmittal

Application Number:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Attorney Docket Number:	47706-0004003

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	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**160**

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 16276235

Filing Date: 14-Feb-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on July 9, 2019

☒ 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:	36515713
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	09-JUL-2019
Filing Date:	14-FEB-2019
Time Stamp:	09:07:39
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$160
RAM confirmation Number	070919INTEFSW00009847061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	34694 179ab59a1a15c6cb9d5cbbc2a08eee7a265d5dcf	no	2
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30653 db3561052ce88d0a09e1c1e94538b93fe699a33b	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	65347		

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

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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NOTICE OF ALLOWANCE AND FEE(S) DUE

159367 7590 08/07/2019

FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
MARTINELL, JAMES	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 08/07/2019

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

TITLE OF INVENTION: Spatially Encoded Biological Assays

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	11/07/2019

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 and an extra copy of the form should be submitted. 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.

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)

159367 7590 08/07/2019
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10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

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.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

TITLE OF INVENTION: Spatially Encoded Biological Assays

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	11/07/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
MARTINELL, JAMES	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 _____
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. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

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EXAMINER	
MARTINELL, JAMES	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 08/07/2019

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.

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.

Notice of Allowability	Application No. 16/276,235	Applicant(s) CHEE, Mark S.	
	Examiner JAMES MARTINELL	Art Unit 1634	AIA (FITF) Status No

-- 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 See Continuation Sheet.
   ☐ 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 2-28 and 30-32 . 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 areply 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 5/16/19.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☑ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/JAMES MARTINELL/
Primary Examiner, Art Unit 1634

Continuation of 1. This communication is responsive to: the communications filed 5/15/19; 5/16/19; 6/6/19; 6/11/19; 6/20/19; 6/21/19; 6/27/19; and 7/9/19

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

EXAMINER'S COMMENT

The Drawings filed February 14, 2019 and June 11, 2019 are accepted by the examiner.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JAMES MARTINELL whose telephone number is (571) 272-0719.

The examiner works a flexible schedule and can be reached by phone and voice mail. Alternatively, a request for a return telephone call may be e-mailed to james.martinell@uspto.gov. Since e-mail communications may not be secure, it is suggested that information in such requests be limited to name, phone number, and the best time to return the call.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave T. Nguyen, can be reached on (571) 272-0731.

OFFICIAL FAX NUMBER

The fax phone number for the organization where this application or proceeding is assigned is (571) 273-8300. Any Official Communication to the USPTO should be faxed to this number.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to (571) 272-0547.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/JAMES MARTINELL/
Primary Examiner, Art Unit 1634

Notice of References Cited	Application/Control No. 16/276,235	Applicant(s)/Patent Under Reexamination CHEE, Mark S.	
	Examiner JAMES MARTINELL	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					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	None cited
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	Art Unit
	JAMES MARTINELL	1634

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* 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
Parent SN 13/080,616 (PN 9,371,598)	04/10/2019	JM
Parent SN 15/187,661 (pending)	04/10/2019	JM
STN search (2 sets of printouts attached)	04/12/2019	JM
EAST text search (printout attached)	04/14/2019	JM
Inventor search (PALM)	04/14/2019	JM
STN search (printout attached)	07/30/2019	JM
EAST text search (printout attached)	07/31/2019	JM
EAST text search (printout attached)	08/01/2019	JM

/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	

Curio EX1010 p.102
Curio v Prognosys

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

Interference Search			
US Class/CPC Symbol	**US Subclass/CPC Group**	**Date**	**Examiner**
	PGPUBS (printout attached)	07/31/2019	JM
	PGPUBS (printout attached)	08/01/2019	JM

/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	

Curio EX1010 p.103
Curio v Prognosys

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	Art Unit
	JAMES MARTINELL	1634

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	04/14/2019	08/01/2019							
	1	-	-							
1	2	✓	=							
2	3	✓	=							
3	4	✓	=							
4	5	✓	=							
5	6	✓	=							
6	7	✓	=							
7	8	✓	=							
8	9	✓	=							
9	10	✓	=							
10	11	✓	=							
11	12	✓	=							
12	13	✓	=							
13	14	✓	=							
14	15	✓	=							
15	16	✓	=							
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17	18	✓	=							
18	19	✓	=							
19	20	✓	=							
20	21	✓	=							
21	22	✓	=							
22	23	✓	=							
23	24	✓	=							
24	25	✓	=							
25	26	✓	=							
26	27	✓	=							
27	28	✓	=							
	29	✓	-							
28	30	✓	=							
29	31	✓	=							
30	32		=							

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

CPC						
Symbol					**Type**	**Version**
C12Q	/	1	/	6837	F	2013-01-01
C40B	/	30	/	04	I	2013-01-01
C40B	/	60	/	04	I	2013-01-01
G01N	/	33	/	6845	I	2013-01-01
C12Q	/	1	/	6809	I	2013-01-01
C12Q	/	1	/	6834	I	2013-01-01
C12Q	/	1	/	6841	I	2013-01-01
C12Q	/	1	/	6869	I	2013-01-01
C12Q	/	1	/	6804	I	2013-01-01
C12Q	/	1	/	6874	I	2013-01-01
G01N	/	33	/	5308	I	2013-01-01
G01N	/	2458	/	10	A	2013-01-01

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
C12Q	/	1	/	6837	I	1	1	2013-01-01
C12Q	/	2565	/	515	A	1	2	2013-01-01
C12Q	/	1	/	6869	I	2	1	2013-01-01
C12Q	/	2535	/	122	A	2	2	2013-01-01
C12Q	/	2543	/	10	A	2	3	2013-01-01
C12Q	/	2563	/	179	A	2	4	2013-01-01
C12Q	/	1	/	6841	I	3	1	2013-01-01
C12Q	/	2535	/	122	A	3	2	2013-01-01
C12Q	/	2543	/	10	A	3	3	2013-01-01
C12Q	/	2563	/	179	A	3	4	2013-01-01
C12Q	/	1	/	6834	I	4	1	2013-01-01
C12Q	/	2535	/	122	A	4	2	2013-01-01
C12Q	/	2543	/	10	A	4	3	2013-01-01
C12Q	/	2563	/	179	A	4	4	2013-01-01
C12Q	/	1	/	6809	I	5	1	2013-01-01

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	01 August 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
C12Q	/	2535	/	122	A	5	2	2013-01-01
C12Q	/	2543	/	10	A	5	3	2013-01-01
C12Q	/	2563	/	179	A	5	4	2013-01-01

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	01 August 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office

Part of Paper No.: 20190801

Curio EX1010-p. 906
Curio v Prognosys

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C12Q	/	1	/	68
G01N	/	33	/	53

NON-CLAIMED				
	/		/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**
435	6.11

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE (Assistant Examiner) (Date)	**Total Claims Allowed:** 30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634 (Primary Examiner) 01 August 2019 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	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	9	10	18	19	27	28								
1	2	10	11	19	20	-	29								
2	3	11	12	20	21	28	30								
3	4	12	13	21	22	29	31								
4	5	13	14	22	23	30	32								
5	6	14	15	23	24										
6	7	15	16	24	25										
7	8	16	17	25	26										
8	9	17	18	26	27										

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634 (Primary Examiner)	01 August 2019 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

U.S. Patent and Trademark Office

Part of Paper No.: 20190801

Curio EX1010-p. 908
Curio v Prognosys

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BIB DATA SHEET

CONFIRMATION NO. 7944

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
16/276,235	02/14/2019	435	1634	47706-0004003

APPLICANTS
Prognosys Biosciences, Inc., San Diego, CA;

INVENTORS
Mark S. CHEE, San Diego, CA;

**** CONTINUING DATA ***************************
This application is a CON of 15/187,661 06/20/2016 PAT 10308982
which is a CON of 13/080,616 04/05/2011 PAT 9371598
which claims benefit of 61/321,124 04/05/2010

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****
03/04/2019

Foreign Priority claimed ❑ Yes ☑ No 35 USC 119(a-d) conditions met ❑ Yes ☑ No Verified and Acknowledged /JAMES MARTINELL/ Examiner's Signature	❑ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	5	1	1

ADDRESS
FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022
UNITED STATES

TITLE
Spatially Encoded Biological Assays

FILING FEE RECEIVED		
1285	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
L1	124	((array$4 or microarray$4 or support$4 or surface) near5 (dna or (nucleic adj acid) or protein or antibod$4)) same (probe near5 pool$6) same (probe near5 sequenc$6)	US-PGPUB; USPAT	OR	ON	2019/08/01 12:05
L2	6681	(((high adj density) or (high adj rsolution)) near5 (array$4 or microarray$4)) near10 (dna or (nucleic adj acid) or protein or antibod$4)	US-PGPUB; USPAT	OR	ON	2019/08/01 12:07
L3	6771	l1 or l2	US-PGPUB; USPAT	OR	ON	2019/08/01 12:23
L4	3339885	@rlad<"20110405"	US-PGPUB; USPAT	OR	ON	2019/08/01 12:24
L5	5018	l3 and l4	US-PGPUB; USPAT	OR	ON	2019/08/01 12:24
L6	248592	@pd>"20190414"	US-PGPUB; USPAT	OR	ON	2019/08/01 12:25
L7	27	l5 and l6	US-PGPUB; USPAT	OR	ON	2019/08/01 12:25

8/ 1/ 2019 12:26:39 PM
C:\ Users\ jmartinell\ Documents\ EAST\ Workspaces\ 16-276235-c.wsp

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 47706-0004003	Application No. 16/276,235
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Prognosys Biosciences, Inc.	
(37 CFR §1.98(b))		Filing Date February 14, 2019	Group Art Unit 1634

cU.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	1.	KR 10-20090081260	7/28/2009	KR				
	2.	WO 2001/090415	11/29/2001	WO				
	3.	WO 2002/059364	8/1/2002	WO				

Abstract and Fig. 4 only

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document

Examiner Signature	/JAMES MARTINELL/	Date Considered	07/29/2019

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.M/

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 NEWS  2  JAN  4  DWPI Manual Code revision in 2019
 NEWS  3  JAN 29  The 2019 Version of MEDLINE Now Available on STN(TM)
 NEWS  4  FEB  4  INPADOC/INPAFAM: Major enhancements for PCT
 NEWS  5  FEB 26  FRFULL and GBFULL reloaded
 NEWS  6  MAR  7  Search Limits Significantly Expanded Exclusively in STNext(R)
 NEWS  7  MAR  7  New Key Terms field supports efficient full-text searching
                  and results evaluation in Patent Full-text Databases
 NEWS  8  MAR  8  CPC Combination Sets Now Fully Available in Derwent
                  World Patents Index
 NEWS  9  MAR 25  Derwent World Patent Index Coverage Expanded to Include
                  Eight Former Soviet Republics and the Eurasian Patent
                  Organization (EAPO)
 NEWS 10  APR  3  Derwent World Patents Index and Derwent Markush
                  Resource (DWPIM) Now Updated Twice Weekly
 NEWS 11  APR 11  Updates in database World Surface Coatings Abstracts
                  (file WSCA) have resumed
 NEWS 12  APR 18  New legal status codes for US patent applications and
                  granted patents in INPADOC
 NEWS 13  APR 30  Derwent Markush Resource (DWPIM) Database Enhancements
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=> file biosis caplus medline
COST IN U.S. DOLLARS                                SINCE FILE       TOTAL
                                                      ENTRY     SESSION
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=> s (spatial(20a)(bead? or microbead? or sphere or microsphere?) and target? and (cod? or barcod? or tag?))/bi,ab
L1             8 (SPATIAL(20A)(BEAD? OR MICROBEAD? OR SPHERE OR MICROSPHERE?)
                 AND TARGET? AND (COD? OR BARCOD? OR TAG?))/BI,AB

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PROCESSING COMPLETED FOR L1
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L4             5 L3 NOT 2018/PY

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L5             5 L4 NOT 2017/PY

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L6             4 L5 NOT 2016/PY

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L9             2 L8 NOT 2013/PY

=> s l9 not 2012/py
L10            1 L9 NOT 2012/PY

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L7             2 S L6 NOT 2015/PY
L8             2 S L7 NOT 2014/PY
L9             2 S L8 NOT 2013/PY
L10            1 S L9 NOT 2012/PY

=> d l10 1 bib ab
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L10 ANSWER 1 OF 1 CAPLUS COPYRIGHT 2019 ACS on STN
AN 2011:246257 CAPLUS Full-text
DN 155:164894
TI SearchFill: A stochastic optimization code for detecting atomic vacancies in crystalline and non-crystalline systems
AU Davis, Sergio M.; Belonoshko, Anatoly B.; Johansson, Boerje
CS Applied Materials Physics, Department of Materials Science and Engineering, KTH, Stockholm, SE-100 44, Swed.
SO Computer Physics Communications (2011), 182(5), 1105-1110
CODEN: CPHCBZ; ISSN: 0010-4655
DOI 10.1016/j.cpc.2010.12.009
PB Elsevier B.V.
DT Journal
LA English
AB We present an implementation of a stochastic optimization algorithm applied to location of atomic vacancies. Our method labels an empty point in space as a vacancy site, if the total spatial overlap of a "virtual sphere", centered around the point, with the surrounding atoms (and other vacancies) falls below a tolerance parameter. A Metropolis-like algorithm displaces the vacancies randomly, using an "overlap temperature" parameter to allow for acceptance of moves into regions with higher overlap, thus avoiding local min. Once the algorithm has targeted a point with low overlap, the overlap temperature is decreased, and the method works as a steepest descent optimization. Our method, with only two free parameters, is able to detect the correct number and coordinates of vacancies in a wide spectrum of condensed-matter systems, from crystals to amorphous solids, in fact in any given set of atomic coordinates, without any need of comparison with a reference initial structure.
OSC.G 3 THERE ARE 3 CAPLUS RECORDS THAT CITE THIS RECORD (3 CITINGS)
RE.CNT 11 THERE ARE 11 CITED REFERENCES AVAILABLE FOR THIS RECORD
ALL CITATIONS AVAILABLE IN THE RE FORMAT

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FILE 'BIOSIS, CAPLUS, MEDLINE' ENTERED AT 10:35:37 ON 30 JUL 2019
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L2 8 DUP REM L1 (0 DUPLICATES REMOVED)
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L4 5 S L3 NOT 2018/PY
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L6 4 S L5 NOT 2016/PY
L7 2 S L6 NOT 2015/PY
L8 2 S L7 NOT 2014/PY
L9 2 S L8 NOT 2013/PY
L10 1 S L9 NOT 2012/PY

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COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	141.47	141.62

STN INTERNATIONAL LOGOFF AT 10:38:07 ON 30 JUL 2019

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
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L2	3339522	@rlad<"20110405"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/07/31 16:29
L3	3137	l1 and l2	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/07/31 16:29
L4	1202	l3 and (code or coded or barcode or barcoded or coding or barcoding or zipcode)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	ON	2019/07/31 16:30

7/ 31/ 2019 5:28:53 PM
C:\ Users\ jmartinell\ Documents\ EAST\ Workspaces\ 16-276235-b.wsp

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/276,235	Filing Date 02/14/2019	☐ 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 $460 =	
☐ 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	08/26/2019	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))	* 1	Minus	*** 3	= 0		x $460 =	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.	SLIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/DEIDRE C KING-WOODWARD/
*** 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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Request for Continued Examination (RCE) Transmittal

Address to:
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P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	16/276,235
Filing Date	February 14, 2019
First Named Inventor	Mark S. Chee
Art Unit	1634
Examiner Name	James Martinell
Attorney Docket Number	47706-0004003

This is a Request for Continued Examination (RCE) under 37 CFR 1 .114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. ☐ Other _____

   b. ☒ Enclosed

      i. ☒ Amendment/Reply

      ii. ☐ Affidavit(s)/ Declaration(s)

      iii. ☒ Information Disclosure Statement (IDS)

      iv. ☐ Other _____

2. Miscellaneous

   a. ☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   The Director is hereby authorized to charge the following fees any underpayment of fees, or credit any overpayments to

   a. ☒ Deposit Account No. 06-1050.

      i. ☒ RCE fee required under 37 CFR 1.17(e)

      ii ☐ Extension of time fee (37 CFR 1.136 and 1.17)

      iii ☒ Other any deficiencies

   b. ☐ Check in the amount of $ _____ enclosed

   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

   d. ☐ Payment by EFS-Web

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SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Teresa A. Lavoie, Ph.D., Reg. No. 42,782/	Date	August 26, 2019
Name (Print/Type)	Teresa A. Lavoie, Ph.D.	Registration No.	42,782

CERTIFICATE OF MAILING OR TRANSMISSION

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Signature			
Name (Print/Type)		Date	

This collection of information is required by 37 CFR 1.114. 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 1.11 and 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: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (03-14)

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

First Named Inventor:	Mark S. Chee	Non provisional Application Number (if known):	16/276,235
Title of Invention:	Spatially Encoded Biological Assays		

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), the prioritized examination fee set forth in 37 CFR 1.17(c), and if not already paid, the publication fee set forth in 37 CFR 1.18(d) have been filed with the request. 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.

3. The applicable box is checked below:

I. ☐ Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)

i. (a) The application is an original non provisional 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 non provisional 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 non provisional 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 /Teresa A. Lavoie, Reg. No. 42,782/	Date August 26, 2019
Name (Print/Typed) Teresa A. Lavoie, Ph.D.	Practitioner Registration Number 42,782

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 _____ forms are submitted.

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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 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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as indicated on the following pages.

Amendment to the Claims begin on page 2 of this paper.

Remarks being on page 7 of this paper.

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

1. (**Canceled**)

2. (**Previously Presented**) A method for determining a spatial location of a biological molecule of a tissue sample comprising

(a) providing a plurality of beads in a spatial pattern, wherein the plurality of beads comprise a plurality of binding agents, wherein a binding agent of the plurality of binding agents (i) comprises a coding identifier having a nucleic acid sequence and (ii) is configured to interact with a biological molecule of a tissue sample, wherein the coding identifier corresponds to a location in the spatial pattern at which the binding agent interacts with a biological molecule of a tissue sample;

(b) contacting the plurality of beads with the tissue sample such that the binding agent interacts with the biological molecule; and

(c) using the coding identifier to identify the location in the spatial pattern where the binding agent interacted with the biological molecule, thereby determining a spatial location of the biological molecule of the tissue sample.

3. (**Previously Presented**) The method of claim 2, wherein the plurality of beads is provided on a substrate surface.

4. (**Previously Presented**) The method of claim 3, wherein the plurality of beads is provided in wells on the substrate surface.

5. (**Previously Presented**) The method of claim 2, wherein the biological molecule is a nucleic acid.

6. (**Previously Presented**) The method of claim 5, wherein the binding agent of the plurality of binding agents comprises a nucleic acid.

7. (**Previously Presented**) The method of claim 6, wherein the binding agent interacts with the biological molecule via hybridization.

8. (**Previously Presented**) The method of claim 2, wherein the binding agent and coding identifier are provided as a single molecule.

9. (**Previously Presented**) The method of claim 8, wherein the binding agent and coding identifier are provided as a single molecule through nucleic acid synthesis.

10. (**Previously Presented**) The method of claim 8, wherein the binding agent and coding identifier are provided as a ligation product.

11. (**Previously Presented**) The method of claim 8, wherein the binding agent and coding identifier are provided as a single molecule through chemical coupling.

12. (**Previously Presented**) The method of claim 2, wherein the plurality of binding agents comprises an additional binding agent, which (i) comprises an additional coding identifier and (ii) is configured to interact with another biological molecule of the tissue sample, wherein the additional coding identifier is different from the coding identifier of step (a)(i).

13. (**Previously Presented**) The method of claim 12, wherein the additional coding identifier corresponds to a location in the spatial pattern at which the additional binding agent of the plurality of binding agents interacts with said another biological molecule of the tissue sample.

14. (**Previously Presented**) The method of claim 13, wherein the location in the spatial pattern at which the binding agent interacts with the biological molecule and the additional binding agent interacts with said another biological molecule is different.

15. (**Previously Presented**) The method of claim 14, wherein the spatial location of the biological molecule and said another biological molecule of the tissue sample is different.

16. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining the spatial locations of a plurality of biological molecules of the tissue sample simultaneously.

17. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining presence or absence of the biological molecule at the location at which it is identified.

18. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining the relative amount of the biological molecule at the location at which it is identified.

19. (**Previously Presented**) The method of claim 2, wherein step (c) comprises determining all or a portion of the nucleic acid sequence of the coding identifier.

20. (**Previously Presented**) The method of claim 2, wherein step (c) comprises sequencing all or a portion of the nucleic acid sequence of the coding identifier.

21. (**Previously Presented**) The method of claim 2, wherein prior to step (c), spatial organization of cells in the tissue sample is preserved.

22. (**Previously Presented**) The method of claim 2, wherein the biological molecule of the biological sample is selected from a protein, a nucleic acid, a lipid, a carbohydrate, and an ion.

23. (**Previously Presented**) The method of claim 22, wherein the biological molecule is part of a multicomponent complex.

24. (**Previously Presented**) The method of claim 2, wherein the tissue sample is a fresh tissue sample.

25. (**Previously Presented**) The method of claim 2, wherein the tissue sample is a preserved tissue sample.

26. (**Previously Presented**) The method of claim 25, wherein the preserved tissue sample is a frozen tissue sample.

27. (**Currently Amended**) The method of ~~claim 26~~ claim 25, wherein the preserved tissue sample is a paraformalin-fixed paraffin-embedded (FFPE) tissue sample.

28. (**Previously Presented**) The method of claim 3 claim 2, further comprising analyzing gene expression in the tissue sample.

29. (**Canceled**)

30. (**Previously Presented**) The method of claim 2, wherein the location of the binding agent is known prior to step (b).

31. (**Previously Presented**) The method of claim 2, wherein the binding agent is immobilized directly or indirectly onto a bead of the plurality of beads.

32. (**Previously Presented**) The method of claim 2, wherein the biological molecule of the biological sample is a protein.

REMARKS

Upon entry of the present amendment, claims 2-28 and 30-32 will be pending in this application. Claims 2-28 and 30-32 were indicated as allowed in the Notice of Allowance mailed August 7, 2019. Claim 27 is amended herein to depend from claim 25. No new matter is added. Applicant respectfully requests reconsideration and allowance of the pending claims.

CONCLUSION

In view of the foregoing, Applicant believes all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance is respectfully requested. If the Examiner believes a telephone conference would expedite prosecution of this application, please contact the undersigned at (612) 335-5070. Please apply any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: August 26, 2019

/Teresa A. Lavoie, Reg. No. 42,782/
Teresa A. Lavoie, Ph.D.
Reg. No. 42,782

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

INFORMATION DISCLOSURE STATEMENT

Please consider the documents listed on the disclosure form submitted with this statement. Copies of the documents are being submitted with this statement to the extent required under 37 C.F.R. § 1.98(a)(2).

This statement is being filed with a Request for Continued Examination. No fee is required.

Certification under 37 C.F.R. § 1.704(d)

Each item of information contained in this information disclosure statement (i) was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in 37 C.F.R. § 1.56(c) more than thirty days prior to the filing of the information disclosure statement; or (ii) is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement. Accordingly, no reduction in patent term adjustment is warranted by the filing of this information disclosure statement.

Apply any necessary charges or credits applicable under 37 C.F.R. § 1.17(p) to deposit account 06-1050, referencing the above attorney docket number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: August 26, 2019

/Teresa A. Lavoie, Ph.D., Reg. No. 42,782/

Teresa A. Lavoie, Ph.D.
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62059642.doc

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary)		47706-0004003	16/276,235
		Applicant: Prognosys Biosciences, Inc.	
(37 CFR §1.98(b))		Filing Date: February 14, 2019	Group Art Unit: 1634

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	5,559,032	9/1/1996	Porneroy			
	2.	6,673,620	1/6/2004	Loeffler			
	3.	6,699,710	3/1/2004	Kononen			
	4.	20020040275	4/4/2002	Cravatt			
	5.	20110275077	11/10/2011	James			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document

Examiner Signature	Date Considered

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Electronic Patent Application Fee Transmittal

Application Number:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Teresa A. Lavoie/Shannon Stadnick
Attorney Docket Number:	47706-0004003

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4000	4000
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
RCE- 1ST REQUEST	1801	1	1300	1300
		Total in USD ($)		**5300**

Electronic Acknowledgement Receipt

EFS ID:	36980777
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Teresa A. Lavoie/Shannon Stadnick
Filer Authorized By:	Teresa A. Lavoie
Attorney Docket Number:	47706-0004003
Receipt Date:	26-AUG-2019
Filing Date:	14-FEB-2019
Time Stamp:	16:54:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$5300
RAM confirmation Number	E20198PG54567360
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	RCE.pdf	117349 361d5a9b2ca9bc72a9983a72d9cda2a0f7d101c4	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
2	TrackOne Request	Track1.pdf	148463 d93bc483e13b0720f9ea6e05d7b5ec0fdda a7cbc	no	2
Warnings:					
Information:					
3		Prelim_Amend.pdf	92853 32d04faf2e6a5a7b85d16ecad398a60c07c6 13af	yes	7

Multipart Description/PDF files in .zip description

Document Description	Start	End
Preliminary Amendment	1	1
Claims	2	6
Applicant Arguments/Remarks Made in an Amendment	7	7

Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Form (SB08)	IDS_Transmittal.pdf	74038 5d243a15d2043dc7057d6960184c1db9d1 82ba20	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					

5	Information Disclosure Statement (IDS) Form (SB08)	IDS_1449.pdf	123430 5992c3bc4deb1f2bcd2b794a3c168a1dedbc0f1f	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
6	Fee Worksheet (SB06)	fee-info.pdf	32191 8e29ac8f50b9fa453d73aef2dd8f211abef2dc07	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	588324		

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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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Conf. No.	:	7944
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

SUPPLEMENTAL REPLY

Please consider the following Supplemental Reply.

Amendments to Claims:
Listing of Claims.

1.-32. (**Canceled**).

33. (**New**) A method for determining a spatial location of a biological molecule of a tissue sample comprising

(a) providing a plurality of beads, wherein the plurality of beads comprise a plurality of binding agents, wherein a binding agent of the plurality of binding agents (i) comprises a coding identifier having a nucleic acid sequence and (ii) is configured to interact with a biological molecule of a tissue sample, wherein the coding identifier corresponds to a location at which the binding agent interacts with a biological molecule of a tissue sample;

(b) contacting the plurality of beads with the tissue sample such that the binding agent interacts with the biological molecule; and

(c) using the coding identifier to identify the location where the binding agent interacted with the biological molecule, thereby determining a spatial location of the biological molecule of the tissue sample.

34. (**New**) The method of claim 33, wherein the plurality of beads is provided on a substrate surface.

35. (**New**) The method of claim 34, wherein the plurality of beads is provided in wells on the substrate surface.

36. (**New**) The method of claim 33, wherein the biological molecule is a nucleic acid.

37. (**New**) The method of claim 36, wherein the binding agent of the plurality of binding agents comprises a nucleic acid.

38. (**New**) The method of claim 37, wherein the binding agent interacts with the biological molecule via hybridization.

39. (**New**) The method of claim 33, wherein the binding agent and coding identifier are provided as a single molecule.

40. (**New**) The method of claim 39, wherein the binding agent and coding identifier are provided as a single molecule through nucleic acid synthesis.

41. (**New**) The method of claim 39, wherein the binding agent and coding identifier are provided as a ligation product.

42. (**New**) The method of claim 39, wherein the binding agent and coding identifier are provided as a single molecule through chemical coupling.

43. (**New**) The method of claim 33, wherein the plurality of binding agents comprises an additional binding agent, which (i) comprises an additional coding identifier and (ii) is configured to interact with another biological molecule of the tissue sample, wherein the additional coding identifier is different from the coding identifier of step (a)(i).

44. (**New**) The method of claim 43, wherein the additional coding identifier corresponds to a location at which the additional binding agent of the plurality of binding agents interacts with said another biological molecule of the tissue sample.

45. (**New**) The method of claim 44, wherein the location at which the binding agent interacts with the biological molecule and the additional binding agent interacts with said another biological molecule is different.

46. (**New**) The method of claim 45, wherein the spatial location of the biological molecule and said another biological molecule of the tissue sample is different.

47. (**New**) The method of claim 33, wherein step (c) comprises determining the spatial locations of a plurality of biological molecules of the tissue sample simultaneously.

48. (**New**) The method of claim 33, wherein step (c) comprises determining presence or absence of the biological molecule at the location at which it is identified.

49. (**New**) The method of claim 33, wherein step (c) comprises determining the relative amount of the biological molecule at the location at which it is identified.

50. (**New**) The method of claim 33, wherein step (c) comprises determining all or a portion of the nucleic acid sequence of the coding identifier.

51. (**New**) The method of claim 33, wherein step (c) comprises sequencing all or a portion of the nucleic acid sequence of the coding identifier.

52. (**New**) The method of claim 33, wherein prior to step (c), spatial organization of cells in the tissue sample is preserved.

53. (**New**) The method of claim 33, wherein the biological molecule of the biological sample is selected from a protein, a nucleic acid, a lipid, a carbohydrate, and an ion.

54. (**New**) The method of claim 53, wherein the biological molecule is part of a multicomponent complex.

55. (**New**) The method of claim 33, wherein the tissue sample is a fresh tissue sample.

56. (**New**) The method of claim 33, wherein the tissue sample is a preserved tissue sample.

57. (**New**) The method of claim 56, wherein the preserved tissue sample is a frozen tissue sample.

58. (**New**) The method of claim 56, wherein the preserved tissue sample is a paraformalin-fixed paraffin-embedded (FFPE) tissue sample.

59. (**New**) The method of claim 33, further comprising analyzing gene expression in the tissue sample.

60. (**New**) The method of claim 33, wherein the location of the binding agent is known prior to step (b).

61. (**New**) The method of claim 33, wherein the binding agent is immobilized directly or indirectly onto a bead of the plurality of beads.

62. (**New**) The method of claim 33, wherein the biological molecule of the biological sample is a protein.

REMARKS

Applicant thanks the Examiner for the courtesy of the telephone call on September 3, 2019. On the call, the Examiner indicated that the claims submitted in the Track 1 RCE filed on August 26, 2019 were allowable, and further requested to perform an Examiner's amendment to (now cancelled) claim 28 to remove the superfluous "claim 3" in the preamble of the claim in order to clarify the proper dependency of the claim (i.e., properly dependent from claim 2, which was also recited in the claim). Applicant agreed with the substance of the Examiner's proposed amendment, noted with appreciation the Examiner's indication that the claims were allowable, and further noted that certain amendments previously submitted with the Response to Office Action dated May 15, 2019 and previously entered by the Office were inadvertently not included in the claims as filed with the Track 1 RCE. Applicant requested clarification on how to best clarify and correct the record regarding the final claim language. The Examiner suggested cancelling the previously pending claims, and submitting new claims which would incorporate 1) the prior amendments of the Response dated May 15, 2019, 2) the amendments in the Track 1 RCE submitted August 26, 2019, and 3) the proposed Examiner's amendment. Applicant has done so with the present claim set. Accordingly, after entry of the present amendment, former claims 1-32 are cancelled, with new claims 33-61 now pending. Claims 33-61 find support throughout the Specification as filed, and directly correlate with the prior pending claims as detailed above. No new matter has been added.

Given all of the above, Applicants respectfully request reconsideration of the newly submitted claims and issuance of a notice of allowance in the present case. The Examiner is invited to telephone the undersigned attorney if such would expedite prosecution.

Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: September 3, 2019

/Teresa A. Lavoie, Reg. No. 42,782/

Teresa A. Lavoie, Ph.D.
Reg. No. 42,782

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 766-2006
Facsimile: (877) 769-7945

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Electronic Acknowledgement Receipt

EFS ID:	37055831
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Teresa A. Lavoie/Lisa Gray
Filer Authorized By:	Teresa A. Lavoie
Attorney Docket Number:	47706-0004003
Receipt Date:	03-SEP-2019
Filing Date:	14-FEB-2019
Time Stamp:	21:05:45
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		47706-0004003suppresponse.pdf	88420 3047a24a6efde7ead3ca2927a63c7bbfdac91bfe	yes	7

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Supplemental Response or Supplemental Amendment	1	1
	Claims	2	5
	Applicant Arguments/Remarks Made in an Amendment	6	7
Warnings:			
Information:			
	Total Files Size (in bytes):	88420	

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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NOTICE OF ALLOWANCE AND FEE(S) DUE

159367 7590 09/09/2019

FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
MARTINELL, JAMES	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 09/09/2019

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

TITLE OF INVENTION: SPATIALLY ENCODED BIOLOGICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	12/09/2019

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 and an extra copy of the form should be submitted. 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.

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)

159367 7590 09/09/2019
FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

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.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

TITLE OF INVENTION: SPATIALLY ENCODED BIOLOGICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	12/09/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
MARTINELL, JAMES	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 _____
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. _____

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.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

159367 7590 09/09/2019

FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
MARTINELL, JAMES	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 09/09/2019

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.

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.

Notice of Allowability	Application No. 16/276,235	Applicant(s) CHEE, Mark S.	
	Examiner JAMES MARTINELL	Art Unit 1634	AIA (FITF) Status No

-- 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 the communication filed 9/3/19.
   ☐ 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 33-62 . 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 8/26/19.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☑ Interview Summary (PTO-413), Paper No./Mail Date. 9/3/19.
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/JAMES MARTINELL/
Primary Examiner, Art Unit 1634

Examiner-Initiated Interview Summary	**Application No.** 16/276,235	**Applicant(s)** CHEE, Mark S.	
	Examiner JAMES MARTINELL	**Art Unit** 1634	**AIA (FITF) Status** No

All participants (applicant, applicant's representative, PTO personnel):

(1) JAMES MARTINELL. (3) _____.

(2) Dr. Lavoie. (4) _____.

Date of Interview: 03 September 2019.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐ 101 ☐ 112 ☐ 102 ☐ 103 ☑ Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 2-28 and 30-32.

Identification of prior art discussed: None.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

See Continuation Sheet.

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.

☐ Attachment

/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: The amendment to the claims filed 8/26/19 contained minor errors in regard to material that had been changed in an earlier amendment (filed 5/15/19); particularly in regard to claims 2,13, 14, and 28. It was agreed that applicant could cancel claims 2-28 and 30-32 and replace them with a clean copy of the claims and numbered 33-62 and in addition to have new claim 58 (corresponding to claim 27 in the 5/15/19 version of the claims) depend from new claim 56 (corresponding to claim 25 in the 5/15/19 version of the claims) as applicants had intended upon filing the Request for Continued Examination filed 8/26/19. The examiner indicated that such changes would put the application in condition for allowance.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	Art Unit
	JAMES MARTINELL	1634

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* 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
Parent SN 13/080,616 (PN 9,371,598)	04/10/2019	JM
Parent SN 15/187,661 (pending)	04/10/2019	JM
STN search (2 sets of printouts attached)	04/12/2019	JM
EAST text search (printout attached)	04/14/2019	JM
Inventor search (PALM)	04/14/2019	JM
STN search (printout attached)	07/30/2019	JM
EAST text search (printout attached)	07/31/2019	JM
EAST text search (printout attached)	08/01/2019	JM
EAST text search (printout attached)	09/03/2019	JM

/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	

Curio EX1010 p.451
Curio v Prognosys

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	Art Unit
	JAMES MARTINELL	1634

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	PGPUBS (printout attached)	07/31/2019	JM
	PGPUBS (printout attached)	08/01/2019	JM
	PGPUBS (printout attached)	09/03/2019	JM

/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	

Curio EX1010p052
Curio v Prognosys

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	Art Unit
	JAMES MARTINELL	1634

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☑ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	04/14/2019	08/01/2019							
	1	-	-							
	2	✓	-							
	3	✓	-							
	4	✓	-							
	5	✓	-							
	6	✓	-							
	7	✓	-							
	8	✓	-							
	9	✓	-							
	10	✓	-							
	11	✓	-							
	12	✓	-							
	13	✓	-							
	14	✓	-							
	15	✓	-							
	16	✓	-							
	17	✓	-							
	18	✓	-							
	19	✓	-							
	20	✓	-							
	21	✓	-							
	22	✓	-							
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	24	✓	-							
	25	✓	-							
	26	✓	-							
	27	✓	-							
	28	✓	-							
	29	✓	-							
	30	✓	-							
	31	✓	-							
	32		-							
1	33		=							
2	34		=							
3	35		=							
4	36		=							
5	37		=							
6	38		=							
7	39		=							
8	40		=							
9	41		=							
10	42		=							

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

CLAIM		DATE								
Final	Original	04/14/2019	08/01/2019							
11	43		=							
12	44		=							
13	45		=							
14	46		=							
15	47		=							
16	48		=							
17	49		=							
18	50		=							
19	51		=							
20	52		=							
21	53		=							
22	54		=							
23	55		=							
24	56		=							
25	57		=							
26	58		=							
27	59		=							
28	60		=							
29	61		=							
30	62		=							

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

CPC						
Symbol					**Type**	**Version**
C12Q	/	1	/	6837	F	2013-01-01
C40B	/	30	/	04	I	2013-01-01
G01N	/	33	/	6845	I	2013-01-01
C12Q	/	1	/	6809	I	2013-01-01
C12Q	/	1	/	6834	I	2013-01-01
C12Q	/	1	/	6841	I	2013-01-01
C12Q	/	1	/	6869	I	2013-01-01
C40B	/	60	/	04	I	2013-01-01
C12Q	/	1	/	6804	I	2013-01-01
C12Q	/	1	/	6874	I	2013-01-01
G01N	/	33	/	5308	I	2013-01-01
G01N	/	2458	/	10	A	2013-01-01

CPC Combination Sets							
Symbol					**Type**	**Set**	**Ranking**	**Version**
C12Q	/	1	/	6837	I	1	1	2013-01-01
C12Q	/	2565	/	515	I	1	2	2013-01-01
C12Q	/	1	/	6869	I	2	1	2013-01-01
C12Q	/	2535	/	122	I	2	2	2013-01-01
C12Q	/	2543	/	10	I	2	3	2013-01-01
C12Q	/	2563	/	179	I	2	4	2013-01-01
C12Q	/	1	/	6841	I	3	1	2013-01-01
C12Q	/	2535	/	122	I	3	2	2013-01-01
C12Q	/	2543	/	10	I	3	3	2013-01-01
C12Q	/	2563	/	179	I	3	4	2013-01-01
C12Q	/	1	/	6834	I	4	1	2013-01-01
C12Q	/	2535	/	122	I	4	2	2013-01-01
C12Q	/	2543	/	10	I	4	3	2013-01-01
C12Q	/	2563	/	179	I	4	4	2013-01-01
C12Q	/	1	/	6809	I	5	1	2013-01-01

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	04 September 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20190904

Curio EX1010-p. 955
Curio v Prognosys

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
C12Q	/	2535	/	122	I	5	2	2013-01-01
C12Q	/	2543	/	10	I	5	3	2013-01-01
C12Q	/	2563	/	179	I	5	4	2013-01-01

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634	04 September 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

U.S. Patent and Trademark Office — Part of Paper No.: 20190904

Curio EX1010-p. 956
Curio v Prognosys

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	1634

INTERNATIONAL CLASSIFICATION				
CLAIMED				
C12Q	/	1	/	68
G01N	/	33	/	53

NON-CLAIMED				
G01N33/68	/	33	/	68
C12Q1/6837	/	1	/	6837
C40B30/04	/	30	/	04
C12Q1/6809	/	1	/	6809
C12Q1/6834	/	1	/	6834
C12Q1/6841	/	1	/	6841
C12Q1/6869	/	1	/	6869
C40B60/04	/	60	/	04
C12Q1/6804	/	1	/	6804
C12Q1/6874	/	1	/	6874
G01N33/53	/	33	/	53
CPCONLY	/	NLY	/	

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**
435	6.11

CROSS REFERENCES(S)							
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**						
435	7.1						

NONE (Assistant Examiner) (Date)	**Total Claims Allowed:** 30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634 (Primary Examiner) 04 September 2019 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/276,235	CHEE, Mark S.
	Examiner	**Art Unit**
	JAMES MARTINELL	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	-	10	-	19	-	28	5	37	14	46	23	55		
-	2	-	11	-	20	-	29	6	38	15	47	24	56		
-	3	-	12	-	21	-	30	7	39	16	48	25	57		
-	4	-	13	-	22	-	31	8	40	17	49	26	58		
-	5	-	14	-	23	-	32	9	41	18	50	27	59		
-	6	-	15	-	24	1	33	10	42	19	51	28	60		
-	7	-	16	-	25	2	34	11	43	20	52	29	61		
-	8	-	17	-	26	3	35	12	44	21	53	30	62		
-	9	-	18	-	27	4	36	13	45	22	54				

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 30	
/JAMES MARTINELL/ Primary Examiner, Art Unit 1634 (Primary Examiner)	04 September 2019 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure None

U.S. Patent and Trademark Office

Part of Paper No.: 20190904

Curio EX1010-p. 958
Curio v Prognosys

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	124	((array$4 or microarray$4 or support$4 or surface) near5 (dna or (nucleic adj acid) or protein or antibod$4)) same (probe near5 pool$6) same (probe near5 sequenc$6)	US-PGPUB; USPAT	OR	ON	2019/09/03 13:20
L2	1520	(((high adj density) or (high adj resolution)) near5 (arrar$4 or microarray$4)) near10 (dna or (nucleic adj acid) or protein or antibod$4)	US-PGPUB; USPAT	OR	ON	2019/09/03 13:21
L3	6992	(((high adj density) or (high adj resolution)) near5 (array$4 or microarray$4)) near10 (dna or (nucleic adj acid) or protein or antibod$4)	US-PGPUB; USPAT	OR	ON	2019/09/03 13:22
L4	7082	l1 or l3	US-PGPUB; USPAT	OR	ON	2019/09/03 13:23
L5	3343805	@rlad< "20110405"	US-PGPUB; USPAT	OR	ON	2019/09/03 13:23
L6	5174	l4 and l5	US-PGPUB; USPAT	OR	ON	2019/09/03 13:23
L7	70045	@pd> "20190801"	US-PGPUB; USPAT	OR	ON	2019/09/03 13:23
L8	8	l6 and l7	US-PGPUB; USPAT	OR	ON	2019/09/03 13:23
L9	5449	(locate or locating or location or address or spat$6 or space) same (bead or microbead or sphere or microsphere) same tissue	US-PGPUB; USPAT	OR	ON	2019/09/03 13:26
L10	3144	l5 and l9	US-PGPUB; USPAT	OR	ON	2019/09/03 13:27
L11	1204	l10 and (code or coded or barcode or barcoded or coding or barcoding or zipcode)	US-PGPUB; USPAT	OR	ON	2019/09/03 13:27
L12	1	l7 and l11	US-PGPUB; USPAT	OR	ON	2019/09/03 13:28

9/ 3/ 2019 1:29:20 PM
C:\ Users\ jmartinell\ Documents\ EAST\ Workspaces\ 16-276235-d.wsp

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No.	Application No.
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))		47706-0004003	16/276,235
		Applicant: Prognosys Biosciences, Inc.	
		Filing Date: February 14, 2019	Group Art Unit: 1634

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	5,559,032	9/17/1996	Porneroy	Published 09/24/1996		
	2.	6,673,620	1/6/2004	Loeffler			
	3.	6,699,710	3/1/2004	Kononen	Published 03/02/2004		
	4.	20020040275	4/4/2002	Cravatt			
	5.	20110275077	11/10/2011	James			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No

Other Documents (include Author, Title, Date, and Place of Publication)

Examiner Initial	Desig. ID	Document

Examiner Signature	Date Considered
/JAMES MARTINELL/	09/03/2019

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.M/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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BIB DATA SHEET

CONFIRMATION NO. 7944

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
16/276,235	02/14/2019	435	1634	47706-0004003

APPLICANTS
Prognosys Biosciences, Inc., San Diego, CA;

INVENTORS
Mark S. CHEE, San Diego, CA;

**** CONTINUING DATA ***************************
This application is a CON of 15/187,661 06/20/2016 PAT 10308982
which is a CON of 13/080,616 04/05/2011 PAT 9371598
which claims benefit of 61/321,124 04/05/2010

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****
03/04/2019

Foreign Priority claimed ❑ Yes ☑ No 35 USC 119(a-d) conditions met ❑ Yes ☑ No Verified and Acknowledged /JAMES MARTINELL/ Examiner's Signature	❑ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	5	1	1

ADDRESS
FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022
UNITED STATES

TITLE
Spatially Encoded Biological Assays

FILING FEE RECEIVED		
1285	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

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Confirmation No.	:	7944
			Notice of Allowance Date: September 09, 2019		

Title : SPATIALLY ENCODED BIOLOGICAL ASSAYS

MAIL STOP ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REPLY TO NOTICE OF ALLOWANCE

In response to the Notice of Allowance dated September 9, 2019, enclosed is a completed Part B - Fee(s) Transmittal.

The issue fee is being paid with this reply on the Electronic Filing System. Apply those fees and any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: September 9, 2019

/Cameron Luitjens/
Cameron M. Luitjens, Ph.D., J.D.
Reg. No. 58,674

Customer Number 159367
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

62094781.doc

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
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Alexandria, Virginia 22314-1450

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)
159367 7590 09/09/2019

FISH & RICHARDSON P.C.
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	02/14/2019	Mark S. Chee	47706-0004003	7944

TITLE OF INVENTION: SPATIALLY ENCODED BIOLOGICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	LARGE	$1000	$0		$1000	12/09/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
MARTINELL, JAMES	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 Fish & Richardson P.C.
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: Prognosys Biosciences, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): San Diego, CA

Please check the appropriate assignee category or categories (will not be printed on the patent): [ ] Individual [X] Corporation or other private group entity [ ] Government

4a. Fees Submitted: [X] 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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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 /Cameron Luitjens/ Date September 9, 2019

Typed or printed name Cameron M. Luitjens, Ph.D., J.D. Registration No. 58,674

62094782.doc

Electronic Patent Application Fee Transmittal

Application Number:	16276235
Filing Date:	14-Feb-2019
Title of Invention:	SPATIALLY ENCODED BIOLOGICAL ASSAYS
First Named Inventor/Applicant Name:	Mark S. CHEE
Filer:	Cameron Mark Luitjens/Anne Roy
Attorney Docket Number:	47706-0004003

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	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1000**

Electronic Acknowledgement Receipt

EFS ID:	37110593
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	SPATIALLY ENCODED BIOLOGICAL ASSAYS
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Anne Roy
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	09-SEP-2019
Filing Date:	14-FEB-2019
Time Stamp:	19:24:46
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1000
RAM confirmation Number	E201999J25034502
Deposit Account	
Authorized User	

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

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Post Allowance Communication - Incoming	47706replyiss.pdf	70990 2ef73f27d7926a51f90601841c34c999a94ff5d5	no	1
Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	PARTB.pdf	159514 7e19f2862f1edcc202cbc104db67e976ef9ae6b1	no	1
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30353 3927d26e80b90f33fccb94c167daa69c18c82812	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	260857		

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 :C47345

Sale Accounting Date:09/16/2019

Sale Item Reference Number	Effective Date
16276235	08/29/2019

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I20199F008512747	1830	PROCESSING FEE, EXCEPT PROV. APPLS.	$140.00	Deposit Account

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

159367 7590 09/18/2019

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10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
MARTINELL, JAMES	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
09/18/2019	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):

PATDOCTC@fr.com
ipadmin@10xgenomics.com

Decision Granting Request for Prioritized Examination (Track I)	**Application No.** 16/276,235	**Applicant(s)** CHEE, Mark S.	
	Examiner BRIAN W BROWN	**Art Unit** OPET	**AIA (FITF) Status** No

1. THE REQUEST FILED 26 August 2019 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 BRIAN BROWN at (571)272-5338.

In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.

/BRIAN W BROWN/ Petitions Examiner, OPET	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Confirmation No.	:	7944
			Notice of Allowance Date: 9/9/2019		
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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Alexandria, VA 22313-1450

AMENDMENT AFTER ALLOWANCE
PURSUANT TO 37 C.F.R. §1.312

Please amend the application as indicated on the following pages.

REMARKS

To facilitate publication, Applicant submits a replacement specification that incorporates all of the amendments to the specification that were made during prosecution of the present application. The present application claims priority to and incorporates by reference the entire text of U.S Provisional Patent Application No. 61/321,124 ("the '124 provisional application"). Many of the amendments made during prosecution of the present application resulted from the explicit incorporation of text from the '124 provisional application into the present application.

A marked-up version of the specification showing all changes made as compared to the original specification that was filed February 14, 2019 is submitted, along with a clean version of the specification.

For the Office's convenience, a timeline of the changes that were made during prosecution of the present application is provided below. Each of these amendments are reflected in the replacement specification submitted herewith.

March 15, 2019

- Paragraph numbering was updated starting at Description of the Figures (not marked as changes). The Description of the Figures had previously restarted at paragraph [0001].
- Paragraphs [0007]-[00023], [00042]-[00080], and [00089] the '124 provisional application were specifically added.

May 15, 2019

- Paragraph [0004] (second instance) of the original specification, now paragraph [00021], was amended to expand the description of Figure 4.
- Paragraph [00029] of the original specification, now paragraph [00046], was amended to refer to specific steps.
- Paragraph [00030] of the original specification, now paragraph [00047], was amended to refer to assay system 200.
- Paragraph [00038] of the original specification, now paragraph [00055], was amended to recite step 306.

June 11, 2019

- Paragraph [00123] of the specification on file as of May 15, 2019, now paragraph [00113], was amended to reference Figure 4 instead of Figure 1. Figure 1 of the '124 provisional application corresponds to Figure 4 of the present application.
- Paragraph [00140] of the specification on file as of May 15, 2019, now paragraph [00121], was amended to reference Figure 3 instead of Figure 2. Figure 2 of the '124 provisional application corresponds to Figure 3 of the present application.

Applicant submits that the replacement specification submitted herewith accurately reflects the changes that were made during prosecution of the present application. No new matter has been added.

CONCLUSION

Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: October 4, 2019

/Cameron Luitjens/

Cameron M. Luitjens, Ph.D., J.D.
Reg. No. 58,674

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62117793.doc

SPATIALLY ENCODED BIOLOGICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Patent Application No. 61/321,124, filed April 5, 2010 and is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] This invention relates to assays of biological molecules, and more particularly to assays for determining spatial distributions of a large number of biological molecules in a solid sample simultaneously.

BACKGROUND OF THE INVENTION

[0003] In the following discussion certain articles and methods will be described for background and introductory purposes. Nothing contained herein is to be construed as an "admission" of prior art. Applicant expressly reserves the right to demonstrate, where appropriate, that the articles and methods referenced herein do not constitute prior art under the applicable statutory provisions.

[0004] Comprehensive gene expression analysis and protein analysis have been useful tools in understanding mechanisms of biology. Use of these tools has allowed the identification of genes and proteins involved in development and in various diseases such as cancer and autoimmune disease. Conventional methods such as *in situ* hybridization and other multiplexed detection of different transcripts have revealed spatial patterns of gene expression and have helped shed light on the molecular basis of development and disease. Other technologies that have enabled the quantitative analysis of many RNA sequences per sample include microarrays (see Shi, et al., Nature Biotechnology, 24(9): 1151-61 (2006); and Slonim and Yanai, Plos Computational Biology, 5(10):e1000543 (2009)); serial analysis of gene expression (SAGE) (see Velculescu, et al., Science, 270(5235):484-87 (1995)), high-throughput implementations of

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Curio v Prognosys

qPCR (see Spurgeon, et al., Plos ONE, 3(2):e1662 (2008)) and *in situ* PCR (see Nuovo, Genome Res., 4:151-67 (1995)). As useful as these methods are, however, they do not enable simultaneous measurement of the expression of many genes or the presence and/or activity of multiple proteins at many spatial locations in a sample. Laser capture microdissection has permitted the analysis of many genes at a small number of locations, but it is very expensive, laborious, and does not scale well, Certain PCR assays in a 2D format preserve spatial information (see Armani, et al., Lab on a Chip, 9(24): 3526-34 (2009)), but these methods have low spatial resolution because they rely on physical transference of tissue into wells, which also prevents random access to tissue samples and high levels of multiplexing.

[0005] At present, no practical method exists to analyze at high resolution the spatial expression patterns of large numbers of genes, proteins, or other biologically active molecules simultaneously. There is thus a need for reproducible, high-resolution spatial maps of biological molecules in tissues. The present invention addresses this need.

SUMMARY OF THE INVENTION

[0006] This Summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This Summary is not intended to identify key or essential features of the claimed subject matter, nor is it intended to be used to limit the scope of the claimed subject matter. Other features, details, utilities, and advantages of the claimed subject matter will be apparent from the following written Detailed Description including those aspects illustrated in the accompanying drawings and defined in the appended claims.

[0007] The invention encompasses assay systems that provide high-resolution spatial maps of biological activity in tissues. The assay system comprises an assay capable of high levels of multiplexing where encoded probes are provided to a biological sample in defined spatial patterns; instrumentation capable of controlled delivery of reagents according to the spatial patterns; and a decoding scheme providing a readout that is digital in nature. In short, the present invention provides the ability to look at many biological targets in many locations, providing the resolution of in situ hybridization with the highly- parallel data analysis of sequencing.

[0008] Thus, in some embodiments, the invention provides an assay system to determine

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spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered; allowing the encoded probes to interact with the biological targets; separating encoded probes that interact with the biological targets from encoded probes that do not interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[0009] In particular aspects of the invention the biological targets comprise nucleic acids and the encoded probes are oligonucleotides, and in some aspects, there are two encoded probes for each of the multiple nucleic acid targets. In some aspects, the multiple biological targets comprise proteins, the probe regions of the encoding probes are proteins and the coding tags comprise oligonucleotides. In some aspects the multiple biological targets comprise enzymes. In some aspects the probe regions of the encoded probes comprise antibodies, aptamers or small molecules.

[00010] Some aspects of the assay system further comprise an amplification step between the separating step and the determining step. In some aspects, the determining step is performed by nucleic acid sequencing, and in preferred aspects, the sequencing is high-throughput digital nucleic acid sequencing.

[00011] In some aspects of the invention, the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 20, in some aspects product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 50, in some aspects the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 75, 100, 150, 500, 750, 1,000, 5,000, 10,000, 25,000, 50,000, 100,000, 500,000, or 1,000,000 or more. In other aspects, the sequence of at least fifty thousand encoding probes are determined in parallel, in other aspects the sequence of at least one hundred thousand encoding probes are determined in parallel, in some aspects the sequence of at least five hundred thousand encoding probes are determined in parallel, and in some aspects the sequence of at least one million, ten million, one hundred million, one billion, ten billion, one hundred billion or more

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encoding probes are determined in parallel.

[00012] In some aspects, the known spatial pattern is determined by histological features of the sample. Also in some aspects, software programmed hardware performs at least two steps of the delivering step, the separation step, the determining step and the associating step.

[00013] In some aspects, the probe regions of the encoded probes are proteins and the separating step is accomplished by encoded probes that interact with the biological targets being captured by an affinity capture agent. In some aspects the probe regions of the encoding probes are nucleic acids and the separating step is accomplished by a washing of the sample.

[00014] In other embodiments there is provided an assay system to determine spatial patterns of abundance or activity or both of multiple nucleic acid targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering oligonucleotide probes for multiple nucleic acid targets to the multiple sites in the sample in a known spatial pattern; allowing the oligonucleotide probes to hybridize with the nucleic acid targets; washing unhybridized encoded oligonucleotide probes from the sample; delivering one or more encoding agents to locations of the multiple sites in the sample according to a known spatial pattern, where the combination of encoding agents delivered to each site is different; coupling the encoding agents and the oligonucleotide probes to form encoded probes; determining all or a portion of a sequence of the encoded probes using high-throughput sequencing, and associating the abundance or activity or both of multiple biological targets to the locations of multiple sites in the sample.

[00015] Other embodiments of the invention provide an assay system to determine spatial patterns of abundance or activity or both of multiple protein targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple protein targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a protein probe region that may interact with the protein targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and the protein probe region of the encoding probe of which the coding tag is part; allowing the encoded probes to interact with the protein targets; separating encoded probes that interact with the protein targets from encoded probes that do not interact with the protein targets; determining all or a portion of a sequence of the encoded probes by high throughput sequencing, and associating the abundance or activity or both of the multiple protein

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targets to the locations of the multiple sites in the sample.

[00016] Other embodiments provide an assay system to determine spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and identifies the biological target; allowing the encoded probes to interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[00017] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

DESCRIPTION OF THE FIGURES

~~[0001]~~**[00018]** Figure 1 provides a simplified overview of the assay system of the present invention.

~~[0002]~~**[00019]** Figure 2 provides a simplified overview of one embodiment of the assay system of the present invention for detecting nucleic acids.

~~[0003]~~**[00020]** Figure 3 is a representational depiction of one embodiment of the assay overviewed in Figure 2.

~~[0004]~~**[00021]** Figure 4A-C illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention. Figure 4A shows two target-specific/encoding oligonucleotide constructs specifically bound to a target nucleic acid of interest in a sample. Figure 4B shows a scheme for delivering twenty different coding tags, a1 through a10 and b1 through b10, to a sample to form a 10x10 coding tag grid. Figure 4C shows a tissue section sample to which the coding tags are delivered, forming the coding tag grid in the sample.

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~~[0005]~~[00022] Figure 5 provides a simplified, specific example of the embodiment of a combinatorial encoding scheme shown in Figure 4.

DEFINITIONS

~~[0006]~~[00023] The terms used herein are intended to have the plain and ordinary meaning as understood by those of ordinary skill in the art. The following definitions are intended to aid the reader in understanding the present invention, but are not intended to vary or otherwise limit the meaning of such terms unless specifically indicated.

~~[0007]~~[00024] The term "antibody" as used herein is intended to refer to an entire immunoglobulin or antibody or any functional fragment of an immunoglobulin molecule which is capable of specific binding to an antigen (antibodies and antigens are "binding partners" as defined herein). "Antibody" as used herein is meant to include the entire antibody as well as any antibody fragments capable of binding the antigen or antigenic fragment of interest. Examples of such peptides include complete antibody molecules, antibody fragments, such as Fab, F(ab')2, CDRS, VL, VH, and any other portion of an antibody which is capable of specifically binding to an antigen. Antibodies for assays of the invention are immunoreactive or immunospecific for, and therefore specifically and selectively bind to, proteins either detected *(i.e.,* biological targets) or used for detection *(i.e.,* probes) in the assays of the invention.

~~[0008]~~[00025] The term "binding agent" as used herein refers to any agent that specifically binds to a biological molecule of interest.

~~[0009]~~[00026] "Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double-stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single-stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the other strand, usually at least about 90% to about 95%, and even about 98% to about 100%.

~~[00010]~~[00027] "Hybridization" refers to the process in which two single-stranded

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polynucleotides bind non-covalently to form a stable double-stranded polynucleotide. The resulting (usually) double-stranded polynucleotide is a "hybrid" or "duplex." "Hybridization conditions" will typically include salt concentrations of approximately less than 1M, often less than about 500 mM and may be less than about 200 mM. A "hybridization buffer" is a buffered salt solution such as 5% SSPE, or other such buffers known in the art. Hybridization temperatures can be as low as 5°C, but are typically greater than 22°C, and more typically greater than about 30°C, and typically in excess of 37°C. Hybridizations are often performed under stringent conditions, *i.e.,* conditions under which a primer will hybridize to its target subsequence but will not hybridize to the other, non-complementary sequences. Stringent conditions are sequence-dependent and are different in different circumstances. For example, longer fragments may require higher hybridization temperatures for specific hybridization than short fragments. As other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one parameter alone. Generally stringent conditions are selected to be about 5°C lower than the Tm for the specific sequence at a defined ionic strength and pH. Exemplary stringent conditions include a salt concentration of at least 0.01 M to no more than 1M sodium ion concentration (or other salt) at a pH of about 7.0 to about 8.3 and a temperature of at least 25°C. For example, conditions of 5xSSPE (750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA at pH 7.4) and a temperature of approximately 30°C are suitable for allele-specific hybridizations, though a suitable temperature depends on the length and/or GC content of the region hybridized.

~~[00011]~~[00028] "Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, *e.g.,* oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon terminal nucleotide of one oligonucleotide with a 3' carbon of another nucleotide.

~~[00012]~~[00029] "Nucleic acid", "oligonucleotide", "oligo" or grammatical equivalents used herein refers generally to at least two nucleotides covalently linked together. A nucleic acid generally will contain phosphodiester bonds, although in some cases nucleic acid analogs may be included that have alternative backbones such as phosphoramidite,

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phosphorodithioate, or methylphophoroamidite linkages; or peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids, positive backbones, non-ionic backbones and non-ribose backbones. Modifications of the ribose-phosphate backbone may be done to increase the stability of the molecules; for example, PNA:DNA hybrids can exhibit higher stability in some environments.

~~[00013]~~[00030] "Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Primers usually are extended by a DNA polymerase.

~~[00014]~~[00031] The term "SNP" or "single nucleotide polymorphism" refers to a genetic variation between individuals; *e.g.,* a single nitrogenous base position in the DNA of organisms that is variable. SNPs are found across the genome; much of the genetic variation between individuals is due to variation at SNP loci, and often this genetic variation results in phenotypic variation between individuals. SNPs for use in the present invention and their respective alleles may be derived from any number of sources, such as public databases (U.C. Santa Cruz Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway) or the NCBI dbSNP website (http://www.ncbi.nlm.nih.gov/SNP/), or may be experimentally determined as described in U.S. Pat No. 6,969,589; and US Pub. No. 2006/0188875 entitled "Human Genomic Polymorphisms." Although the use of SNPs is described in some of the embodiments presented herein, it will be understood that other biallelic or multi-allelic genetic markers may also be used. A biallelic genetic marker is one that has two polymorphic forms, or alleles. As mentioned above, for a biallelic genetic marker that is associated with a trait, the allele that is more abundant in the genetic composition of a case group as compared to a control group is termed the "associated allele," and the other allele may be referred to as the "unassociated allele." Thus, for each biallelic polymorphism that is associated with a given trait *(e.g.,* a disease or drug response), there is a corresponding associated allele. Other biallelic polymorphisms that may be used with the methods presented herein include, but are not limited to multinucleotide changes, insertions, deletions, and translocations. It will be further appreciated that references to DNA herein may include genomic DNA, mitochondrial DNA, episomal DNA, and/or derivatives

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of DNA such as amplicons, RNA transcripts, cDNA, DNA analogs, etc. The polymorphic loci that are screened in an association study may be in a diploid or a haploid state and, ideally, would be from sites across the genome.

~~[00015]~~[00032] The term "selectively binds", "selective binding" and the like as used herein, when referring to a binding partner (*e.g.*, protein, nucleic acid, antibody or other affinity capture agent, etc.), refers to a binding reaction of two or more binding partners with high affinity and/or complementarity to ensure selective hybridization under designated assay conditions. Typically, specific binding will be at least three times the standard deviation of the background signal. Thus, under designated conditions the binding partner binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

~~[00016]~~[00033] "Sequencing", "sequence determination" and the like means determination of information relating to the nucleotide base sequence of a nucleic acid. Such information may include the identification or determination of partial as well as full sequence information of the nucleic acid. Sequence information may be determined with varying degrees of statistical reliability or confidence. In one aspect, the term includes the determination of the identity and ordering of a plurality of contiguous nucleotides in a nucleic acid. "High throughput digital sequencing" or "next generation sequencing" means sequence determination using methods that determine many (typically thousands to billions) of nucleic acid sequences in an intrinsically parallel manner, *i.e.* where DNA templates are prepared for sequencing not one at a time, but in a bulk process, and where many sequences are read out preferably in parallel, or alternatively using an ultra-high throughput serial process that itself may be parallelized. Such methods include but are not limited to pyrosequencing (for example, as commercialized by 454 Life Sciences, Inc., Branford, CT); sequencing by ligation (for example, as commercialized in the SOLiD™ technology, Life Technology, Inc., Carlsbad, CA); sequencing by synthesis using modified nucleotides (such as commercialized in TruSeq™ and HiSeq™ technology by Illumina, Inc., San Diego, CA, HeliScope™ by Helicos Biosciences Corporation, Cambridge, MA, and PacBio RS by Pacific Biosciences of California, Inc., Menlo Park, CA), sequencing by ion detection technologies (Ion Torrent, Inc., South San Francisco, CA); sequencing of DNA nanoballs (Complete Genomics, Inc., Mountain View, CA); nanopore-based sequencing technologies (for example, as developed by Oxford Nanopore Technologies, LTD, Oxford, UK),

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and like highly parallelized sequencing methods.

~~[00017]~~[00034] The term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation, T_m=81.5+0.41 (% G+C), when a nucleic acid is in aqueous solution at 1M NaCl (see *e.g.*, Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985)). Other references (*e.g.*, Allawi and SantaLucia, Jr., Biochemistry, 36:10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

DETAILED DESCRIPTION OF THE INVENTION

~~[00018]~~[00035] The practice of the techniques described herein may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and sequencing technology, which are within the skill of those who practice in the art. Such conventional techniques include polymer array synthesis, hybridization and ligation of polynucleotides, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the examples herein. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Green, et al., Eds., *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (1999); Weiner, Gabriel, Stephens, Eds., *Genetic Variation: A Laboratory Manual* (2007); Dieffenbach, Dveksler, Eds., *PCR Primer: A Laboratory Manual* (2003); Bowtell and Sambrook, *DNA Microarrays: A Molecular Cloning Manual* (2003); Mount, *Bioinformatics: Sequence and Genome Analysis* (2004); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); Stryer, *Biochemistry* (4th Ed.) (1995) W.H. Freeman, New York N.Y.; Gait, *"Oligonucleotide Synthesis: A Practical Approach"* (2002) IRL Press, London; Nelson and

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Cox, *Lehninger, Principles of Biochemistry* (2000) 3rd Ed., W. H. Freeman Pub., New York, N.Y.; and Berg, et al., *Biochemistry* (2002) 5th Ed., W.H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

~~[00019]~~[00036] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" refers to one or more nucleic acids, and reference to "the assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

~~[00020]~~[00037] 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 this invention belongs. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing devices, formulations and methodologies that may be used in connection with the presently described invention.

~~[00021]~~[00038] Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

~~[00022]~~[00039] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention.

The Invention in General

~~[00023]~~[00040] The assay systems of the invention provide spatially-encoded, multiplexed assays comprising 1) an assay capable of high levels of multiplexing with an efficient spatial encoding scheme; 2) instrumentation capable of delivering reagents according to a spatial

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pattern; and 3) decoding determined by a readout that is digital in nature. The assay systems of the invention detect the presence or absence and relative amount of a biological target or biological activity indicative of a biological target, as well as the location of the biological target or activity in a biological sample, *e.g.*, a tissue section or other biological structure disposed upon a support such as a microscope slide or culture dish.

~~[00024]~~[00041] The assay system further provides instrumentation with an ability to deliver reagents in a spatially-defined pattern. This instrumentation, together with software, reagents and protocols, provides a key component of the highly innovative assay system of the invention, allowing for measurement of numerous biological targets or activities in a meaningful spatial environment, including gene expression and peptide localization. An encoding scheme used in these assay systems allows one to determine the location of biological targets or activity (or lack thereof) in the biological samples after the products of the multiplexed assay are removed from the biological sample and pooled for analysis. Decoding of the encoding scheme can be performed by, *e.g.*, next-generation sequencing, which easily provides millions to trillions of datapoints at low cost. The assay results such as the amount or activity of biological targets can then be mapped back to specific location in the biological sample. The assay systems open a new analytical window into the complex spatial patterns of cellular function and regulation in biological samples.

~~[00025]~~[00042] A simplified overview of the assay system 100 of the present invention is provided at Figure 1. At step 110, a biological sample affixed to a support is provided. The biological sample contains biological targets of interest. Biological targets can include any molecule of interest, such as nucleic acids (including, *e.g.* RNA transcripts, genomic DNA sequences, cDNAs, amplicons, or other nucleic acid sequences) and proteins, enzymes and the like. At step 120, encoded probes are delivered to the biological sample according to a known spatial pattern. Encoded probes comprise probes, which can interact with biological targets of interest, and coding tags, which identify the positions in the sample of the biological targets being assayed, and thus can be used to link assay results back to locations in the sample. Coding tags in most embodiments are oligonucleotides. However, coding tags may also be mass tags, fluorescent labels, or other moieties.

~~[00026]~~[00043] In some embodiments, the probe and coding tag portions of the encoded probe are pre-coupled before being delivered to the biological sample, For example, in

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the case where the encoded probes are oligonucleotides, both the probe and coding tag sequence can be synthesized as a single oligonucleotide. Alternatively, the probe and coding tag portions of the encoding probes can be synthesized or obtained separately and combined before delivery to the biological sample (*e.g.*,two separate oligonucleotides can be synthesized and coupled by, *e.g.*, ligation; or an antibody and an oligonucleotide can be prepared separately and conjugated before delivery to the biological sample). Also, as is described in Figures 2-5, the probes and the coding tags (in encoding oligonucleotides) are synthesized separately, and are delivered to the biological sample at different steps (*e.g.*, probes first and coding tags thereafter, or vice versa) in the assay.

~~[00027]~~[00044] At step 130, the encoded probes are allowed to react or interact with the biological targets, *i.e.*, conditions are provided to allow *e.g.*, oligonucleotides to hybridize to nucleic acid targets, enzymes to catalyze reactions with protein targets, antibodies to bind epitopes, etc. In the case where the biological targets are nucleic acids, the encoded probes are typically oligonucleotides and hybridize to the target nucleic acids. In the case that the biological targets are proteins, the encoded probes typically are aptamers, small molecules, or oligonucleotide-conjugated proteins that interact with target proteins by binding to them or by reacting with them (that is, one of the proteins is a substrate for the other). Encoding oligonucleotides may be coupled to the probes (proteins) by conjugation, chemical or photo-crosslinking via suitable groups and the like.

~~[00028]~~[00045] Once encoded probes interact with the biological targets, the encoded probes that interacted with the biological targets must be separated from the encoded probes that did not interact with the biological targets at step 140. In the case where the biological targets are nucleic acids and the encoded probes are oligonucleotides, the separation can be accomplished by, *e.g.*, washing the unhybridized encoded probes from the sample. Similarly, for other assays that are based on affinity binding, including those using aptamer, small molecule, and protein probes, washing steps can be used to remove low affinity binders. In the case where the probe is transformed via interaction with the target, *e.g.*, in the case of a peptide, *e.g.*, via cleavage by a protease or phosphorylation by a kinase, it is convenient to collect, all encoded probes--both encoded probes that interacted with the biological targets and were transformed and encoded probes that were not transformed. After collection or pooling, an antibody or other affinity capture agent can be used to capture probes that were transformed by addition of a moiety

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(*e.g.*, a phosphate group). In cases where probes have been transformed via cleavage, the transformed probes can be separated, *e.g.*, by capturing the non-transformed probes via a tag that is removed from the transformed probes during the transformation (*e.g.*, by cleavage), or by adding a new tag at the site of cleavage.

~~[00029]~~[00046] Once the reacted (transformed) or interacted encoded probes are separated from the unreacted or un-interacted encoded probes, the sequence of the reacted and/or interacted encoded probes is determined at step 150 by, preferably, sequencing. The sequence of the encoded probes allows the mapping of the assay results at step 160 back to locations in the biological sample.

~~[00030]~~[00047] Figure 2 provides a simplified overview of an assay system 200 of the present invention embodying an efficient implementation of a combinatorial coding scheme for the encoding of spatial information. For purposes of this overview, the probes are oligonucleotides, but as explained elsewhere, other types of probes can also be used. In step 210, a biological sample affixed to a support, *e.g.*, a tissue sample or other biological structure, is provided. In step 220, one or more oligonucleotide probes are delivered to the biological sample, where the oligonucleotide probes are capable of hybridizing with biological targets in the biological sample. In step 230, the oligonucleotide probes are allowed to interact with (hybridize to) the nucleic acid targets; that is, appropriate conditions are provided where oligonucleotide probes can hybridize to the target nucleic acids.

~~[00031]~~[00048] In step 240, the oligonucleotide probes that did not hybridize to target nucleic acids are removed, and thereby separated from oligonucleotide probes that did hybridize to target nucleic acids. In this embodiment, separation can be accomplished by, *e.g.*, washing the sample to remove unhybridized oligonucleotide probes. Next, in step 250, encoding oligonucleotides (the encoding agents) are delivered to the biological sample according to a chosen spatial pattern, where the encoding oligonucleotides comprise coding tags that are used to encode the location of biological targets in the biological sample. Note that in contrast to the assay system of Figure 1, here the probes and encoding agents (encoding oligonucleotides) are delivered in separate steps. In step 260, the encoding oligonucleotides are coupled to the oligonucleotide probes to create encoded probes. In this case where the probes are oligonucleotides, the encoding oligonucleotides may be coupled to the oligonucleotides probes by, *e.g.*, ligation. Alternatively, the information in the encoding oligonucleotides can be

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transferred by using a DNA polymerase to extend a probe oligonucleotide that acts as a primer, and thereby copy and incorporate the sequence of the encoding oligonucleotides.

~~[00032]~~[00049] In step 270, the sequence of the coding tags in the encoded probes as well as the sequence or a portion of the sequence of the probe itself is determined, and in step 280, the target nucleic acids are mapped back to the biological sample. In some embodiments, the abundance of sequences reveals the relative quantity of biological targets at the location. Although this embodiment shows the individual steps in a particular order, so as to better explain the invention, the precise order of the steps can be varied. For example, steps 220 and 250 can be combined, so that a mixture of the probes and encoding oligonucleotides is delivered according to a chosen spatial pattern. Coupling step 260 can then be carried out immediately after the combined steps 220 and 250, or concomitantly with them. In this case, step 240 would then occur after step 260. It can therefore be appreciated that the two key results of this series of steps, *i.e.*, the location-specific encoding of probe molecules and the separation of probe molecules based on their ability to interact with corresponding target molecules, can be accomplished with some flexibility in the implementation of the particular steps. Similarly, there is considerable flexibility in the design of the coding scheme. As described *infra*, the assays of the invention are particularly amenable to combinatorial methods.

~~[00033]~~[00050] Thus, the present invention provides an ability to look at many different biological targets in many locations, providing the resolution of in situ hybridization with the highly-parallel data analysis of sequencing. In some embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 20, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 50, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 100, greater than 500, 1,000, 10,000, 25,000, 100,000, 500,000, 1,000,000. It will be appreciated that, due to the spatial encoding dimension of the invention, even much larger numbers can be contemplated. For example, assaying 10,000 targets per location x 10,000 locations would generate 10^8 different assays, and even larger numbers than these can easily be contemplated, particularly if spatial locations with resolution on the order of that of single cells are utilized. Further, in embodiments where high-throughput digital sequencing is employed, the sequences of at least 1,000 encoding probes are typically determined in parallel. More typically,

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using a digital readout, it is desirable to obtain multiple sequence reads for each assay (defined by a probe and a spatial location code). It is desirable to obtain an average of at least 3 copies per assay, and more typically at least 10 or at least 30 copies per assay, depending on the design of the experiment and requirements of the assay. For a quantitative readout with suitable dynamic range, it may be desirable to obtain at least 1,000 reads per assay. Therefore, if 1,000,000 assays are carried out, the number of sequence reads may be 1 billion or more. With high-throughput digital sequencing, and allowing for redundancy, the sequence of at least 10,000 encoding probes are determined in parallel, or the sequence of at least 100,000, 500,000, 1,000,000, 10,000,000, 100,000,000, 1,000,000,000 or more encoding probes are determined in parallel.

Assays

~~[00034]~~[00051] The assay portion of the assay systems of the present invention comprise the following general steps: delivering probes and encoding agents where the encoding agents (in some embodiments pre-coupled to the probes) are delivered to the sample according to a known spatial pattern, allowing the probes to interact or react with biological targets in the sample, and, if the probes and encoding agents have not been pre- coupled, coupling the encoding agents to probes.

~~[00035]~~[00052] The samples of the present invention include virtually any biological sample or samples that can be affixed to a support or provided essentially in a two-dimensional manner, where the ability to tie an assayed biological target or activity back to the location within the biological sample is important. Exemplary biological samples include tissue sections (*e.g.*,including whole animal sectioning and tissue biopsies), cell populations on slides or culture dishes, and the like. The assay systems of the invention are particularly advantageous in that they are compatible with numerous biological sample types, including fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the biological samples are immobilized on a substrate surface having discrete, independently measureable areas.

~~[00036]~~[00053] The biological targets to be detected can be any biological molecules including but not limited to proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Examples of subcellular targets include

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organelles, *e.g.*, mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplasts, endocytic vesicles, exocytic vesicles, vacuoles, lysosomes, etc.

~~[00037]~~[00054] In some particular embodiments, the assay system is used to analyze nucleic acids, *e.g.*, by genotyping, quantitation of DNA copy number or RNA transcripts, localization of particular transcripts within samples, and the like. Figure 3 illustrates an overall scheme for an exemplary assay for, *e.g.*, detecting single nucleotide polymorphisms (SNPs) that can be used with the assay system of the invention. In Figure 3, two oligonucleotide probes are provided. Each oligonucleotide probe comprises a target-specific region (located on either side of the SNP to be analyzed) seen at 305 and 307, and ligation regions, seen at 301 and 303. The oligonucleotide probes are allowed to hybridize to a target nucleic acid (not shown) in the biological sample. At step 302, one of the oligonucleotide probes is extended to incorporate the SNP sequence and ligated to the other probe to form an extended probe comprising target nucleic acid region 309 and ligation regions 301 and 303.

~~[00038]~~[00055] Two encoding agents, both comprising a coding tag (seen at 315 and 317), a ligation region (seen at 311 and 313), and a primer region (seen at 319 and 321) are combined with and ligated to the extended probe at step 304 to form an encoded target- specific oligonucleotide. Again, in contrast with Figure 1, the probes and encoding agents are delivered at separate steps. Doing so allows use of the combinatorial embodiments described *infra*. In preferred embodiments, the encoding oligonucleotides within a pair of encoding oligonucleotides ligate specifically to one side of the target sequence or the other *(i.e.,5'* or 3' of the target sequence) in step 306. Also, typically, the ligation and primer regions of the encoding oligonucleotides and probes are universal; that is, the set of ligation and primer regions used in constructing the probes and encoding oligonucleotides are constant, and only the target-specific regions of the probes and the coding tags of the encoding oligonucleotides differ. However, again in alternative embodiments, the ligation and primer regions are not universal and differ between probes and encoding agents.

~~[00039]~~[00056] Following ligation, the encoded probes are eluted, pooled, and, optionally, sequencing adapters are added to the encoded probes via PCR. In alternative embodiments, sequencing primers may be ligated to the encoding oligonucleotides, or sequencing primer sequences can be included as part of the encoding oligonucleotide. As seen in Figure 3, each sequencing adapter comprises primer region 319 or 321, compatible with the primer regions

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319 and 321 on the encoded probes. The final construct comprising first adapter 327, first primer region 319, first coding tag 315, ligation regions 311 and 301, target region 309, ligation regions 313 and 303, second coding tag 317, second primer region 325 and second adapter 329 is now ready for input into a digital high-throughput sequencing process.

~~[00040]~~[00057] A combination of extension and ligation reactions are exemplified in Figure 3, but it should be appreciated that a variety of reactions may be used to couple the encoding oligonucleotides to the target-specific oligonucleotides, including ligation only (*e.g.*, for oligonucleotides that hybridize to contiguous portions of the target nucleic acid sequence). Alternatively, an assay utilizing an additional oligonucleotide, such as in the GOLDENGATE® assay (see Fan, et al., Cold Spring Symp. Quant. Biol., 68:69-78 (2003); (Illumina, Inc., San Diego, CA)), may be employed.

~~[00041]~~[00058] In other embodiments, the assay system of the invention also can be used to analyze peptides or proteins, the presence of antibodies, enzymatic and other protein activities, posttranslational modifications, active and non-active forms of peptides, as well as peptide isoforms in a biological sample. Accordingly, the probes may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, aptamers and the like.

~~[00042]~~[00059] In certain aspects, the probes are substrates for enzymes or proenzymes, *e.g.*, kinases, phosphatases, zymogens, proteases, or fragments thereof. In certain aspects, the probes are phosphorylation substrates used to detect proteins involved in one or more signal transduction pathways, *e.g.*, a kinase or a phosphatase. In another specific aspect of the invention, the probes are specific protease substrates that associate only with individual proteases or classes of proteases. In other aspects, the probes are different processed forms, isoforms and/or domains of an enzyme. Protein-based probes are typically conjugated or otherwise linked to oligonucleotide encoding agents. The oligonucleotide encoding agents in this case would also include a nucleotide sequence component that allows for identification of the protein probe.

~~[00043]~~[00060] In certain aspects, the present invention provides assays for evaluating differences in the amount and/or activity of biological targets between different locations in a sample and/or between samples. The method includes determining a plurality of encoded results from the biological sample and evaluating the differences in quantity of the biological targets at each location in the biological sample.

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Combinatorial Embodiments

~~[00044]~~[00061] To maximize the efficiency of encoding, a combinatorial approach using pairs of coding tags in the encoding oligonucleotides can be used. By de-coupling the target-specific information and the coding tags, the number of oligonucleotides required is dramatically reduced, with a concomitant decrease in cost.

~~[00045]~~[00062] Figure 4 illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention[[.]], where nucleic acids in a representative tissue section (shown at 416) are assayed. Figure 4 at A shows two target-specific/encoding oligonucleotide constructs 420 and 422 *(e.g.,* formed between steps 302 and 304 of Figure 3) specifically bound to a target nucleic acid 402 of interest, The first encoded probe 420 comprises coding tag 408, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 404. The second encoded probe 422 comprises coding tag 406, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 410.

~~[00046]~~[00063] Figure 4 at B shows the spatial pattern that may be used for twenty different coding tags, a1 through a10 (coding tag 406 on encoded probe 420) and b1 through b10 (coding tag 408 encoded probe 422). Coding tag a1, for example, is deposited on the biological sample in ten discrete areas or spots (shown as the first horizontal line of spots in 412). Coding tag a2 is deposited on the biological sample in ten spots on the second horizontal line in 412. Coding tag a3 is deposited on the biological sample in ten spots on the third horizontal line in 412, and so on. Whereas the "a" tags are deposited in ten horizontal rows, the "b" tags are deposited in ten vertical rows as shown in 414. For example, coding tag b1 is deposited on the biological sample in ten discrete spots in the first vertical row of 414, coding tag b2 is deposited on the biological sample in ten discrete spots in the second vertical row of 414, and so on. Using such a configuration allows for twenty coding tags to uniquely define 100 different locations on the biological sample.

~~[00047]~~[00064] Figure 4 at C shows a representative tissue section 416 coincident with coding tag grid 418. The arrows show how the "a" coding tags and the "b" coding tags are deposited on grid 418 that is coincident with tissue section 416. If, once sequenced, coding tags

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a1 and b4, *e.g.*, are associated with a target nucleic acid sequence, then that target nucleic acid sequence *(i.e.*, biological target) was present in the tissue section at location a1, b4.

~~[00048]~~[00065] Figure 5 provides a simplified, specific example of the encoding scheme of the assay systems of the invention. Figure 5 shows encoding oligonucleotides 510, comprising a1, a2, a3, a4 and b1, [[b3]]b2, b3 and b4. Target-specific oligonucleotides (TSOs) (probes) 1 and 2 are shown at 520. A deposit or dispensing scheme is shown at 530. Like the grid exemplified in Figure 4, encoding oligonucleotides al through a4 are deposited in spots in a pattern (here, in a vertical pattern), and encoding oligonucleotides b1 through b4 are deposited in spots in a pattern (here, a horizontal pattern). The grid though shown as a square with spots is actually a deposition pattern on a biological sample (not shown) such as tissue section 416 shown in Figure 4.

~~[00049]~~[00066] The target-specific oligonucleotides are delivered to the biological sample, where the target-specific oligonucleotides hybridize to target nucleic acids in the biological sample if target nucleic acids are present. Unhybridized target-specific oligonucleotides are then removed, *e.g.*, by washing. The encoding oligonucleotides are then delivered to the biological sample according to the spatial pattern shown at 530. The encoding oligonucleotides are ligated (or, *e.g.*, extended and ligated) to any target-specific oligonucleotides that hybridized to the target nucleic acid in the biological sample, the ligated constructs are then eluted from the biological sample, pooled, and sequencing adapters are added through, *e.g.*, PCR or ligation, if the sequences were not previously included in the encoding oligonucleotides. The ligated constructs are sequenced by, *e.g.*, high throughput or "next generation" sequencing.

~~[00050]~~[00067] The pool of resulting sequences is shown at 540. A sequence readout was obtained for target-specific oligonucleotide 1 only at a4b1, a4b2, a1b3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at a1b1 (position shown with vertical lines). A sequence readout was obtained for both target-specific oligonucleotides 1 and 2 at positions a2b1, a3b1, a1b2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at a1b4, a2b4 or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample,

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and neither target nucleic acid was detected in the upper right portion of the biological sample, as shown in 550. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample.

~~[00051]~~[00068] In addition to location information, information relating to relative abundance of the encoded tags can be obtained. For example, if it is found that there are ten times as many a4T1b1 sequences occurring in the data set as compared to a4T1b2 sequences, this would indicate that target nucleic acid sequence 1 is ten times more abundant at the a4T1b1 location than at the a4T1b2 location.

~~[00052]~~[00069] In the case of nucleotide analysis as shown in Figure 3, by ligating the coding tags directly to target-specific oligonucleotides, only 2n target-specific oligonucleotides are needed for n targets. For example, using the combinatorial approach outlined in Figure 2, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 target-specific oligonucleotides and 2 x 100 encoding oligonucleotides. The total count of assay oligonucleotides would be only 400 (200 target-specific and 200 encoding), not counting universal primers. In contrast, if the coding oligonucleotides were not decoupled from the target-specific oligonucleotides, (n x X positional codes) + (n x Y positional codes) would he needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences. Moreover, though the embodiments shown in Figures 2- 5 depict a combinatorial scheme using two encoding agents (coding tags), three, four or more encoding agents and coding tags may be used, and attached to the probe or one another by varying means and in varying combinations of steps.

~~[00053]~~[00070] Due to the spatial encoding aspect of the assay system of the invention, a large amount of information can be generated with even a modest number of assays. For example, five or more biological targets assayed at five or more positions in the sample generates 25 or more combinations. Using digital sequencing as a readout, the optimum number of sequence reads per combination depends on the sensitivity and dynamic range required, and can be adjusted. For example, if for each combination on average 100 reads are sampled, the total for 25 combination is 25,000 reads. If 1,000 targets are assayed at 1,000 locations with an average sampling depth of 1,000, then 10^9 reads are required. These numbers, although large, are within the capacity of intrinsically parallel digital sequencing methods, which can generate datasets of

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billions or even trillions of reads in a reasonable timeframe and at a very low cost per read. Therefore, by varying the numbers of positions interrogated or biological targets assayed, or both, and using digital sequencing, large amounts of information can be obtained. In specific aspects, multiple locations are interrogated for two or more biological molecules.

Reagent Delivery Systems

~~[00054]~~[00071] The reagent delivery system of the invention includes instrumentation that allows the delivery of reagents to discrete portions of the biological sample, maintaining the integrity of the spatial patterns of the encoding scheme. Reagent delivery systems of the assay systems of the invention comprise optional imaging means, reagent delivery hardware and control software. Reagent delivery can be achieved in a number of different ways. It should be noted that reagent delivery may be to many different biological samples at one time. A single tissue section has been exemplified herein; however, multiple biological samples may be affixed and analyzed simultaneously. For example, serial sections of a tissue sample can be analyzed in parallel and the data combined to build a 3D map.

~~[00055]~~[00072] Integral to the assay system of the invention is instrumentation that allows for spatial patterning of reagents onto the biological sample. Technologies for formulating and delivering both biological molecules (*e.g.* oligonucleotides or antibodies) and chemical reagents (*e.g.*, small molecules or dNTPs) are known in the art, and uses of these instrument systems are known to one skilled in the art and easily adaptable to the assay systems of the invention. One example of a suitable reagent delivery system is the Labcyte™ Echo acoustic liquid handier, which can he used to deliver nanoliter scale droplets containing biological molecules with high precision and reproducibility. One skilled in the art could incorporate this reagent delivery device into the overall system, using software to specify the locations to which reagents should be delivered.

~~[00056]~~[00073] Other instruments that can be used for the deposition of agents and/or coding identifiers onto biological samples include, but are not limited to, ink jet spotting; mechanical spotting by means of pin, pen or capillary; micro contact printing; photochemical or photolithographic methods; and the like. For several applications, it may be preferred to segment or sequester certain areas of the biological samples into one or more assay areas for different reagent distributions and/or biological target determination. The assay areas may be physically

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separated using barriers or channels.

~~[00057]~~[00074] In one exemplary aspect, the reagent delivery system may be a flow-based system. The flow-based systems for reagent delivery in the present invention can include instrumentation such as one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Reagent delivery systems are configured to move fluid to contact a discrete section of the biological sample. Movement of the reagents can be driven by a pump disposed, for example, downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, reagents may be driven through the fluid by gravity. US Pub. Nos. 20070166725 and 20050239192 disclose certain general-purpose fluidics tools that can be used with the assay systems of the invention, allowing for the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

~~[00058]~~[00075] In a more specific example, one or more flow-cells can be attached to the substrate-affixed biological sample from above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump is used to deliver reagents to the flow-cell and across the biological sample. The flow cells are configured to deliver reagents only to certain portions of the biological sample, restricting the amount and type of reagent delivered to any specific section of the biological sample.

~~[00059]~~[00076] In another aspect, a microfluidic system can be integrated into the substrate upon which the biological sample is disposed or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid may he formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected and delivered according to selective opening and closing of valves disposed between reagent reservoirs.

~~[00060]~~[00077] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes (*i.e.*, microfluidic structures). The pump can operate mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among other means. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. Mechanical pumps may be micromachined, molded, etc.

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Exemplary electrical pumps may include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

~~[00061]~~[00078] Valves generally include any mechanism for regulating the passage of fluid through a channel. Valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into a channel to partially or completely block a channel, an electrocapillary structure, and/or the like.

~~[00062]~~[00079] An open gasket can be attached to the top of the biological sample and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber may be formed by a gasket sandwiched between the biological sample on the substrate and a chemically inert, water resistant material such as, but not limited to, black-anodized aluminum, thermoplastics (*e.g.*, polystyrene, polycarbonate, etc), glass, etc.

~~[00063]~~[00080] In an optional embodiment, the assay system comprises imaging means to determine features and organization of the biological sample of interest. The images obtained, *e.g.*, may be used to design the deposition pattern of the reagents. Imaging means are optional, as an individual can instead view the biological sample using, *e.g.*, a microscope, analyze the organization of the biological sample, and specify a spatial pattern for delivery assay reagents. If included, the delivery system can comprise a microcircuit arrangement including an imager, such as a CCD or IGFET-based *(e.g.*, CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Pub. No. 20090197326, which is incorporated herein by reference. Also, it should be noted that although Figures 4 and 5 illustrate using a x,y grid configuration, other configurations can be used, such as, *e.g.*, following the topology of a tissue sample; targeting certain groups of cells, cell layers and/or cell types in a tissue, and the like.

~~[00064]~~[00081] In yet another alternative, the reagent delivery system controls the delivery of reagents to specific patterns on a biological sample surface using semiconductor techniques such as masking and spraying. Specific areas of a biological sample can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the biological sample using conventional techniques such as spraying or fluid flow. The use of masked delivery results in a patterned delivery scheme on the

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substrate surface.

~~[00065]~~[00082] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms (*e.g.*, thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions are be achieved.

~~[00066]~~[00083] In order to target specific sites of interest, an informative image of the biological sample to be assayed may be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions of the biological sample can be identified using image processing (*e.g.*, images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with other features of the assay system. In some aspects, software is used to automatically translate image information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample for reagent delivery is thus an important component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and/or other very accurate physical positioning systems can be adapted to this purpose.

~~[00067]~~[00084] The invention preferably comprises a complete suite of software tailored to the assay system. Optionally, oligonucleotide design software is used to design the encoding nucleotides (and in embodiments where nucleic acids are assayed, the target-specific oligonucleotides) for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for reagent delivery and data analysis (*i.e.*, sequence analysis) may be integrated to determine assay results. Integrated data analysis is particularly useful, as the type of dataset that is generated may be massive as a consequence of scale. Algorithms and software tools that are specifically designed for analysis of the spatially-associated data generated by the assay systems, including pattern-analysis software and visualization tools, enhance the value of the data generated by the assay systems.

~~[00068]~~[00085] In certain aspects, the assay system comprises processes for making and carrying out the quality control of reagents, *e.g.*, the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents are formulated according to factors such as volatility, stability at key temperatures, and chemical compatibility for compatibility with the reagent delivery instrumentation and may be analyzed by instrumentation integrated within the assay system.

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Sequencing

~~[00069]~~[00086] Numerous methods can be used to identify the coding tags and probe sequences in the encoded probes of the assay systems of the invention. The coding tags can be detected using techniques such as mass spectroscopy (*e.g.*, Maldi-T of, LC-MS/MS), nuclear magnetic resonance imaging, or, preferably, nucleic acid sequencing. Examples of techniques for decoding the coding tags of the present invention can be found, for example, in US Pub. No. 20080220434, which is incorporated herein by reference. For example, the coding tags may be oligonucleotide mass tags (OMTs or massTags). Such tags are described, *e.g.*, in US Pub. No. 20090305237, which is incorporated by reference in its entirety. In yet another alternative, the encoded probes can be amplified and hybridized to a microarray. This would require separate amplification reactions to be carried out, in which each amplification is specific to a particular spatial code or subset of codes, accomplished by using code-specific primers. Each amplification would also incorporate a different resolvable label (e.g. fluorophor). Following hybridization, the relative amounts of a particular target mapping to different spatial locations in the sample can he determined by the relative abundances of the resolvable labels.

~~[00070]~~[00087] In one particularly preferred aspect, the resulting coding tags according to the assay system are substrates for high-throughput, next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of the coding tags, for example, with SOLiD™ technology (Life Technologies, Inc.) or Genome Ananlyzer (Illumina, Inc.). Such next-generation sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in *e.g.*, Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et al, U.S. patent publication 2005/0191656; sequencing-by- synthesis methods, *e.g.*, U.S. Pat Nos. 6,210,891; 6,828,100; 6,969,488; 6,897,023; 6,833,246; 6,911,345; 6,787,308; 7,297,518; 7,462,449 and 7,501,245; US Publication Application Nos. 20110059436; 20040106110; 20030064398; and 20030022207; Ronaghi, et al, Science, 281: 363-365 (1998); and Li, et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); ligation-based methods, *e.g.*, U.S. Patent Nos. 5,912,148 and 6,130,073; and U.S. Pat. Appln Nos. 20100105052, 20070207482 and 20090018024; nanopore sequencing *e.g.*, U.S. Pat. Appln Nos. 20070036511; 20080032301; 20080128627; 20090082212; and Soni and

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Meller, Clin Chem 53: 1996-2001 (2007)), as well as other methods, *e.g.*, U.S. Pat. Appln Nos. 20110033854; 20090264299; 20090155781; and 20090005252; also, see, McKernan, et al., Genome Res., 19:1527-41 (2009) and Bentley, et al., Nature 456:53-59 (2008), all of which are incorporated herein in their entirety for all purposes.

Applications of Assay System

~~[00071]~~[00088] It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous important areas of biological research, diagnostics, and drug development that will benefit from a high throughput multiplexed assay system that can measure simultaneously the amount and spatial location of a biological target in a biological sample. For example, combining the ability to estimate the relative abundance of different RNA transcripts with the ability to reconstruct an image of spatial patterns of abundance across many locations, which may be as small as or even smaller than individual cells, in a tissue enables many different areas of basic research. The following are exemplary uses and are by no means meant to be limiting in scope.

~~[00072]~~[00089] In one example, 3-dimensional patterns of gene expression are determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning. Such a method can be used to measure changes in gene expression in disease pathology, *e.g.*, in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues is obtained, leading to new insights into the function and regulation both in normal and diseased states, and provides new hypotheses that can be tested. For example, an assay system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney, et al., Nature, 447:799-816 (2007)) and modENCODE to be integrated at the tissue level. The assay systems also aid computational efforts to model interacting networks of gene expression in the field of systems biology.

~~[00073]~~[00090] The assay systems also provide a novel approach to analysis of somatic variation, *e.g.*, somatic mutations in cancer or variability in response to infectious

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organisms. For example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer-related processes in both cancer and genetically normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers. In another example, individuals have varying susceptibility to infectious organisms, and the assay systems of the invention can be used to study the interaction between microbes and tissues or the various cell types within the tissue.

~~[00074]~~[00091] Importantly, in addition to providing spatially-associated information, the invention allows a great increase in the sensitivity of detecting rare mutations, as signal to noise can he dramatically increased since only a small location is assayed in any given reaction. In a typical assay for rare mutations in a mixed sample, the sample is treated in bulk, *i.e.*, nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in one cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the assay systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, in the assay systems of the present invention, background is reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed, which may be particularly important in detecting key mutations in tissue sections in cancer. Already molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics. The technology of the invention promises to greatly increase the power of such approaches.

[00092] The present invention provides assays, assay systems, and methods of using such assays in spatially encoded biological assays. The invention provides an assay system comprising one or more agents provided in defined spatial patterns on a substrate surface, and a detection system for identifying the presence or absence, relative amount, and location of a biological molecule. Such biological molecules include, but are not limited to, nucleic acids, peptides, carbohydrates, cellular components, and the like. The assay system is a novel multiplexing

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approach, as it allows multiple molecules and their respective multiple locations to be identified in a single system using a unique encoding scheme. This encoding scheme uses both molecule-specific binding agents and coding identifiers to provide a practical and cost-effective determination of information on multiple biological molecules, including specific positional information of such molecules in a biological sample, *e.g.*, a tissue section. The single molecule detection analysis using the encoding system also allows relative amounts of biological molecules to be detected, thus providing information on expression levels, sequestering in specific locales, and the like.

[00093] The assay systems detect the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. In addition, the assays provide methods for doing this for multiple biological molecules simultaneously. The assay systems utilize one or more binding agents that specifically bind to the biological molecule of interest and unique coding identifiers associated with specific binding agents. The detection system utilizes a method for identifying the presence and spatial address of the agent binding based on the positive and/or negative results that are obtained using detection of the agent and identifier and the encoding scheme of the spatial patterns on the substrate surface. In a specific aspect, the encoding scheme employs limited reagent delivery to the spatial patterns on the substrate surface, and access of the coding identifiers and/or binding agents to portions of the sample is controlled through such limited delivery.

[00094] In one aspect, the assay system detects the presence or absence and spatial location of a biological molecule based on the positive and/or negative results that are obtained using limited reagent delivery and the encoding scheme of the spatial patterns on the substrate surface.

[00095] The assay system and methods of the invention are based on relational, solid-state substrates with positions that represent specific spatial locations within a biological sample, *e.g.*, a cell, organelle or tissue. The ability to use encoding features to represent locations allows high-throughput analysis of the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. The encoding features also allow provide assaying of multiple biological molecules at these multiple locations simultaneously.

[00096] A primary feature of the invention is the preservation of the spatial organization of elements in a sample of interest through the use of an encoding scheme. For example, the assay may be designed to preserve the relative position of cells in a tissue, and the assay may interrogate

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the individual cells for genomic DNA variation (including epigenetic modifications), and RNA and protein expression.

[00097] In one specific aspect, the encoding scheme of the assay system comprises the use of two or more coding patterns, each comprising regions defined by spatial patterns on the substrate surface. For example, the assay system can utilize an encoding scheme that comprises a 2-dimensional grid format based on the discrete positioning of the binding agents in the substrate surfaces. In another example, the spatial patterns may be based on more randomized cell locations, *e.g.*, the patterns on the substrate surface follow an underlying biological structure rather than a strict, x,y grid pattern. This aspect includes systems with two or more substantially identical spatial patterns using different binding agents and/or coding identifiers, as well as systems having different patterns for different agents and/or coding identifiers. The encoding scheme of the systems can be controlled by delivery of different reagents to discrete regions on the substrate surfaces, which allows different reactions to take place on substantially similar agents of known location on the substrate surfaces.

[00098] In one specific aspect, the invention provides high resolution, high-throughput analysis of nucleic acids and/or expression levels that provides both detection and spatial identification of large numbers of nucleic acids, *e.g.*, DNA or RNA.

[00099] In another specific aspect, the invention provides high resolution, high-throughput analysis of proteins that provides both detection and spatial identification of large numbers of such proteins, *e.g.*, kinases or proteases.

[00100] Numerous reagent delivery systems can be used with the assay system of the invention. The primary criteria of such reagent delivery systems is the ability to direct delivery of specific agents based on spatial patterns on the substrate surface.

[00101] In one preferred aspect, the encoding scheme utilizes a reagent delivery system based on printing and informatics technologies to implement the spatial patterns used for identification and localization of the biological materials. For example, the patterns found in the encoding scheme may be created using ink jet printing technology to provide reagents at specific locations on one or more substrate surfaces. The desired patterns are set out in specific coding patterns on the substrate surface.

[00102] In certain aspects of the invention, the binding agents are immobilized directly to the substrate surface, and the location of the binding agents is known or determined prior to use

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of the substrate surface in the assay system. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, e.g., provided in wells or channels.

[00103] In specific aspects of the invention, the binding agents are nucleic acids immobilized directly or indirectly to the substrate surface, *e.g.*, directly through the use of amino groups on the substrate surface or indirectly through the use of a linker. The location of the nucleic acid sequences is known or determined prior to use of the substrate surface in the assay system. In another specific aspect, the nucleic acids may be immobilized directly or indirectly onto beads that are then provided in known locations on the substrate surface. In yet another aspect, the nucleic acids may be provided in or on features of the substrate surface, *e.g.*, provided in wells.

[00104] In these aspects involving nucleic acid agents, any methods of sequence determination can be used, *e.g.*, sequencing, hybridization and the like. In a preferred aspect, nucleic acid sequencing, and preferably next-generation sequencing, is used to decode the spatial encoding scheme in the assay system of the invention. This provides a very wide dynamic range for very large numbers of assays, allowing for efficient multiplexing.

[00105] In some aspects, the assay utilizes two or more oligonucleotides, the oligonucleotides comprising a universal primer region and a region that correlates specifically to a single spatial pattern within the spatial encoding scheme. In a specific aspect, the assay comprises two allele specific oligonucleotides and one locus specific oligonucleotides. These oligonucleotides allow the identification of specific SNPs, indels or mutations within an allele. This is useful in the identification of genetic changes in somatic cells, genotyping of tissues, and the like.

[00106] In other specific aspects of the invention, the binding agents are peptides. In one aspect, these peptides are associated directly or indirectly to known locations on a substrate surface, *e.g.*, using binding protein pairs or through oligonucleotide linkers complementary to oligonucleotides on the substrate surface. In another aspect, the binding agents are peptides are immobilized directly or indirectly onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the peptides may be provided in or on features of the substrate surface, *e.g.*, provided in wells.

[00107] In yet other specific aspects of the invention, the binding agents are chemical

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entities (*e.g.*, small molecules) that are coded, e.g. using sequence tags or mass spectroscopy tags as coding identifiers. In one aspect, these chemical entities can be are immobilized directly to the substrate surface. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, *e.g.*, provided in wells.

[00108] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

The Invention in General

[00109] The assay system and methods of the invention are based on relational methods that allow extraction of data to detect the presence or absence and relative amount of a biological molecule, and the location of this molecule in a sample having a distinct structure, *e.g.*, a tissue section or other biological structure with distinct locations of specific biological molecules. The encoding scheme used in these systems corresponds to the structural elements of the sample, and the information obtained using a two-dimensional coding system is indicative of the spatial addresses of these molecules in a sample of interest.

[00110] Integral to the assay system of the invention is a method for spatial patterning of reagents. Technologies for formulating and delivering both biological molecules (*e.g.* DNA or antibodies) and chemical reagents (*e.g.*, small molecules or dNTPs) have already been demonstrated, and use of these systems will be available to one skilled in the art and easily adaptable upon reading this specification.

[00111] The assay design of the invention provides an accurate and easily scalable spatial encoding system. The ability to deliver reagents in a spatially defined pattern together with software, reagents and protocols comprises a novel and highly innovative assay system for spatial analysis of various biological molecules and activities. This allows the assays to measure numerous biological functions in a meaningful spatial environment, including functions such as gene expression and peptide localization. The systems provide the potential to open a new

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analytical window into the complex spatial patterns of cellular function and regulation in biological systems.

[00112] The biological molecules to be detected can be any biological molecules such as proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Further examples of subcellular objects include organelles, *e.g.*, mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplast, endocytic vesicle, exocytic vesicles, vacuole, lysosome, etc.

[00113] Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system 401, two reagents 420, 422 that specifically bind to a biological molecule of interest are associated with coding identifiers 406, 408 that encode for a spatial location in the sample. These coding identifiers 406, 408 are optionally associated with sites that assist in their identification in the assay format, *e.g.*, universal priming sites 404, 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section 416 is encoded using the combination of the patterns 412, 414 created using the separate coding identifiers 406, 408 which provide a two dimensional code 418 that shows the location of any positive detection of the biological molecule 402 as well as quantifying the biological molecule 402 at each location assayed in the tissue.

[00114] The assay systems of the invention are particularly advantageous in that they are compatible with numerous samples types, such as fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the binding agents are immobilized on a substrate surface in discrete, independently measureable areas. These discrete areas can be formed by spatially selective deposition of the binding agents on the substrate surface. Numerous methods can be used for the deposition of the agent and the coding identifiers associates with the agent. For example, the coding identifiers can be delivered together or separately from the agent. If delivered together they can be attached (*e.g.*, synthesized as a single molecule or attached through ligation or a chemical coupling mechanism) or simply mixed together to be attached after delivery to the substrate. In a preferred aspect, the agent and the coding identifier are made separately, mixed together for attachment, and delivered either attached or as a mixture to be attached on the surface. In a specific aspect the binding agents

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are delivered generally over the substrate surface and the coding identifiers are delivered in a pattern-specific manner.

[00115] Examples of methods that can be used for deposition of agents and/or coding identifiers onto the substrate surface include, but are not limited to, ink jet spotting, mechanical spotting by means of pin, pen or capillary, micro contact printing, fluidically contacting the measurement areas with the biological or biochemical or synthetic recognition elements upon their supply in parallel or crossed micro channels, upon exposure to pressure differences or to electric or electromagnetic potentials, and photochemical or photolithographic immobilization methods.

[00116] For several applications, it may be preferred to arrange the substrates into segments of one or more measurement areas for reagent distribution and agent determination. These regions may be physically separated using barriers or channels. They may still comprise several additional discrete measurement areas with agents that are different or in different combination from each other.

[00117] In certain aspects, the present invention provides a method, *e.g.*, a machine-based method, for evaluating changes in the presence and/or location of a biological molecule over time. The method includes providing a plurality of encoded array results representative of the biological molecule over time and evaluating the differences in detection and/or localization of the biological molecules.

Nucleic Acid Detection and Localization

[00118] In a particular aspect, the assay system is used to analyze nucleic acids, *e.g* genotyping, gene expression analysis, localization of particular transcripts within samples, and the like.

[00119] Genotyping may be performed using any technique known to those of skill in the art. Preferred techniques permit rapid, accurate determination of multiple variations with a minimum of sample handling. Some examples of suitable techniques involve but are not limited to direct DNA sequencing, capillary electrophoresis, hybridization, allele-specific probes or primers, single-strand conformation polymorphism analysis, nucleic acid arrays, bead arrays, restriction fragment length polymorphism analysis, cleavage fragment length polymorphism

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analysis, random amplified polymorphic DNA, ligase detection reaction, heteroduplex or fragment analysis, differential sequencing with mass spectrometry, atomic force microscopy, pyrosequencing, FRET (*e.g.*, TaqMan (Applied Biosystems, Inc., Foster City, Calif.) and Molecular Beacon (Stratagene, La Jolla, Calif.) assays), and other related techniques. Several methods for DNA sequencing are well known and generally available in the art. See, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, New York) (2001); Ausubel, et al., Current Protocols in Molecular Biology (John Wiley and Sons, New York) (1997), Twyman, et al. (2003) "Techniques Patents for SNP Genotyping", Pharmacogenomics 4(1):67-79; and Kristensen, et al. (2001) "High-Throughput Methods for Detection of Genetic Variation", BioTechniques 30(2):318-332. For details on the use of nucleic acid arrays (DNA chips) for the detection of, for example, SNPs, see U.S. Pat. No. 6,300,063 issued to Lipshultz, et al., and U.S. Pat. No. 5,837,832 to Chee, et al., HuSNP Mapping Assay, reagent kit and user manual, Affymetrix Part No. 90094 (Affymetrix, Santa Clara, Calif.). The molecular inversion probe (MIP) assay format (Hardenbol et al., 2003) is another example of a highly multiplexable assay that may be used with the assay systems of the invention.

[00120] In one exemplary and preferred method for analyzing nucleic acids using the assay system of the invention, the detection of nucleic acids uses two allele- specific oligonucleotides and a locus specific oligonucleotide. The assay methods are carried out according to the strategy outlined in Fig. 2 using next-generation sequencing or another highly parallel nucleic acid assay technology. In this assay, a set of two oligonucleotides is designed to hybridize to each target sequence, with a common oligonucleotide and two unique coding identifiers. The allele can be determined, *e.g.* by primer extension of the locus specific oligonucleotide. Following primer extension and ligation, an amplifiable template is formed with universal primer sequences at either end. Assay oligonucleotides are annealed to a template and enzymatic reactions are used to join the two oligonucleotides only when both are correctly annealed. The detection techniques and read out parameters used in this system of the invention include a much shorter tag than the oligonucleotides used in the assays that are based on capture by hybridization. These shorter tags are designed to be read out by sequencing or, preferably, used to ligate codes onto both ends of the fragment as illustrated in Fig. 2.

[00121] In Figure 3, two target-specific assay oligonucleotides are ligated together 302 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides

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containing tag sequence sets X and Y are ligated 304 to the target specific oligonucleotides. Oligonucleotides containing X ligate specifically to one side of the targeting construct and oligonucleotides containing Y ligate to the other. The oligonucleotides contain universal primer sites P1 and P2. Following ligation, the constructs are eluted and, optionally, sequencing adapters can be attached 306, *e.g.*, via PCR.

[00122] In one preferred aspect, the final construct created from the assay method is a substrate for next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of constructs. Such sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et al, U.S. patent publication 2005/0191656, and sequencing by synthesis methods, *e.g.*, Nyren et al, U.S. Pat. No. 6,210,891; Ronaghi, U.S. Pat. No. 6,828,100; Ronaghi et al (1998), Science, 281: 363-365; Balasubramanian, U.S. Pat. No. 6,833,246; Quake, U.S. Pat. No. 6,911,345; Li et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); Smith et al, PCT publication WO 2006/074351; use of reversible extension terminators, *e.g.*, Turner, U.S. Pat. No. 6,833,246 and Turner, U.S. Pat. No. 6,833,246 and ligation-based methods, *e.g.*, Shendure et al (2005), Science, 309: 1728-1739, Macevicz, U.S. Pat. No. 6,306,597; which references are incorporated by reference. Soddart et al., PNAS U S A. 2009 Apr 20; Xiao et al., Nat Methods. 2009 Mar;6(3):199-201. Epub 2009 Feb 8.

[00123] To maximize the efficiency of encoding, a combinatorial approach using pairs of oligonucleotides can be used. For example, with only two sets of 100 codes, a substrate can theoretically encode up to 10,000 locations. The number of assay oligonucleotides required is dramatically reduced, the cost decreased, and the robustness of the approach increased by decoupling the coding sequences from the genome-specific sequences. Alternative assay formats can also be used (e.g. ligation or primer extension followed by ligation).

[00124] By ligating the codes on separately, only 2n target-specific assay oligonucleotides are needed for n targets. For example, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 targeting oligonucleotides and 2 x 100 encoding oligonucleotides, using a combinatorial approach outlined in Fig. 2. The total count of assay oligonucleotides would be only 400 (200 target-specific and 200 encoding), not counting universal primers. In contrast, if

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the coding oligonucleotides were not decoupled, (n x X positional codes) + (n x Y positional codes) would be needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences.

[00125] Due to the matrix system of the invention, a large amount of information can be obtained even using five or more positions interrogated for five or more biological molecules. By varying one or the other of these, large amounts of information can be obtained, both in terms of locations and/or specific biological In specific aspects, multiple locations are interrogated for two or more biological molecules. As an example, for each datapoint ~1,000 reads may be sampled, for a total of -10E9 reads for 10E6 datapoints.

Peptide Detection Systems

[00126] The assay system of the invention can be used to analyze biological molecules using peptide agents that are associated with the substrate surface in a spatial pattern. Such peptides may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, etc.

[00127] The assay system of the invention allows the identification and spatial location of various forms of peptides, including isoforms and peptides that have undergone posttranslational modification. Importantly, certain aspects of the invention allow the identification of active versus non-active forms of such peptides in a sample. This allows the identification of the presence or absence of specific peptide isoforms, and also acts as a proxy for identification of peptide activity in a sample.

[00128] In certain aspects of the invention, the binding agents associated with the substrate surfaces of the assay system include substrates for enzymes or proenzymes, *e.g.*, a kinase, a phosphatase, a zymogen, a protease, or a fragment thereof. In certain aspects, the binding agents associated with the substrate surfaces are phosphorylation substrates used to detect proteins involved with one or more signal transduction pathways, *e.g.*, a kinase or a phosphatase. In another specific aspect of the invention, the binding agents are specific protease substrates that associate only with individual or classes of proteases. In other aspects, the binding agents on the substrate surface are different processed forms, isoforms and/or domains of an enzyme.

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Reagent Delivery

[00129] The reagent delivery system of the invention can be any system that allows the delivery of reagents to discrete portions of the array in order to keep the integrity of the defined spatial patterns of the encoding scheme. Such discrete delivery can be achieved in a number of different ways.

[00130] In one exemplary aspect, the reagent delivery system can be a flow-based system. The flow-based systems for reagent delivery in the present invention can include one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Such a reagent delivery system is configured to move fluid in contact with a discrete section of the substrate surface. Movement of the reagents can be driven through a fluid by a pump disposed, for example, downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, the reagents may be driven through the fluid by gravity.

[00131] US Appln Nos. 20070166725 and 20050239192 disclose certain general- purpose fluidics tools that can be used with the assay systems of the invention. These allow the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

[00132] In a more specific example, one or more flow-cells can be attached to the substrate from above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump can be used to deliver the sample or reagents to the flow-cell and across the substrate. The flow cell is configured to deliver reagents only to certain portions of the array, restricting the amount and type of reagent delivered to any specific section of the array.

[00133] In another aspect, a microfluidic system can be integrated into the substrate or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid can be formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected according to selective opening and closing of valves disposed between reagent reservoirs.

[00134] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes (i.e., microfluidic structures). The pump can operate

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mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among others. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. The mechanical pumps may be micromachined, molded, etc. Exemplary electrical pumps can include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[00135] Valves generally include any mechanism for regulating the passage of fluid through a channel. The valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into the channel to partially or completely block the channel, an electrocapillary structure, and/or the like.

[00136] In yet another aspect, an open gasket can be attached to the top of the substrate and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber formed by a gasket sandwiched between the substrate and a chemically inert, water resistant material such as, but not limited to, black-anodized aluminum, thermoplastics (e.g., polystyrene, polycarbonate, etc), glass, etc.

[00137] In a specific aspect of the present invention, the delivery system can comprise a microcircuit arrangement including an imager, such as a CCD or IGFET-based (e.g., CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Appln No. 20090197326, which is incorporated herein by reference.

[00138] In yet another aspect of the invention, the reagent delivery system controls the delivery of reagents to specific patterns on a substrate surface using semiconductor techniques such as masking and spraying. Specific areas of a substrate surface can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the substrate using conventional techniques such as spraying or fluid flow. The use of the masked substrate delivery results in a patterned delivery scheme on the substrate surface.

[00139] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms (*e.g.*,

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thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions can be achieved.

Software for use in the Assay System

[00140] In order to target specific sites of interest, an informative image of the biological section to be analyzed can be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions can be identified using image processing (*e.g.*, images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with the other features of the assay system. In some aspects, software is used to automatically translate this information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample in a targeting system is thus a preferred component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and other very accurate physical positioning systems can be adapted to this purpose.

[00141] Additional software components will also be key components that will be part of a complete assay system. The invention thus preferably comprises a complete suite of software tailored to the assay system. Optionally, oligonucleotide design software will be customized for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for data analysis may be integrated to assist in determination of results of the assays. This can be especially useful, as the type of dataset that will be generated will be novel, particularly as a consequence of scale. The ability to provide algorithms and software tools that are specifically designed for analysis of spatially-associated data for significant patterns, including pattern-analysis software and visualization tools, is a novel feature that will enhance the value of the data generated by the assay systems.

[00142] In certain aspects, the assay system will comprise processes for making and carrying out quality control of reagents, *e.g.*, the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents will need to be formulated for compatibility with the reagent delivery instrumentation. Factors such as volatility, stability at key temperatures, and chemical compatibility can be optimized by those skilled in the art upon reading the present disclosure.

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Applications of Assay System

[00143] It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous very important areas of biological research, diagnostics, and drug development that will benefit from a high throughput means to simultaneously measure the presence or absence and spatial location of a biological molecule in a sample. For example, this technology combining the ability to analyze semi-quantitatively the expression of many different genes with the ability to image the spatial organization of expression across many cells in a tissue is enabling for many different areas of basic research. The following are exemplary uses and are by no means meant to be limiting in scope.

[00144] In one example, 3-dimensional patterns of expression can be determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning. This can be used to measure changes in gene expression in disease pathology, *e.g.*, in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues can be obtained. This may lead to new insights into the function and regulation both in normal and diseased states, and is likely to provide new hypotheses that can be tested. For example, a system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney et al., 2007) and modENCODE to be integrated at the tissue level. The assay systems will also aid in computational efforts to model interacting networks of gene expression in the field of systems biology.

[00145] The assay systems also provide a novel approach that enables the analysis of somatic variation, *e.g.*, somatic mutations in cancer or variability in response to infectious organisms. For example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer related processes in both cancer and genetically

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normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers.

[00146] In another example, different people have varying susceptibility to infectious organisms, and much of this may be to underlying genetic differences in individuals and/or populations. Identifying these differences will aid in an understanding of the underlying disease pathologies and assist in the development of vaccines or therapeutics to prevent or ameliorate these disease states.

[00147] Importantly, in addition to providing spatially associated information, the technology of the invention will allow a great increase in the sensitivity of detecting rare mutations. The reason is that signal to noise can be dramatically increased because the approach of the invention assays a small location in any given reaction. In a typical assay for rare mutations in a mixed sample, the sample is treated in bulk, i.e. nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in 1 cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, the background can be reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed. This may be particularly important in detecting key mutations in tissue sections in cancer. Already, molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics (Choe et al., 2003). The technology of the invention promises to greatly increase the power of such approaches.

EXAMPLES

~~[00075]~~[00148] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventor regards as his invention, nor are they intended to represent or imply that the experiments below are all of or the only experiments performed. It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific

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embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

~~[00076]~~[00149] Efforts have been made to ensure accuracy with respect to numbers used *(e.g.,* amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees centigrade, and pressure is at or near atmospheric.

EXAMPLE 1: Initial Proof of Concept of Encoding Scheme

~~[00077]~~[00150] As an initial proof of concept, a model system is developed using a microarray to demonstrate a working single-plex assay. The basic design validates the concept of the assay, and establishes a working assay prior to addressing issues related to the analysis of a more complicated biological sample. Conventional sequencing is used as a readout for this proof of concept.

~~[00078]~~[00151] A microarray is used as a proxy for a tissue section. The target sequences of the microarray are fully specified, so that the composition of the targets are known and can be varied systematically. Synthetic oligonucleotide templates are attached to a glass slide via a 5' amino modification. Each slide has a single oligonucleotide template sequence, and the assays that are carried out may employ either ligation, or extension followed by ligation as this may be useful in determining certain polymorphisms.

~~[00079]~~[00152] Once the *in situ* part of the assay is complete, the reaction products are eluted and analyzed by qPCR to determine presence or absence of a product and estimate yield, and by conventional sequencing to determine the structure of the assay products. The single-plex assays that are tested include appropriate positive and negative controls, and a single nucleotide variant (SNV) to check ability to discriminate single base variants.

EXAMPLE 2: Scalability

~~[00080]~~[00153] The complexity of the assay system is increased to establish scalability of the assay for use in high throughput studies. Scalability of both the spatial encoding

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and assay systems is demonstrated by carrying out a 24-plex x 24-site assay using a microarray model system.

~~[00081]~~[00154] The amount of biological target, here a DNA target sequence, at each assay location is systematically varied on microarray substrate. For example, in a microarray with 50 micron spot size (center to center), a $1mm^2$ area contains ~400 spots. The region around each site is optionally occupied by a region that is devoid of these spots to allow individual resolvability of the target sequences. Alternatively, the spots may be clustered, with two or more directly adjacent spots surrounded by or adjacent to a region that is devoid of target sequences.

~~[00082]~~[00155] In order to demonstrate that spatial encoding is accurate, the sites comprise different target compositions to show that the assay readout matches the expected composition of each site. With 24 target sequences, a simple digital pattern is made with each site having a different set of 12 targets present and 12 targets absent, to make a binary code (0 = absent, 1 = present). The assay readout is then determined to show that the detected regions match the expected signal after spatial decoding. In this particular example, the code space is large enough (2^{24}) so that even a few errors would not result in different codes being mixed up. Moreover, this design allows identification of errors and allows an estimation not only of accuracy of spatial encoding but also of accuracy calling the presence or absence of target sequences.

[00156] In an exemplary aspect, a 4 x 4 arrangement of 16 sequences is used for the array configuration. A white square indicates that the sequence is absent and a black square that it is present, i.e. 8 of the 16 possible sequences are present in this sample. In a different sample, a different pattern of absent and present sequences can be constructed. In this way, unique patterns are associated with spatial locations so that the accuracy of spatial encoding can be measured.

~~[00083]~~[00157] The ability to detect quantitative differences is evaluated by generating dose- response curves for each of the 24 assays that are carried out at each site in a 24-site assay. This allows estimation of the limit of detection, dynamic range, and power to detect a given fold-change across the range.

~~[00084]~~[00158] In one aspect, a latin square design is used to represent individual targets at different ratios by varying the number of features for each target. In other words, with multiple spots in a site, the number of spots allocated to each of the 24 target sequences can be varied and each of the 24 sites can have a different composition. A 1 x 3 inch microarray is

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sufficiently large to permit multiple replicates. This larger set of 24 sequences will require deconvolution, and this is accomplished using high throughput techniques such as next-generation sequencing technologies *(e.g.,* SOLiD™ technology (Life Technologies, Inc., Carlsbad, CA) or Genome Analyzer (Illumina, Inc., San Diego, CA)). The use of the 24-plex assay demonstrates both the accuracy of spatial encoding and decoding, and the quantitative response of the assay system.

EXAMPLE 3: Adaptation of the assay to preserved samples.

~~[00085]~~[00159] Genomic DNA is assayed as a proof of concept for assaying RNA, as it provides a way to establish a single-copy reference signal Once a working assay is developed for FFPE samples, it is adapted to an RNA assay. To this end, assay oligonucleotide concentrations are assayed to ensure compatibility with high multiplexing. Assuming a cell diameter of 10 microns, and delivery of a 10 micron diameter reagent droplet to an individual cell, the volume of the droplet will be ~500 µl and can contain ~3 x 10^{11} molecules at a 1 µM concentration. To assay 1,000 target sequences in 10,000 cells, ~2,000 targeting oligonucleotides would be required in a droplet. Therefore, each droplet could contain ~160 million copies of each assay oligo, a vast excess over the few thousand target sequences in a cell.

~~[00086]~~[00160] The handling of small absolute numbers of product molecules generated from very small or compromised samples are enhanced to counter the issue of low recovery efficiency; that is, elution is efficient and losses resulting from adsorption of molecules to surfaces are prevented. An approach to addressing the latter issue is to include a carrier material, such as glycogen or carrier nucleic acids.

Example 4: Adapting the Assay to a Biological Sample.

~~[00087]~~[00161] A control RNA template is immobilized to a solid support in order to create an artificial system. The assay is performed using T4 DNA ligase, which can repair nicks in DNA/RNA hybrids. Assays are carried out on matched slides, or different sections of the same slide, where in one case gDNA is assayed and in the other RNA is assayed. When assaying gDNA the slide can be pretreated with RNase, and when assaying RNA the slide is pretreated

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with DNase. Results of the assay are confirmed by extracting gDNA or RNA and quantitating the relative amounts by qPCR or RT-qPCR respectively.

~~[00088]~~[00162] In order make the tissue section RNA assays as informative as possible, pre- existing information on expression levels in specific tissues to target transcripts across a range of abundances are used in the assay design. Both high abundance transcripts, as well as some medium and low abundance transcripts, are targeted to enable an initial assessment of the quantitative performance characteristics of the assay.

~~[00089]~~[00163] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims. In the claims that follow, unless the term "means" is used, none of the features or elements recited therein should be construed as means-plus-function limitations pursuant to 35 U.S.C. §112, ¶6.

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SPATIALLY ENCODED BIOLOGICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Patent Application No. 61/321,124, filed April 5, 2010 and is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] This invention relates to assays of biological molecules, and more particularly to assays for determining spatial distributions of a large number of biological molecules in a solid sample simultaneously.

BACKGROUND OF THE INVENTION

[0003] In the following discussion certain articles and methods will be described for background and introductory purposes. Nothing contained herein is to be construed as an "admission" of prior art. Applicant expressly reserves the right to demonstrate, where appropriate, that the articles and methods referenced herein do not constitute prior art under the applicable statutory provisions.

[0004] Comprehensive gene expression analysis and protein analysis have been useful tools in understanding mechanisms of biology. Use of these tools has allowed the identification of genes and proteins involved in development and in various diseases such as cancer and autoimmune disease. Conventional methods such as *in situ* hybridization and other multiplexed detection of different transcripts have revealed spatial patterns of gene expression and have helped shed light on the molecular basis of development and disease. Other technologies that have enabled the quantitative analysis of many RNA sequences per sample include microarrays (see Shi, et al., Nature Biotechnology, 24(9): 1151-61 (2006); and Slonim and Yanai, Plos Computational Biology, 5(10):e1000543 (2009)); serial analysis of gene expression (SAGE) (see Velculescu, et al., Science, 270(5235):484-87 (1995)), high-throughput implementations of

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qPCR (see Spurgeon, et al., Plos ONE, 3(2):e1662 (2008)) and *in situ* PCR (see Nuovo, Genome Res., 4:151-67 (1995)). As useful as these methods are, however, they do not enable simultaneous measurement of the expression of many genes or the presence and/or activity of multiple proteins at many spatial locations in a sample. Laser capture microdissection has permitted the analysis of many genes at a small number of locations, but it is very expensive, laborious, and does not scale well, Certain PCR assays in a 2D format preserve spatial information (see Armani, et al., Lab on a Chip, 9(24): 3526-34 (2009)), but these methods have low spatial resolution because they rely on physical transference of tissue into wells, which also prevents random access to tissue samples and high levels of multiplexing.

[0005] At present, no practical method exists to analyze at high resolution the spatial expression patterns of large numbers of genes, proteins, or other biologically active molecules simultaneously. There is thus a need for reproducible, high-resolution spatial maps of biological molecules in tissues. The present invention addresses this need.

SUMMARY OF THE INVENTION

[0006] This Summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This Summary is not intended to identify key or essential features of the claimed subject matter, nor is it intended to be used to limit the scope of the claimed subject matter. Other features, details, utilities, and advantages of the claimed subject matter will be apparent from the following written Detailed Description including those aspects illustrated in the accompanying drawings and defined in the appended claims.

[0007] The invention encompasses assay systems that provide high-resolution spatial maps of biological activity in tissues. The assay system comprises an assay capable of high levels of multiplexing where encoded probes are provided to a biological sample in defined spatial patterns; instrumentation capable of controlled delivery of reagents according to the spatial patterns; and a decoding scheme providing a readout that is digital in nature. In short, the present invention provides the ability to look at many biological targets in many locations, providing the resolution of in situ hybridization with the highly- parallel data analysis of sequencing.

[0008] Thus, in some embodiments, the invention provides an assay system to determine

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spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered; allowing the encoded probes to interact with the biological targets; separating encoded probes that interact with the biological targets from encoded probes that do not interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[0009] In particular aspects of the invention the biological targets comprise nucleic acids and the encoded probes are oligonucleotides, and in some aspects, there are two encoded probes for each of the multiple nucleic acid targets. In some aspects, the multiple biological targets comprise proteins, the probe regions of the encoding probes are proteins and the coding tags comprise oligonucleotides. In some aspects the multiple biological targets comprise enzymes. In some aspects the probe regions of the encoded probes comprise antibodies, aptamers or small molecules.

[00010] Some aspects of the assay system further comprise an amplification step between the separating step and the determining step. In some aspects, the determining step is performed by nucleic acid sequencing, and in preferred aspects, the sequencing is high-throughput digital nucleic acid sequencing.

[00011] In some aspects of the invention, the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 20, in some aspects product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 50, in some aspects the product of the multiple biological targets being assayed and the multiple sites in the sample is greater than 75, 100, 150, 500, 750, 1,000, 5,000, 10,000, 25,000, 50,000, 100,000, 500,000, or 1,000,000 or more. In other aspects, the sequence of at least fifty thousand encoding probes are determined in parallel, in other aspects the sequence of at least one hundred thousand encoding probes are determined in parallel, in some aspects the sequence of at least five hundred thousand encoding probes are determined in parallel, and in some aspects the sequence of at least one million, ten million, one hundred million, one billion, ten billion, one hundred billion or more

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encoding probes are determined in parallel.

[00012] In some aspects, the known spatial pattern is determined by histological features of the sample. Also in some aspects, software programmed hardware performs at least two steps of the delivering step, the separation step, the determining step and the associating step.

[00013] In some aspects, the probe regions of the encoded probes are proteins and the separating step is accomplished by encoded probes that interact with the biological targets being captured by an affinity capture agent. In some aspects the probe regions of the encoding probes are nucleic acids and the separating step is accomplished by a washing of the sample.

[00014] In other embodiments there is provided an assay system to determine spatial patterns of abundance or activity or both of multiple nucleic acid targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering oligonucleotide probes for multiple nucleic acid targets to the multiple sites in the sample in a known spatial pattern; allowing the oligonucleotide probes to hybridize with the nucleic acid targets; washing unhybridized encoded oligonucleotide probes from the sample; delivering one or more encoding agents to locations of the multiple sites in the sample according to a known spatial pattern, where the combination of encoding agents delivered to each site is different; coupling the encoding agents and the oligonucleotide probes to form encoded probes; determining all or a portion of a sequence of the encoded probes using high-throughput sequencing, and associating the abundance or activity or both of multiple biological targets to the locations of multiple sites in the sample.

[00015] Other embodiments of the invention provide an assay system to determine spatial patterns of abundance or activity or both of multiple protein targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple protein targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a protein probe region that may interact with the protein targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and the protein probe region of the encoding probe of which the coding tag is part; allowing the encoded probes to interact with the protein targets; separating encoded probes that interact with the protein targets from encoded probes that do not interact with the protein targets; determining all or a portion of a sequence of the encoded probes by high throughput sequencing, and associating the abundance or activity or both of the multiple protein

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targets to the locations of the multiple sites in the sample.

[00016] Other embodiments provide an assay system to determine spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, where the assay system performs the following steps: providing a sample affixed to a support; delivering encoded probes for the multiple biological targets to the multiple sites in the sample in a known spatial pattern, where each encoded probe comprises a probe region that may interact with the biological targets and a coding tag that identifies a location of the site to which the encoded probe was delivered and identifies the biological target; allowing the encoded probes to interact with the biological targets; determining all or a portion of a sequence of the encoded probes, and associating the abundance or activity or both of the multiple biological targets to the locations of the sites in the sample.

[00017] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

DESCRIPTION OF THE FIGURES

[00018] Figure 1 provides a simplified overview of the assay system of the present invention.

[00019] Figure 2 provides a simplified overview of one embodiment of the assay system of the present invention for detecting nucleic acids.

[00020] Figure 3 is a representational depiction of one embodiment of the assay overviewed in Figure 2.

[00021] Figure 4A-C illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention. Figure 4A shows two target-specific/encoding oligonucleotide constructs specifically bound to a target nucleic acid of interest in a sample. Figure 4B shows a scheme for delivering twenty different coding tags, a1 through a10 and b1 through b10, to a sample to form a 10x10 coding tag grid. Figure 4C shows a tissue section sample to which the coding tags are delivered, forming the coding tag grid in the sample.

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[00022] Figure 5 provides a simplified, specific example of the embodiment of a combinatorial encoding scheme shown in Figure 4.

DEFINITIONS

[00023] The terms used herein are intended to have the plain and ordinary meaning as understood by those of ordinary skill in the art. The following definitions are intended to aid the reader in understanding the present invention, but are not intended to vary or otherwise limit the meaning of such terms unless specifically indicated.

[00024] The term "antibody" as used herein is intended to refer to an entire immunoglobulin or antibody or any functional fragment of an immunoglobulin molecule which is capable of specific binding to an antigen (antibodies and antigens are "binding partners" as defined herein). "Antibody" as used herein is meant to include the entire antibody as well as any antibody fragments capable of binding the antigen or antigenic fragment of interest. Examples of such peptides include complete antibody molecules, antibody fragments, such as Fab, F(ab')2, CDRS, VL, VH, and any other portion of an antibody which is capable of specifically binding to an antigen. Antibodies for assays of the invention are immunoreactive or immunospecific for, and therefore specifically and selectively bind to, proteins either detected *(i.e.,* biological targets) or used for detection *(i.e.,* probes) in the assays of the invention.

[00025] The term "binding agent" as used herein refers to any agent that specifically binds to a biological molecule of interest.

[00026] "Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double-stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single-stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single-stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the other strand, usually at least about 90% to about 95%, and even about 98% to about 100%.

[00027] "Hybridization" refers to the process in which two single-stranded polynucleotides

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bind non-covalently to form a stable double-stranded polynucleotide. The resulting (usually) double-stranded polynucleotide is a "hybrid" or "duplex." "Hybridization conditions" will typically include salt concentrations of approximately less than 1M, often less than about 500 mM and may be less than about 200 mM. A "hybridization buffer" is a buffered salt solution such as 5% SSPE, or other such buffers known in the art. Hybridization temperatures can be as low as 5°C, but are typically greater than 22°C, and more typically greater than about 30°C, and typically in excess of 37°C. Hybridizations are often performed under stringent conditions, *i.e.*, conditions under which a primer will hybridize to its target subsequence but will not hybridize to the other, non-complementary sequences. Stringent conditions are sequence-dependent and are different in different circumstances. For example, longer fragments may require higher hybridization temperatures for specific hybridization than short fragments. As other factors may affect the stringency of hybridization, including base composition and length of the complementary strands, presence of organic solvents, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one parameter alone. Generally stringent conditions are selected to be about 5°C lower than the Tm for the specific sequence at a defined ionic strength and pH. Exemplary stringent conditions include a salt concentration of at least 0.01 M to no more than 1M sodium ion concentration (or other salt) at a pH of about 7.0 to about 8.3 and a temperature of at least 25°C. For example, conditions of 5xSSPE (750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA at pH 7.4) and a temperature of approximately 30°C are suitable for allele-specific hybridizations, though a suitable temperature depends on the length and/or GC content of the region hybridized.

[00028] "Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, *e.g.*, oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon terminal nucleotide of one oligonucleotide with a 3' carbon of another nucleotide.

[00029] "Nucleic acid", "oligonucleotide", "oligo" or grammatical equivalents used herein refers generally to at least two nucleotides covalently linked together. A nucleic acid generally will contain phosphodiester bonds, although in some cases nucleic acid analogs may be included that have alternative backbones such as phosphoramidite, phosphorodithioate, or

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methylphophoroamidite linkages; or peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids, positive backbones, non-ionic backbones and non-ribose backbones. Modifications of the ribose-phosphate backbone may be done to increase the stability of the molecules; for example, PNA:DNA hybrids can exhibit higher stability in some environments.

[00030] "Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is fom1ed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Primers usually are extended by a DNA polymerase.

[00031] The term "SNP" or "single nucleotide polymorphism" refers to a genetic variation between individuals; *e.g.,* a single nitrogenous base position in the DNA of organisms that is variable. SNPs are found across the genome; much of the genetic variation between individuals is due to variation at SNP loci, and often this genetic variation results in phenotypic variation between individuals. SNPs for use in the present invention and their respective alleles may be derived from any number of sources, such as public databases (U.C. Santa Cruz Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway) or the NCBI dbSNP website (http://www.ncbi.nlm.nih.gov/SNP/), or may be experimentally determined as described in U.S. Pat No. 6,969,589; and US Pub. No. 2006/0188875 entitled "Human Genomic Polymorphisms." Although the use of SNPs is described in some of the embodiments presented herein, it will be understood that other biallelic or multi-allelic genetic markers may also be used. A biallelic genetic marker is one that has two polymorphic forms, or alleles. As mentioned above, for a biallelic genetic marker that is associated with a trait, the allele that is more abundant in the genetic composition of a case group as compared to a control group is termed the "associated allele," and the other allele may be referred to as the "unassociated allele." Thus, for each biallelic polymorphism that is associated with a given trait *(e.g.,* a disease or drug response), there is a corresponding associated allele. Other biallelic polymorphisms that may be used with the methods presented herein include, but are not limited to multinucleotide changes, insertions, deletions, and translocations. It will be further appreciated that references to DNA herein may include genomic DNA, mitochondrial DNA, episomal DNA, and/or derivatives of DNA such as amplicons, RNA transcripts, cDNA, DNA analogs, etc. The polymorphic loci that are screened

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in an association study may be in a diploid or a haploid state and, ideally, would be from sites across the genome.

[00032] The term "selectively binds", "selective binding" and the like as used herein, when referring to a binding partner (*e.g.*, protein, nucleic acid, antibody or other affinity capture agent, etc.), refers to a binding reaction of two or more binding partners with high affinity and/or complementarity to ensure selective hybridization under designated assay conditions. Typically, specific binding will be at least three times the standard deviation of the background signal. Thus, under designated conditions the binding partner binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

[00033] "Sequencing", "sequence determination" and the like means determination of information relating to the nucleotide base sequence of a nucleic acid. Such information may include the identification or determination of partial as well as full sequence information of the nucleic acid. Sequence information may be determined with varying degrees of statistical reliability or confidence. In one aspect, the term includes the determination of the identity and ordering of a plurality of contiguous nucleotides in a nucleic acid. "High throughput digital sequencing" or "next generation sequencing" means sequence determination using methods that determine many (typically thousands to billions) of nucleic acid sequences in an intrinsically parallel manner, *i.e.* where DNA templates are prepared for sequencing not one at a time, but in a bulk process, and where many sequences are read out preferably in parallel, or alternatively using an ultra-high throughput serial process that itself may be parallelized. Such methods include but are not limited to pyrosequencing (for example, as commercialized by 454 Life Sciences, Inc., Branford, CT); sequencing by ligation (for example, as commercialized in the SOLiD™ technology, Life Technology, Inc., Carlsbad, CA); sequencing by synthesis using modified nucleotides (such as commercialized in TruSeq™ and HiSeq™ technology by Illumina, Inc., San Diego, CA, HeliScope™ by Helicos Biosciences Corporation, Cambridge, MA, and PacBio RS by Pacific Biosciences of California, Inc., Menlo Park, CA), sequencing by ion detection technologies (Ion Torrent, Inc., South San Francisco, CA); sequencing of DNA nanoballs (Complete Genomics, Inc., Mountain View, CA); nanopore-based sequencing technologies (for example, as developed by Oxford Nanopore Technologies, LTD, Oxford, UK), and like highly parallelized sequencing methods.

[00034] The term "T_m" is used in reference to the "melting temperature." The melting

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temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation, T_m=81.5+0.41 (% G+C), when a nucleic acid is in aqueous solution at 1M NaCl (see *e.g.,* Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985)). Other references (*e.g.,* Allawi and SantaLucia, Jr., Biochemistry, 36:10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

DETAILED DESCRIPTION OF THE INVENTION

[00035] The practice of the techniques described herein may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and sequencing technology, which are within the skill of those who practice in the art. Such conventional techniques include polymer array synthesis, hybridization and ligation of polynucleotides, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the examples herein. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Green, et al., Eds., *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (1999); Weiner, Gabriel, Stephens, Eds., *Genetic Variation: A Laboratory Manual* (2007); Dieffenbach, Dveksler, Eds., *PCR Primer: A Laboratory Manual* (2003); Bowtell and Sambrook, *DNA Microarrays: A Molecular Cloning Manual* (2003); Mount, *Bioinformatics: Sequence and Genome Analysis* (2004); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); Stryer, *Biochemistry* (4th Ed.) (1995) W.H. Freeman, New York N.Y.; Gait, *"Oligonucleotide Synthesis: A Practical Approach"* (2002) IRL Press, London; Nelson and Cox, *Lehninger, Principles of Biochemistry* (2000) 3rd Ed., W. H. Freeman Pub., New York, N.Y.; and Berg, et al., *Biochemistry* (2002) 5th Ed., W.H. Freeman Pub., New York, N.Y., all of

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which are herein incorporated in their entirety by reference for all purposes.

[00036] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" refers to one or more nucleic acids, and reference to "the assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[00037] 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 this invention belongs. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing devices, formulations and methodologies that may be used in connection with the presently described invention.

[00038] Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

[00039] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention.

The Invention in General

[00040] The assay systems of the invention provide spatially-encoded, multiplexed assays comprising 1) an assay capable of high levels of multiplexing with an efficient spatial encoding scheme; 2) instrumentation capable of delivering reagents according to a spatial pattern; and 3) decoding determined by a readout that is digital in nature. The assay systems of the invention detect the presence or absence and relative amount of a biological target or biological activity indicative of a biological target, as well as the location of the biological target or activity in a

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biological sample, *e.g.*, a tissue section or other biological structure disposed upon a support such as a microscope slide or culture dish.

[00041] The assay system further provides instrumentation with an ability to deliver reagents in a spatially-defined pattern. This instrumentation, together with software, reagents and protocols, provides a key component of the highly innovative assay system of the invention, allowing for measurement of numerous biological targets or activities in a meaningful spatial environment, including gene expression and peptide localization. An encoding scheme used in these assay systems allows one to determine the location of biological targets or activity (or lack thereof) in the biological samples after the products of the multiplexed assay are removed from the biological sample and pooled for analysis. Decoding of the encoding scheme can be performed by, *e.g.*, next-generation sequencing, which easily provides millions to trillions of datapoints at low cost. The assay results such as the amount or activity of biological targets can then be mapped back to specific location in the biological sample. The assay systems open a new analytical window into the complex spatial patterns of cellular function and regulation in biological samples.

[00042] A simplified overview of the assay system 100 of the present invention is provided at Figure 1. At step 110, a biological sample affixed to a support is provided. The biological sample contains biological targets of interest. Biological targets can include any molecule of interest, such as nucleic acids (including, *e.g*, RNA transcripts, genomic DNA sequences, cDNAs, amplicons, or other nucleic acid sequences) and proteins, enzymes and the like. At step 120, encoded probes are delivered to the biological sample according to a known spatial pattern. Encoded probes comprise probes, which can interact with biological targets of interest, and coding tags, which identify the positions in the sample of the biological targets being assayed, and thus can be used to link assay results back to locations in the sample. Coding tags in most embodiments are oligonucleotides. However, coding tags may also be mass tags, fluorescent labels, or other moieties.

[00043] In some embodiments, the probe and coding tag portions of the encoded probe are pre-coupled before being delivered to the biological sample, For example, in the case where the encoded probes are oligonucleotides, both the probe and coding tag sequence can be synthesized as a single oligonucleotide. Alternatively, the probe and coding tag portions of the encoding probes can be synthesized or obtained separately and combined before delivery to the biological

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sample (*e.g.*,two separate oligonucleotides can be synthesized and coupled by, *e.g.*, ligation; or an antibody and an oligonucleotide can be prepared separately and conjugated before delivery to the biological sample). Also, as is described in Figures 2-5, the probes and the coding tags (in encoding oligonucleotides) are synthesized separately, and are delivered to the biological sample at different steps (*e.g.*, probes first and coding tags thereafter, or vice versa) in the assay.

[00044] At step 130, the encoded probes are allowed to react or interact with the biological targets, *i.e.*, conditions are provided to allow *e.g.*, oligonucleotides to hybridize to nucleic acid targets, enzymes to catalyze reactions with protein targets, antibodies to bind epitopes, etc. In the case where the biological targets are nucleic acids, the encoded probes are typically oligonucleotides and hybridize to the target nucleic acids. In the case that the biological targets are proteins, the encoded probes typically are aptamers, small molecules, or oligonucleotide-conjugated proteins that interact with target proteins by binding to them or by reacting with them (that is, one of the proteins is a substrate for the other). Encoding oligonucleotides may be coupled to the probes (proteins) by conjugation, chemical or photo-crosslinking via suitable groups and the like.

[00045] Once encoded probes interact with the biological targets, the encoded probes that interacted with the biological targets must be separated from the encoded probes that did not interact with the biological targets at step 140. In the case where the biological targets are nucleic acids and the encoded probes are oligonucleotides, the separation can be accomplished by, *e.g.*, washing the unhybridized encoded probes from the sample. Similarly, for other assays that are based on affinity binding, including those using aptamer, small molecule, and protein probes, washing steps can be used to remove low affinity binders. In the case where the probe is transformed via interaction with the target, *e.g.*, in the case of a peptide, *e.g.*, via cleavage by a protease or phosphorylation by a kinase, it is convenient to collect, all encoded probes--both encoded probes that interacted with the biological targets and were transformed and encoded probes that were not transformed. After collection or pooling, an antibody or other affinity capture agent can be used to capture probes that were transformed by addition of a moiety (*e.g.*, a phosphate group). In cases where probes have been transformed via cleavage, the transformed probes can be separated, *e.g.*, by capturing the non-transformed probes via a tag that is removed from the transformed probes during the transformation *(e.g.*, by cleavage), or by adding a new tag at the site of cleavage.

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[00046] Once the reacted (transformed) or interacted encoded probes are separated from the unreacted or un-interacted encoded probes, the sequence of the reacted and/or interacted encoded probes is determined at step 150 by, preferably, sequencing. The sequence of the encoded probes allows the mapping of the assay results at step 160 back to locations in the biological sample.

[00047] Figure 2 provides a simplified overview of an assay system 200 of the present invention embodying an efficient implementation of a combinatorial coding scheme for the encoding of spatial information. For purposes of this overview, the probes are oligonucleotides, but as explained elsewhere, other types of probes can also be used. In step 210, a biological sample affixed to a support, *e.g.*, a tissue sample or other biological structure, is provided. In step 220, one or more oligonucleotide probes are delivered to the biological sample, where the oligonucleotide probes are capable of hybridizing with biological targets in the biological sample. In step 230, the oligonucleotide probes are allowed to interact with (hybridize to) the nucleic acid targets; that is, appropriate conditions are provided where oligonucleotide probes can hybridize to the target nucleic acids.

[00048] In step 240, the oligonucleotide probes that did not hybridize to target nucleic acids are removed, and thereby separated from oligonucleotide probes that did hybridize to target nucleic acids. In this embodiment, separation can be accomplished by, *e.g.*, washing the sample to remove unhybridized oligonucleotide probes. Next, in step 250, encoding oligonucleotides (the encoding agents) are delivered to the biological sample according to a chosen spatial pattern, where the encoding oligonucleotides comprise coding tags that are used to encode the location of biological targets in the biological sample. Note that in contrast to the assay system of Figure 1, here the probes and encoding agents (encoding oligonucleotides) are delivered in separate steps. In step 260, the encoding oligonucleotides are coupled to the oligonucleotide probes to create encoded probes. In this case where the probes are oligonucleotides, the encoding oligonucleotides may be coupled to the oligonucleotides probes by, *e.g.*, ligation. Alternatively, the information in the encoding oligonucleotides can be transferred by using a DNA polymerase to extend a probe oligonucleotide that acts as a primer, and thereby copy and incorporate the sequence of the encoding oligonucleotides.

[00049] In step 270, the sequence of the coding tags in the encoded probes as well as the sequence or a portion of the sequence of the probe itself is determined, and in step 280, the target

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nucleic acids are mapped back to the biological sample. In some embodiments, the abundance of sequences reveals the relative quantity of biological targets at the location. Although this embodiment shows the individual steps in a particular order, so as to better explain the invention, the precise order of the steps can be varied. For example, steps 220 and 250 can be combined, so that a mixture of the probes and encoding oligonucleotides is delivered according to a chosen spatial pattern. Coupling step 260 can then be carried out immediately after the combined steps 220 and 250, or concomitantly with them. In this case, step 240 would then occur after step 260. It can therefore be appreciated that the two key results of this series of steps, *i.e.*, the location-specific encoding of probe molecules and the separation of probe molecules based on their ability to interact with corresponding target molecules, can be accomplished with some flexibility in the implementation of the particular steps. Similarly, there is considerable flexibility in the design of the coding scheme. As described *infra*, the assays of the invention are particularly amenable to combinatorial methods.

[00050] Thus, the present invention provides an ability to look at many different biological targets in many locations, providing the resolution of in situ hybridization with the highly-parallel data analysis of sequencing. In some embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 20, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 50, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 100, greater than 500, 1,000, 10,000, 25,000, 100,000, 500,000, 1,000,000. It will be appreciated that, due to the spatial encoding dimension of the invention, even much larger numbers can be contemplated. For example, assaying 10,000 targets per location x 10,000 locations would generate 10^8 different assays, and even larger numbers than these can easily be contemplated, particularly if spatial locations with resolution on the order of that of single cells are utilized. Further, in embodiments where high-throughput digital sequencing is employed, the sequences of at least 1,000 encoding probes are typically determined in parallel. More typically, using a digital readout, it is desirable to obtain multiple sequence reads for each assay (defined by a probe and a spatial location code). It is desirable to obtain an average of at least 3 copies per assay, and more typically at least 10 or at least 30 copies per assay, depending on the design of the experiment and requirements of the assay. For a quantitative readout with suitable dynamic range, it may be desirable to obtain at

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least 1,000 reads per assay. Therefore, if 1,000,000 assays are carried out, the number of sequence reads may be 1 billion or more. With high-throughput digital sequencing, and allowing for redundancy, the sequence of at least 10,000 encoding probes are determined in parallel, or the sequence of at least 100,000, 500,000, 1,000,000, 10,000,000, 100,000,000, 1,000,000,000 or more encoding probes are determined in parallel.

Assays

[00051] The assay portion of the assay systems of the present invention comprise the following general steps: delivering probes and encoding agents where the encoding agents (in some embodiments pre-coupled to the probes) are delivered to the sample according to a known spatial pattern, allowing the probes to interact or react with biological targets in the sample, and, if the probes and encoding agents have not been pre- coupled, coupling the encoding agents to probes.

[00052] The samples of the present invention include virtually any biological sample or samples that can be affixed to a support or provided essentially in a two-dimensional manner, where the ability to tie an assayed biological target or activity back to the location within the biological sample is important. Exemplary biological samples include tissue sections (*e.g.*, including whole animal sectioning and tissue biopsies), cell populations on slides or culture dishes, and the like. The assay systems of the invention are particularly advantageous in that they are compatible with numerous biological sample types, including fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the biological samples are immobilized on a substrate surface having discrete, independently measureable areas.

[00053] The biological targets to be detected can be any biological molecules including but not limited to proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Examples of subcellular targets include organelles, *e.g.*, mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplasts, endocytic vesicles, exocytic vesicles, vacuoles, lysosomes, etc.

[00054] In some particular embodiments, the assay system is used to analyze nucleic acids, *e.g.*, by genotyping, quantitation of DNA copy number or RNA transcripts, localization of

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particular transcripts within samples, and the like. Figure 3 illustrates an overall scheme for an exemplary assay for, *e.g.*, detecting single nucleotide polymorphisms (SNPs) that can be used with the assay system of the invention. In Figure 3, two oligonucleotide probes are provided. Each oligonucleotide probe comprises a target-specific region (located on either side of the SNP to be analyzed) seen at 305 and 307, and ligation regions, seen at 301 and 303. The oligonucleotide probes are allowed to hybridize to a target nucleic acid (not shown) in the biological sample. At step 302, one of the oligonucleotide probes is extended to incorporate the SNP sequence and ligated to the other probe to form an extended probe comprising target nucleic acid region 309 and ligation regions 301 and 303.

[00055] Two encoding agents, both comprising a coding tag (seen at 315 and 317), a ligation region (seen at 311 and 313), and a primer region (seen at 319 and 321) are combined with and ligated to the extended probe at step 304 to form an encoded target- specific oligonucleotide. Again, in contrast with Figure 1, the probes and encoding agents are delivered at separate steps. Doing so allows use of the combinatorial embodiments described *infra*. In preferred embodiments, the encoding oligonucleotides within a pair of encoding oligonucleotides ligate specifically to one side of the target sequence or the other *(i.e.,5'* or 3' of the target sequence) in step 306. Also, typically, the ligation and primer regions of the encoding oligonucleotides and probes are universal; that is, the set of ligation and primer regions used in constructing the probes and encoding oligonucleotides are constant, and only the target-specific regions of the probes and the coding tags of the encoding oligonucleotides differ. However, again in alternative embodiments, the ligation and primer regions are not universal and differ between probes and encoding agents.

[00056] Following ligation, the encoded probes are eluted, pooled, and, optionally, sequencing adapters are added to the encoded probes via PCR. In alternative embodiments, sequencing primers may be ligated to the encoding oligonucleotides, or sequencing primer sequences can be included as part of the encoding oligonucleotide. As seen in Figure 3, each sequencing adapter comprises primer region 319 or 321, compatible with the primer regions 319 and 321 on the encoded probes. The final construct comprising first adapter 327, first primer region 319, first coding tag 315, ligation regions 311 and 301, target region 309, ligation regions 313 and 303, second coding tag 317, second primer region 325 and second adapter 329 is now ready for input into a digital high-throughput sequencing process.

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[00057] A combination of extension and ligation reactions are exemplified in Figure 3, but it should be appreciated that a variety of reactions may be used to couple the encoding oligonucleotides to the target-specific oligonucleotides, including ligation only (*e.g.*,for oligonucleotides that hybridize to contiguous portions of the target nucleic acid sequence). Alternatively, an assay utilizing an additional oligonucleotide, such as in the GOLDENGATE® assay (see Fan, et al., Cold Spring Symp. Quant. Biol., 68:69-78 (2003); (Illumina, Inc., San Diego, CA)), may be employed.

[00058] In other embodiments, the assay system of the invention also can be used to analyze peptides or proteins, the presence of antibodies, enzymatic and other protein activities, posttranslational modifications, active and non-active forms of peptides, as well as peptide isoforms in a biological sample. Accordingly, the probes may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, aptamers and the like.

[00059] In certain aspects, the probes are substrates for enzymes or proenzymes, *e.g.*, kinases, phosphatases, zymogens, proteases, or fragments thereof. In certain aspects, the probes are phosphorylation substrates used to detect proteins involved in one or more signal transduction pathways, *e.g.*, a kinase or a phosphatase. In another specific aspect of the invention, the probes are specific protease substrates that associate only with individual proteases or classes of proteases. In other aspects, the probes are different processed forms, isoforms and/or domains of an enzyme. Protein-based probes are typically conjugated or otherwise linked to oligonucleotide encoding agents. The oligonucleotide encoding agents in this case would also include a nucleotide sequence component that allows for identification of the protein probe.

[00060] In certain aspects, the present invention provides assays for evaluating differences in the amount and/or activity of biological targets between different locations in a sample and/or between samples. The method includes determining a plurality of encoded results from the biological sample and evaluating the differences in quantity of the biological targets at each location in the biological sample.

Combinatorial Embodiments

[00061] To maximize the efficiency of encoding, a combinatorial approach using pairs of coding tags in the encoding oligonucleotides can be used. By de-coupling the target-specific

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information and the coding tags, the number of oligonucleotides required is dramatically reduced, with a concomitant decrease in cost.

[00062] Figure 4 illustrates a general mechanism for one embodiment of a combinatorial encoding scheme of the assay systems of the invention, where nucleic acids in a representative tissue section (shown at 416) are assayed. Figure 4 at A shows two target-specific/encoding oligonucleotide constructs 420 and 422 *(e.g.,* formed between steps 302 and 304 of Figure 3) specifically bound to a target nucleic acid 402 of interest, The first encoded probe 420 comprises coding tag 408, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 404. The second encoded probe 422 comprises coding tag 406, associated with, *e.g.,* a universal priming site for amplification of the assay products or an adapter to enable identification of the coding identifiers using sequencing technologies 410.

[00063] Figure 4 at B shows the spatial pattern that may be used for twenty different coding tags, a1 through a10 (coding tag 406 on encoded probe 420) and b1 through b10 (coding tag 408 encoded probe 422). Coding tag a1, for example, is deposited on the biological sample in ten discrete areas or spots (shown as the first horizontal line of spots in 412). Coding tag a2 is deposited on the biological sample in ten spots on the second horizontal line in 412. Coding tag a3 is deposited on the biological sample in ten spots on the third horizontal line in 412, and so on. Whereas the "a" tags are deposited in ten horizontal rows, the "b" tags are deposited in ten vertical rows as shown in 414. For example, coding tag b1 is deposited on the biological sample in ten discrete spots in the first vertical row of 414, coding tag b2 is deposited on the biological sample in ten discrete spots in the second vertical row of 414, and so on. Using such a configuration allows for twenty coding tags to uniquely define 100 different locations on the biological sample.

[00064] Figure 4 at C shows a representative tissue section 416 coincident with coding tag grid 418. The arrows show how the "a" coding tags and the "b" coding tags are deposited on grid 418 that is coincident with tissue section 416. If, once sequenced, coding tags a1 and b4, *e.g.,* are associated with a target nucleic acid sequence, then that target nucleic acid sequence *(i.e.,* biological target) was present in the tissue section at location a1, b4.

[00065] Figure 5 provides a simplified, specific example of the encoding scheme of the assay systems of the invention. Figure 5 shows encoding oligonucleotides 510, comprising a1,

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a2, a3, a4 and b1, b2, b3 and b4. Target-specific oligonucleotides (TSOs) (probes) 1 and 2 are shown at 520. A deposit or dispensing scheme is shown at 530. Like the grid exemplified in Figure 4, encoding oligonucleotides al through a4 are deposited in spots in a pattern (here, in a vertical pattern), and encoding oligonucleotides b1 through b4 are deposited in spots in a pattern (here, a horizontal pattern). The grid though shown as a square with spots is actually a deposition pattern on a biological sample (not shown) such as tissue section 416 shown in Figure 4.

[00066] The target-specific oligonucleotides are delivered to the biological sample, where the target-specific oligonucleotides hybridize to target nucleic acids in the biological sample if target nucleic acids are present. Unhybridized target-specific oligonucleotides are then removed, *e.g.*, by washing. The encoding oligonucleotides are then delivered to the biological sample according to the spatial pattern shown at 530. The encoding oligonucleotides are ligated (or, *e.g.*, extended and ligated) to any target-specific oligonucleotides that hybridized to the target nucleic acid in the biological sample, the ligated constructs are then eluted from the biological sample, pooled, and sequencing adapters are added through, *e.g.*, PCR or ligation, if the sequences were not previously included in the encoding oligonucleotides. The ligated constructs are sequenced by, *e.g.*, high throughput or "next generation" sequencing.

[00067] The pool of resulting sequences is shown at 540. A sequence readout was obtained for target-specific oligonucleotide 1 only at a4b1, a4b2, a1b3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at a1b1 (position shown with vertical lines). A sequence readout was obtained for both target-specific oligonucleotides 1 and 2 at positions a2b1, a3b1, a1b2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at a1b4, a2b4 or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample, and neither target nucleic acid was detected in the upper right portion of the biological sample, as shown in 550. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample.

[00068] In addition to location information, information relating to relative abundance of

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the encoded tags can be obtained. For example, if it is found that there are ten times as many a4T1b1 sequences occurring in the data set as compared to a4T1b2 sequences, this would indicate that target nucleic acid sequence 1 is ten times more abundant at the a4T1b1 location than at the a4T1b2 location.

[00069] In the case of nucleotide analysis as shown in Figure 3, by ligating the coding tags directly to target-specific oligonucleotides, only 2n target-specific oligonucleotides are needed for n targets. For example, using the combinatorial approach outlined in Figure 2, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 target- specific oligonucleotides and 2 x 100 encoding oligonucleotides. The total count of assay oligonucleotides would be only 400 (200 target-specific and 200 encoding), not counting universal primers. In contrast, if the coding oligonucleotides were not decoupled from the target-specific oligonucleotides, (n x X positional codes) + (n x Y positional codes) would he needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences. Moreover, though the embodiments shown in Figures 2- 5 depict a combinatorial scheme using two encoding agents (coding tags), three, four or more encoding agents and coding tags may be used, and attached to the probe or one another by varying means and in varying combinations of steps.

[00070] Due to the spatial encoding aspect of the assay system of the invention, a large amount of information can be generated with even a modest number of assays. For example, five or more biological targets assayed at five or more positions in the sample generates 25 or more combinations. Using digital sequencing as a readout, the optimum number of sequence reads per combination depends on the sensitivity and dynamic range required, and can be adjusted. For example, if for each combination on average 100 reads are sampled, the total for 25 combination is 25,000 reads. If 1,000 targets are assayed at 1,000 locations with an average sampling depth of 1,000, then 10^9 reads are required. These numbers, although large, are within the capacity of intrinsically parallel digital sequencing methods, which can generate datasets of billions or even trillions of reads in a reasonable timeframe and at a very low cost per read. Therefore, by varying the numbers of positions interrogated or biological targets assayed, or both, and using digital sequencing, large amounts of information can be obtained. In specific aspects, multiple locations are interrogated for two or more biological molecules.

Reagent Delivery Systems

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[00071] The reagent delivery system of the invention includes instrumentation that allows the delivery of reagents to discrete portions of the biological sample, maintaining the integrity of the spatial patterns of the encoding scheme. Reagent delivery systems of the assay systems of the invention comprise optional imaging means, reagent delivery hardware and control software. Reagent delivery can be achieved in a number of different ways. It should be noted that reagent delivery may be to many different biological samples at one time. A single tissue section has been exemplified herein; however, multiple biological samples may be affixed and analyzed simultaneously. For example, serial sections of a tissue sample can be analyzed in parallel and the data combined to build a 3D map.

[00072] Integral to the assay system of the invention is instrumentation that allows for spatial patterning of reagents onto the biological sample. Technologies for formulating and delivering both biological molecules (*e.g.* oligonucleotides or antibodies) and chemical reagents (*e.g.*, small molecules or dNTPs) are known in the art, and uses of these instrument systems are known to one skilled in the art and easily adaptable to the assay systems of the invention. One example of a suitable reagent delivery system is the Labcyte™ Echo acoustic liquid handier, which can he used to deliver nanoliter scale droplets containing biological molecules with high precision and reproducibility. One skilled in the art could incorporate this reagent delivery device into the overall system, using software to specify the locations to which reagents should be delivered.

[00073] Other instruments that can be used for the deposition of agents and/or coding identifiers onto biological samples include, but are not limited to, ink jet spotting; mechanical spotting by means of pin, pen or capillary; micro contact printing; photochemical or photolithographic methods; and the like. For several applications, it may be preferred to segment or sequester certain areas of the biological samples into one or more assay areas for different reagent distributions and/or biological target determination. The assay areas may be physically separated using barriers or channels.

[00074] In one exemplary aspect, the reagent delivery system may be a flow-based system. The flow-based systems for reagent delivery in the present invention can include instrumentation such as one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Reagent delivery systems are configured to move fluid to contact a discrete section of the biological sample. Movement of the reagents can be driven by a pump disposed, for example,

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downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, reagents may be driven through the fluid by gravity. US Pub. Nos. 20070166725 and 20050239192 disclose certain general-purpose fluidics tools that can be used with the assay systems of the invention, allowing for the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

[00075] In a more specific example, one or more flow-cells can be attached to the substrate-affixed biological sample from above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump is used to deliver reagents to the flow-cell and across the biological sample. The flow cells are configured to deliver reagents only to certain portions of the biological sample, restricting the amount and type of reagent delivered to any specific section of the biological sample.

[00076] In another aspect, a microfluidic system can be integrated into the substrate upon which the biological sample is disposed or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid may he formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected and delivered according to selective opening and closing of valves disposed between reagent reservoirs.

[00077] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes (*i.e.*, microfluidic structures). The pump can operate mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among other means. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. Mechanical pumps may be micromachined, molded, etc. Exemplary electrical pumps may include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[00078] Valves generally include any mechanism for regulating the passage of fluid through a channel. Valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into a channel to partially or completely block a channel, an electrocapillary structure, and/or the like.

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[00079] An open gasket can be attached to the top of the biological sample and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber may be formed by a gasket sandwiched between the biological sample on the substrate and a chemically inert, water resistant material such as, but not limited to, black-anodized aluminum, thermoplastics (*e.g.,* polystyrene, polycarbonate, etc), glass, etc.

[00080] In an optional embodiment, the assay system comprises imaging means to determine features and organization of the biological sample of interest. The images obtained, *e.g.,* may be used to design the deposition pattern of the reagents. Imaging means are optional, as an individual can instead view the biological sample using, *e.g.,* a microscope, analyze the organization of the biological sample, and specify a spatial pattern for delivery assay reagents. If included, the delivery system can comprise a microcircuit arrangement including an imager, such as a CCD or IGFET-based *(e.g.,* CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Pub. No. 20090197326, which is incorporated herein by reference. Also, it should be noted that although Figures 4 and 5 illustrate using a x,y grid configuration, other configurations can be used, such as, *e.g.,* following the topology of a tissue sample; targeting certain groups of cells, cell layers and/or cell types in a tissue, and the like.

[00081] In yet another alternative, the reagent delivery system controls the delivery of reagents to specific patterns on a biological sample surface using semiconductor techniques such as masking and spraying. Specific areas of a biological sample can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the biological sample using conventional techniques such as spraying or fluid flow. The use of masked delivery results in a patterned delivery scheme on the substrate surface.

[00082] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms (*e.g.,* thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions are be achieved.

[00083] In order to target specific sites of interest, an informative image of the biological sample to be assayed may be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions of the biological sample can be identified using image

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processing (*e.g.*, images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with other features of the assay system. In some aspects, software is used to automatically translate image information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample for reagent delivery is thus an important component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and/or other very accurate physical positioning systems can be adapted to this purpose.

[00084] The invention preferably comprises a complete suite of software tailored to the assay system. Optionally, oligonucleotide design software is used to design the encoding nucleotides (and in embodiments where nucleic acids are assayed, the target-specific oligonucleotides) for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for reagent delivery and data analysis (*i.e.*, sequence analysis) may be integrated to determine assay results. Integrated data analysis is particularly useful, as the type of dataset that is generated may be massive as a consequence of scale. Algorithms and software tools that are specifically designed for analysis of the spatially-associated data generated by the assay systems, including pattern-analysis software and visualization tools, enhance the value of the data generated by the assay systems.

[00085] In certain aspects, the assay system comprises processes for making and carrying out the quality control of reagents, *e.g.*, the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents are formulated according to factors such as volatility, stability at key temperatures, and chemical compatibility for compatibility with the reagent delivery instrumentation and may be analyzed by instrumentation integrated within the assay system.

Sequencing

[00086] Numerous methods can be used to identify the coding tags and probe sequences in the encoded probes of the assay systems of the invention. The coding tags can be detected using techniques such as mass spectroscopy (*e.g.*, Maldi-T of, LC-MS/MS), nuclear magnetic resonance imaging, or, preferably, nucleic acid sequencing. Examples of techniques for decoding the coding tags of the present invention can be found, for example, in US Pub. No. 20080220434, which is incorporated herein by reference. For example, the coding tags may be oligonucleotide mass tags (OMTs or massTags). Such tags are described, *e.g.*, in US Pub. No. 20090305237, which is incorporated by reference in its entirety. In yet another alternative, the encoded probes

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can be amplified and hybridized to a microarray. This would require separate amplification reactions to be carried out, in which each amplification is specific to a particular spatial code or subset of codes, accomplished by using code-specific primers. Each amplification would also incorporate a different resolvable label (e.g. fluorophor). Following hybridization, the relative amounts of a particular target mapping to different spatial locations in the sample can he determined by the relative abundances of the resolvable labels.

[00087] In one particularly preferred aspect, the resulting coding tags according to the assay system are substrates for high-throughput, next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of the coding tags, for example, with SOLiD™ technology (Life Technologies, Inc.) or Genome Ananlyzer (Illumina, Inc.). Such next-generation sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in *e.g.*, Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et al, U.S. patent publication 2005/0191656; sequencing-by- synthesis methods, *e.g.*, U.S. Pat Nos. 6,210,891; 6,828,100; 6,969,488; 6,897,023; 6,833,246; 6,911,345; 6,787,308; 7,297,518; 7,462,449 and 7,501,245; US Publication Application Nos. 20110059436; 20040106110; 20030064398; and 20030022207; Ronaghi, et al, Science, 281: 363-365 (1998); and Li, et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); ligation-based methods, *e.g.*, U.S. Patent Nos. 5,912,148 and 6,130,073; and U.S. Pat. Appln Nos. 20100105052, 20070207482 and 20090018024; nanopore sequencing *e.g.*, U.S. Pat. Appln Nos. 20070036511; 20080032301; 20080128627; 20090082212; and Soni and Meller, Clin Chem 53: 1996-2001 (2007)), as well as other methods, *e.g.*, U.S. Pat. Appln Nos. 20110033854; 20090264299; 20090155781; and 20090005252; also, see, McKernan, et al., Genome Res., 19:1527-41 (2009) and Bentley, et al., Nature 456:53-59 (2008), all of which are incorporated herein in their entirety for all purposes.

Applications of Assay System

[00088] It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous important areas of biological research, diagnostics, and drug development

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that will benefit from a high throughput multiplexed assay system that can measure simultaneously the amount and spatial location of a biological target in a biological sample. For example, combining the ability to estimate the relative abundance of different RNA transcripts with the ability to reconstruct an image of spatial patterns of abundance across many locations, which may be as small as or even smaller than individual cells, in a tissue enables many different areas of basic research. The following are exemplary uses and are by no means meant to be limiting in scope.

[00089] In one example, 3-dimensional patterns of gene expression are determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning. Such a method can be used to measure changes in gene expression in disease pathology, *e.g.*, in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues is obtained, leading to new insights into the function and regulation both in normal and diseased states, and provides new hypotheses that can be tested. For example, an assay system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney, et al., Nature, 447:799-816 (2007)) and modENCODE to be integrated at the tissue level. The assay systems also aid computational efforts to model interacting networks of gene expression in the field of systems biology.

[00090] The assay systems also provide a novel approach to analysis of somatic variation, *e.g.*, somatic mutations in cancer or variability in response to infectious organisms. For example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer-related processes in both cancer and genetically normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers. In another example, individuals have varying susceptibility to infectious organisms, and the assay systems of the invention can be used to study the interaction between microbes and tissues or the various cell types within the tissue.

[00091] Importantly, in addition to providing spatially-associated information, the

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invention allows a great increase in the sensitivity of detecting rare mutations, as signal to noise can he dramatically increased since only a small location is assayed in any given reaction. In a typical assay for rare mutations in a mixed sample, the sample is treated in bulk, *i.e.*, nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in one cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the assay systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, in the assay systems of the present invention, background is reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed, which may be particularly important in detecting key mutations in tissue sections in cancer. Already molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics. The technology of the invention promises to greatly increase the power of such approaches.

[00092] The present invention provides assays, assay systems, and methods of using such assays in spatially encoded biological assays. The invention provides an assay system comprising one or more agents provided in defined spatial patterns on a substrate surface, and a detection system for identifying the presence or absence, relative amount, and location of a biological molecule. Such biological molecules include, but are not limited to, nucleic acids, peptides, carbohydrates, cellular components, and the like. The assay system is a novel multiplexing approach, as it allows multiple molecules and their respective multiple locations to be identified in a single system using a unique encoding scheme. This encoding scheme uses both molecule-specific binding agents and coding identifiers to provide a practical and cost-effective determination of information on multiple biological molecules, including specific positional information of such molecules in a biological sample, *e.g.*, a tissue section. The single molecule detection analysis using the encoding system also allows relative amounts of biological molecules to be detected, thus providing information on expression levels, sequestering in specific locales, and the like.

[00093] The assay systems detect the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. In addition, the assays provide methods for doing this for multiple biological molecules simultaneously. The assay systems

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utilize one or more binding agents that specifically bind to the biological molecule of interest and unique coding identifiers associated with specific binding agents. The detection system utilizes a method for identifying the presence and spatial address of the agent binding based on the positive and/or negative results that are obtained using detection of the agent and identifier and the encoding scheme of the spatial patterns on the substrate surface. In a specific aspect, the encoding scheme employs limited reagent delivery to the spatial patterns on the substrate surface, and access of the coding identifiers and/or binding agents to portions of the sample is controlled through such limited delivery.

[00094] In one aspect, the assay system detects the presence or absence and spatial location of a biological molecule based on the positive and/or negative results that are obtained using limited reagent delivery and the encoding scheme of the spatial patterns on the substrate surface.

[00095] The assay system and methods of the invention are based on relational, solid-state substrates with positions that represent specific spatial locations within a biological sample, *e.g.*, a cell, organelle or tissue. The ability to use encoding features to represent locations allows high-throughput analysis of the presence or absence, and relative amount, of a biological molecule at more than one spatial location in a sample. The encoding features also allow provide assaying of multiple biological molecules at these multiple locations simultaneously.

[00096] A primary feature of the invention is the preservation of the spatial organization of elements in a sample of interest through the use of an encoding scheme. For example, the assay may be designed to preserve the relative position of cells in a tissue, and the assay may interrogate the individual cells for genomic DNA variation (including epigenetic modifications), and RNA and protein expression.

[00097] In one specific aspect, the encoding scheme of the assay system comprises the use of two or more coding patterns, each comprising regions defined by spatial patterns on the substrate surface. For example, the assay system can utilize an encoding scheme that comprises a 2-dimensional grid format based on the discrete positioning of the binding agents in the substrate surfaces. In another example, the spatial patterns may be based on more randomized cell locations, *e.g.*, the patterns on the substrate surface follow an underlying biological structure rather than a strict, x,y grid pattern. This aspect includes systems with two or more substantially identical spatial patterns using different binding agents and/or coding identifiers, as well as systems having different patterns for different agents and/or coding identifiers. The encoding

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scheme of the systems can be controlled by delivery of different reagents to discrete regions on the substrate surfaces, which allows different reactions to take place on substantially similar agents of known location on the substrate surfaces.

[00098] In one specific aspect, the invention provides high resolution, high-throughput analysis of nucleic acids and/or expression levels that provides both detection and spatial identification of large numbers of nucleic acids, *e.g.*, DNA or RNA.

[00099] In another specific aspect, the invention provides high resolution, high-throughput analysis of proteins that provides both detection and spatial identification of large numbers of such proteins, *e.g.*, kinases or proteases.

[00100] Numerous reagent delivery systems can be used with the assay system of the invention. The primary criteria of such reagent delivery systems is the ability to direct delivery of specific agents based on spatial patterns on the substrate surface.

[00101] In one preferred aspect, the encoding scheme utilizes a reagent delivery system based on printing and informatics technologies to implement the spatial patterns used for identification and localization of the biological materials. For example, the patterns found in the encoding scheme may be created using ink jet printing technology to provide reagents at specific locations on one or more substrate surfaces. The desired patterns are set out in specific coding patterns on the substrate surface.

[00102] In certain aspects of the invention, the binding agents are immobilized directly to the substrate surface, and the location of the binding agents is known or determined prior to use of the substrate surface in the assay system. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, e.g., provided in wells or channels.

[00103] In specific aspects of the invention, the binding agents are nucleic acids immobilized directly or indirectly to the substrate surface, *e.g.*, directly through the use of amino groups on the substrate surface or indirectly through the use of a linker. The location of the nucleic acid sequences is known or determined prior to use of the substrate surface in the assay system. In another specific aspect, the nucleic acids may be immobilized directly or indirectly onto beads that are then provided in known locations on the substrate surface. In yet another aspect, the nucleic acids may be provided in or on features of the substrate surface, *e.g.*, provided in wells.

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[00104] In these aspects involving nucleic acid agents, any methods of sequence determination can be used, *e.g.*, sequencing, hybridization and the like. In a preferred aspect, nucleic acid sequencing, and preferably next-generation sequencing, is used to decode the spatial encoding scheme in the assay system of the invention. This provides a very wide dynamic range for very large numbers of assays, allowing for efficient multiplexing.

[00105] In some aspects, the assay utilizes two or more oligonucleotides, the oligonucleotides comprising a universal primer region and a region that correlates specifically to a single spatial pattern within the spatial encoding scheme. In a specific aspect, the assay comprises two allele specific oligonucleotides and one locus specific oligonucleotides. These oligonucleotides allow the identification of specific SNPs, indels or mutations within an allele. This is useful in the identification of genetic changes in somatic cells, genotyping of tissues, and the like.

[00106] In other specific aspects of the invention, the binding agents are peptides. In one aspect, these peptides are associated directly or indirectly to known locations on a substrate surface, *e.g.*, using binding protein pairs or through oligonucleotide linkers complementary to oligonucleotides on the substrate surface. In another aspect, the binding agents are peptides are immobilized directly or indirectly onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the peptides may be provided in or on features of the substrate surface, *e.g.*, provided in wells.

[00107] In yet other specific aspects of the invention, the binding agents are chemical entities (*e.g.*, small molecules) that are coded, e.g. using sequence tags or mass spectroscopy tags as coding identifiers. In one aspect, these chemical entities can be are immobilized directly to the substrate surface. In another aspect, the binding agents are immobilized onto beads or other separate structural elements that are then provided in known locations on the substrate surface. In yet another aspect, the binding agents may be provided in or on features of the substrate surface, *e.g.*, provided in wells.

[00108] The assay system of the invention can utilize various detection mechanisms, based on the molecules to be detected and the reagents needed for such detection system. Exemplary methods that can be used with the assay systems of the invention are described in more detail below.

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The Invention in General

[00109] The assay system and methods of the invention are based on relational methods that allow extraction of data to detect the presence or absence and relative amount of a biological molecule, and the location of this molecule in a sample having a distinct structure, *e.g.*, a tissue section or other biological structure with distinct locations of specific biological molecules. The encoding scheme used in these systems corresponds to the structural elements of the sample, and the information obtained using a two-dimensional coding system is indicative of the spatial addresses of these molecules in a sample of interest.

[00110] Integral to the assay system of the invention is a method for spatial patterning of reagents. Technologies for formulating and delivering both biological molecules (*e.g.* DNA or antibodies) and chemical reagents (*e.g.*, small molecules or dNTPs) have already been demonstrated, and use of these systems will be available to one skilled in the art and easily adaptable upon reading this specification.

[00111] The assay design of the invention provides an accurate and easily scalable spatial encoding system. The ability to deliver reagents in a spatially defined pattern together with software, reagents and protocols comprises a novel and highly innovative assay system for spatial analysis of various biological molecules and activities. This allows the assays to measure numerous biological functions in a meaningful spatial environment, including functions such as gene expression and peptide localization. The systems provide the potential to open a new analytical window into the complex spatial patterns of cellular function and regulation in biological systems.

[00112] The biological molecules to be detected can be any biological molecules such as proteins, nucleic acids, lipids, carbohydrates, ions, or multicomponent complexes containing any of the above. Further examples of subcellular objects include organelles, *e.g.*, mitochondria, Golgi apparatus, endoplasmic reticulum, chloroplast, endocytic vesicle, exocytic vesicles, vacuole, lysosome, etc.

[00113] Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system 401, two reagents 420, 422 that specifically bind to a biological molecule of interest are associated with coding identifiers 406, 408 that encode for a spatial location in the sample. These coding identifiers 406, 408 are optionally associated with

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sites that assist in their identification in the assay format, *e.g.*, universal priming sites 404, 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section 416 is encoded using the combination of the patterns 412, 414 created using the separate coding identifiers 406, 408 which provide a two dimensional code 418 that shows the location of any positive detection of the biological molecule 402 as well as quantifying the biological molecule 402 at each location assayed in the tissue.

[00114] The assay systems of the invention are particularly advantageous in that they are compatible with numerous samples types, such as fresh samples, such as primary tissue sections, and preserved samples including but not limited to frozen samples and paraformalin-fixed, paraffin-embedded (FFPE) samples. An important aspect of the assay systems of the invention is that the binding agents are immobilized on a substrate surface in discrete, independently measureable areas. These discrete areas can be formed by spatially selective deposition of the binding agents on the substrate surface. Numerous methods can be used for the deposition of the agent and the coding identifiers associates with the agent. For example, the coding identifiers can be delivered together or separately from the agent. If delivered together they can be attached (*e.g.*, synthesized as a single molecule or attached through ligation or a chemical coupling mechanism) or simply mixed together to be attached after delivery to the substrate. In a preferred aspect, the agent and the coding identifier are made separately, mixed together for attachment, and delivered either attached or as a mixture to be attached on the surface. In a specific aspect the binding agents are delivered generally over the substrate surface and the coding identifiers are delivered in a pattern-specific manner.

[00115] Examples of methods that can be used for deposition of agents and/or coding identifiers onto the substrate surface include, but are not limited to, ink jet spotting, mechanical spotting by means of pin, pen or capillary, micro contact printing, fluidically contacting the measurement areas with the biological or biochemical or synthetic recognition elements upon their supply in parallel or crossed micro channels, upon exposure to pressure differences or to electric or electromagnetic potentials, and photochemical or photolithographic immobilization methods.

[00116] For several applications, it may be preferred to arrange the substrates into segments of one or more measurement areas for reagent distribution and agent determination.

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These regions may be physically separated using barriers or channels. They may still comprise several additional discrete measurement areas with agents that are different or in different combination from each other.

[00117] In certain aspects, the present invention provides a method, *e.g.*, a machine-based method, for evaluating changes in the presence and/or location of a biological molecule over time. The method includes providing a plurality of encoded array results representative of the biological molecule over time and evaluating the differences in detection and/or localization of the biological molecules.

Nucleic Acid Detection and Localization

[00118] In a particular aspect, the assay system is used to analyze nucleic acids, *e.g* genotyping, gene expression analysis, localization of particular transcripts within samples, and the like.

[00119] Genotyping may be performed using any technique known to those of skill in the art. Preferred techniques permit rapid, accurate determination of multiple variations with a minimum of sample handling. Some examples of suitable techniques involve but are not limited to direct DNA sequencing, capillary electrophoresis, hybridization, allele-specific probes or primers, single-strand conformation polymorphism analysis, nucleic acid arrays, bead arrays, restriction fragment length polymorphism analysis, cleavage fragment length polymorphism analysis, random amplified polymorphic DNA, ligase detection reaction, heteroduplex or fragment analysis, differential sequencing with mass spectrometry, atomic force microscopy, pyrosequencing, FRET (*e.g.*, TaqMan (Applied Biosystems, Inc., Foster City, Calif.) and Molecular Beacon (Stratagene, La Jolla, Calif.) assays), and other related techniques. Several methods for DNA sequencing are well known and generally available in the art. See, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, New York) (2001); Ausubel, et al., Current Protocols in Molecular Biology (John Wiley and Sons, New York) (1997), Twyman, et al. (2003) "Techniques Patents for SNP Genotyping", Pharmacogenomics 4(1):67-79; and Kristensen, et al. (2001) "High-Throughput Methods for Detection of Genetic Variation", BioTechniques 30(2):318-332. For details on the use of nucleic acid arrays (DNA chips) for the detection of, for example, SNPs, see U.S. Pat. No. 6,300,063

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issued to Lipshultz, et al., and U.S. Pat. No. 5,837,832 to Chee, et al., HuSNP Mapping Assay, reagent kit and user manual, Affymetrix Part No. 90094 (Affymetrix, Santa Clara, Calif.). The molecular inversion probe (MIP) assay format (Hardenbol et al., 2003) is another example of a highly multiplexable assay that may be used with the assay systems of the invention.

[00120] In one exemplary and preferred method for analyzing nucleic acids using the assay system of the invention, the detection of nucleic acids uses two allele- specific oligonucleotides and a locus specific oligonucleotide. The assay methods are carried out according to the strategy outlined in Fig. 2 using next-generation sequencing or another highly parallel nucleic acid assay technology. In this assay, a set of two oligonucleotides is designed to hybridize to each target sequence, with a common oligonucleotide and two unique coding identifiers. The allele can be determined, *e,g,* by primer extension of the locus specific oligonucleotide. Following primer extension and ligation, an amplifiable template is formed with universal primer sequences at either end. Assay oligonucleotides are annealed to a template and enzymatic reactions are used to join the two oligonucleotides only when both are correctly annealed. The detection techniques and read out parameters used in this system of the invention include a much shorter tag than the oligonucleotides used in the assays that are based on capture by hybridization. These shorter tags are designed to be read out by sequencing or, preferably, used to ligate codes onto both ends of the fragment as illustrated in Fig. 2.

[00121] In Figure 3, two target-specific assay oligonucleotides are ligated together 302 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides containing tag sequence sets X and Y are ligated 304 to the target specific oligonucleotides. Oligonucleotides containing X ligate specifically to one side of the targeting construct and oligonucleotides containing Y ligate to the other. The oligonucleotides contain universal primer sites P1 and P2. Following ligation, the constructs are eluted and, optionally, sequencing adapters can be attached 306, *e.g.,* via PCR.

[00122] In one preferred aspect, the final construct created from the assay method is a substrate for next-generation sequencing, and highly parallel next-generation sequencing methods are used to confirm the sequence of constructs. Such sequencing methods can be carried out, for example, using a one pass sequencing method or using paired-end sequencing. Next generation sequencing methods include, but are not limited to, hybridization-based methods, such as disclosed in Drmanac, U.S. Pat. Nos. 6,864,052; 6,309,824; and 6,401,267; and Drmanac et

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al, U.S. patent publication 2005/0191656, and sequencing by synthesis methods, *e.g.*, Nyren et al, U.S. Pat. No. 6,210,891; Ronaghi, U.S. Pat. No. 6,828,100; Ronaghi et al (1998), Science, 281: 363-365; Balasubramanian, U.S. Pat. No. 6,833,246; Quake, U.S. Pat. No. 6,911,345; Li et al, Proc. Natl. Acad. Sci., 100: 414-419 (2003); Smith et al, PCT publication WO 2006/074351; use of reversible extension terminators, *e.g.*, Turner, U.S. Pat. No. 6,833,246 and Turner, U.S. Pat. No. 6,833,246 and ligation-based methods, *e.g.*, Shendure et al (2005), Science, 309: 1728-1739, Macevicz, U.S. Pat. No. 6,306,597; which references are incorporated by reference. Soddart et al., PNAS U S A. 2009 Apr 20; Xiao et al., Nat Methods. 2009 Mar;6(3):199-201. Epub 2009 Feb 8.

[00123] To maximize the efficiency of encoding, a combinatorial approach using pairs of oligonucleotides can be used. For example, with only two sets of 100 codes, a substrate can theoretically encode up to 10,000 locations. The number of assay oligonucleotides required is dramatically reduced, the cost decreased, and the robustness of the approach increased by decoupling the coding sequences from the genome-specific sequences. Alternative assay formats can also be used (e.g. ligation or primer extension followed by ligation).

[00124] By ligating the codes on separately, only 2n target-specific assay oligonucleotides are needed for n targets. For example, assaying 100 different targets at 10,000 spatial locations would require 2 x 100 targeting oligonucleotides and 2 x 100 encoding oligonucleotides, using a combinatorial approach outlined in Fig. 2. The total count of assay oligonucleotides would be only 400 (200 target-specific and 200 encoding), not counting universal primers. In contrast, if the coding oligonucleotides were not decoupled, (n x X positional codes) + (n x Y positional codes) would be needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences.

[00125] Due to the matrix system of the invention, a large amount of information can be obtained even using five or more positions interrogated for five or more biological molecules. By varying one or the other of these, large amounts of information can be obtained, both in terms of locations and/or specific biological In specific aspects, multiple locations are interrogated for two or more biological molecules. As an example, for each datapoint ~1,000 reads may be sampled, for a total of ~10E9 reads for 10E6 datapoints.

Peptide Detection Systems

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[00126] The assay system of the invention can be used to analyze biological molecules using peptide agents that are associated with the substrate surface in a spatial pattern. Such peptides may comprise an active region of an enzyme, a binding domain of an immunoglobulin, defined domains of proteins, whole proteins, synthetic peptides, peptides with introduced mutations, etc.

[00127] The assay system of the invention allows the identification and spatial location of various forms of peptides, including isoforms and peptides that have undergone posttranslational modification. Importantly, certain aspects of the invention allow the identification of active versus non-active forms of such peptides in a sample. This allows the identification of the presence or absence of specific peptide isoforms, and also acts as a proxy for identification of peptide activity in a sample.

[00128] In certain aspects of the invention, the binding agents associated with the substrate surfaces of the assay system include substrates for enzymes or proenzymes, *e.g.*, a kinase, a phosphatase, a zymogen, a protease, or a fragment thereof. In certain aspects, the binding agents associated with the substrate surfaces are phosphorylation substrates used to detect proteins involved with one or more signal transduction pathways, *e.g.*, a kinase or a phosphatase. In another specific aspect of the invention, the binding agents are specific protease substrates that associate only with individual or classes of proteases. In other aspects, the binding agents on the substrate surface are different processed forms, isoforms and/or domains of an enzyme.

Reagent Delivery

[00129] The reagent delivery system of the invention can be any system that allows the delivery of reagents to discrete portions of the array in order to keep the integrity of the defined spatial patterns of the encoding scheme. Such discrete delivery can be achieved in a number of different ways.

[00130] In one exemplary aspect, the reagent delivery system can be a flow-based system. The flow-based systems for reagent delivery in the present invention can include one or more pumps, valves, fluid reservoirs, channels, and/or reagent storage cells. Such a reagent delivery system is configured to move fluid in contact with a discrete section of the substrate surface.

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Movement of the reagents can be driven through a fluid by a pump disposed, for example, downstream of the fluid reagents. The pump can drive each fluid reagent to (and past) the reaction compartment. Alternatively, the reagents may be driven through the fluid by gravity.

[00131] US Appln Nos. 20070166725 and 20050239192 disclose certain general- purpose fluidics tools that can be used with the assay systems of the invention. These allow the precise manipulation of gases, liquids and solids to accomplish very complex analytical manipulations with relatively simple hardware.

[00132] In a more specific example, one or more flow-cells can be attached to the substrate from above. The flow-cell can include inlet and outlet tubes connected thereto and optionally an external pump can be used to deliver the sample or reagents to the flow-cell and across the substrate. The flow cell is configured to deliver reagents only to certain portions of the array, restricting the amount and type of reagent delivered to any specific section of the array.

[00133] In another aspect, a microfluidic system can be integrated into the substrate or externally attached on top of the substrate. Microfluidic passages for holding and carrying fluid can be formed on and/or above the planar substrate by a fluidics layer abutted to the substrate. Fluid reagents can be selected according to selective opening and closing of valves disposed between reagent reservoirs.

[00134] Pumps generally include any mechanism for moving fluid and/or reagents disposed in fluid. In some examples, the pump can be configured to move fluid and/or reagents through passages with small volumes (i.e., microfluidic structures). The pump can operate mechanically by exerting a positive or negative pressure on fluid and/or on a structure carrying fluid, electrically by appropriate application of an electric field(s), or both, among others. Exemplary mechanical pumps may include syringe pumps, peristaltic pumps, rotary pumps, pressurized gas, pipettors, etc. The mechanical pumps may be micromachined, molded, etc. Exemplary electrical pumps can include electrodes and may operate by electrophoresis, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[00135] Valves generally include any mechanism for regulating the passage of fluid through a channel. The valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into the channel to partially or completely block the channel, an

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electrocapillary structure, and/or the like.

[00136] In yet another aspect, an open gasket can be attached to the top of the substrate and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber formed by a gasket sandwiched between the substrate and a chemically inert, water resistant material such as, but not limited to, black-anodized aluminum, thermoplastics (e.g., polystyrene, polycarbonate, etc), glass, etc.

[00137] In a specific aspect of the present invention, the delivery system can comprise a microcircuit arrangement including an imager, such as a CCD or IGFET-based (e.g., CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Appln No. 20090197326, which is incorporated herein by reference.

[00138] In yet another aspect of the invention, the reagent delivery system controls the delivery of reagents to specific patterns on a substrate surface using semiconductor techniques such as masking and spraying. Specific areas of a substrate surface can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the substrate using conventional techniques such as spraying or fluid flow. The use of the masked substrate delivery results in a patterned delivery scheme on the substrate surface.

[00139] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms (*e.g.*, thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions can be achieved.

Software for use in the Assay System

[00140] In order to target specific sites of interest, an informative image of the biological section to be analyzed can be used to assist in the reagent delivery methods and associated encoding scheme. Sample regions can be identified using image processing (*e.g.*, images of cell types differentiated by immunohistochemistry or other staining chemistries) integrated with the

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other features of the assay system. In some aspects, software is used to automatically translate this information into a reagent delivery pattern. A mechanism to register and align very precisely the biological sample in a targeting system is thus a preferred component of the assay systems of the invention. Mechanisms such as the use of fiducial markers on slides and other very accurate physical positioning systems can be adapted to this purpose.

[00141] Additional software components will also be key components that will be part of a complete assay system. The invention thus preferably comprises a complete suite of software tailored to the assay system. Optionally, oligonucleotide design software will be customized for the specific assay to be run, and may be integrated as a part of the system. Also optionally, algorithms and software for data analysis may be integrated to assist in determination of results of the assays. This can be especially useful, as the type of dataset that will be generated will be novel, particularly as a consequence of scale. The ability to provide algorithms and software tools that are specifically designed for analysis of spatially-associated data for significant patterns, including pattern-analysis software and visualization tools, is a novel feature that will enhance the value of the data generated by the assay systems.

[00142] In certain aspects, the assay system will comprise processes for making and carrying out quality control of reagents, *e.g.,* the integrity and sequence fidelity of oligonucleotide pools. In particular, reagents will need to be formulated for compatibility with the reagent delivery instrumentation. Factors such as volatility, stability at key temperatures, and chemical compatibility can be optimized by those skilled in the art upon reading the present disclosure.

Applications of Assay System

[00143] It will be apparent to one skilled in the art upon reading the present disclosure that there are numerous very important areas of biological research, diagnostics, and drug development that will benefit from a high throughput means to simultaneously measure the presence or absence and spatial location of a biological molecule in a sample. For example, this technology combining the ability to analyze semi-quantitatively the expression of many different genes with the ability to image the spatial organization of expression across many cells in a tissue is enabling for many different areas of basic research. The following are exemplary uses and are

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by no means meant to be limiting in scope.

[00144] In one example, 3-dimensional patterns of expression can be determined by analyzing a series of tissue sections, in a manner analogous to image reconstruction in CT scanning. This can be used to measure changes in gene expression in disease pathology, *e.g.*, in cancerous tissue and/or a tissue upon injury, inflammation or infection. With the assay systems of the invention, more detailed information on gene expression and protein localization in complex tissues can be obtained. This may lead to new insights into the function and regulation both in normal and diseased states, and is likely to provide new hypotheses that can be tested. For example, a system of the invention may enable some of the insights gained from many individual studies and larger programs like ENCODE (Birney et al., 2007) and modENCODE to be integrated at the tissue level. The assay systems will also aid in computational efforts to model interacting networks of gene expression in the field of systems biology.

[00145] The assay systems also provide a novel approach that enables the analysis of somatic variation, *e.g.*, somatic mutations in cancer or variability in response to infectious organisms. For example, tumors are typically highly heterogeneous, containing cancer cells as well as genetically normal cells in an abnormal local environment. Cancer cells undergo mutation and selection, and in this process it is not unusual for local clones to develop. Identifying relatively rare somatic mutations in the context of tumors may enable the study of the role of key mutations in the selection of clonal variants. Transcriptional patterns associated with angiogenesis, inflammation, or other cancer related processes in both cancer and genetically normal cells can be analyzed for insights into cancer biology and assist in the development of new therapeutic agents for the treatment of cancers.

[00146] In another example, different people have varying susceptibility to infectious organisms, and much of this may be to underlying genetic differences in individuals and/or populations. Identifying these differences will aid in an understanding of the underlying disease pathologies and assist in the development of vaccines or therapeutics to prevent or ameliorate these disease states.

[00147] Importantly, in addition to providing spatially associated information, the technology of the invention will allow a great increase in the sensitivity of detecting rare mutations. The reason is that signal to noise can be dramatically increased because the approach of the invention assays a small location in any given reaction. In a typical assay for rare mutations

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in a mixed sample, the sample is treated in bulk, i.e. nucleic acids are extracted from many cells into a single pool. Thus, if a mutation is present in 1 cell in 10,000, it must be detected against a background of normal DNA from ~10,000 cells. In contrast, with the systems of the invention many cells can be analyzed, but individual cells or small groups of cells would be identified by the spatial coding system. Therefore, the background can be reduced by orders of magnitude, greatly increasing sensitivity. Furthermore, the spatial organization of mutant cells can be observed. This may be particularly important in detecting key mutations in tissue sections in cancer. Already, molecular histological analyses are yielding insights into cancer biology and may have potential for use in diagnostics (Choe et al., 2003). The technology of the invention promises to greatly increase the power of such approaches.

EXAMPLES

[00148] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventor regards as his invention, nor are they intended to represent or imply that the experiments below are all of or the only experiments performed. It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

[00149] Efforts have been made to ensure accuracy with respect to numbers used *(e.g.,* amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees centigrade, and pressure is at or near atmospheric.

EXAMPLE 1: Initial Proof of Concept of Encoding Scheme

[00150] As an initial proof of concept, a model system is developed using a microarray to demonstrate a working single-plex assay. The basic design validates the concept of the assay, and

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establishes a working assay prior to addressing issues related to the analysis of a more complicated biological sample. Conventional sequencing is used as a readout for this proof of concept.

[00151] A microarray is used as a proxy for a tissue section. The target sequences of the microarray are fully specified, so that the composition of the targets are known and can be varied systematically. Synthetic oligonucleotide templates are attached to a glass slide via a 5' amino modification. Each slide has a single oligonucleotide template sequence, and the assays that are carried out may employ either ligation, or extension followed by ligation as this may be useful in determining certain polymorphisms.

[00152] Once the *in situ* part of the assay is complete, the reaction products are eluted and analyzed by qPCR to determine presence or absence of a product and estimate yield, and by conventional sequencing to determine the structure of the assay products. The single-plex assays that are tested include appropriate positive and negative controls, and a single nucleotide variant (SNV) to check ability to discriminate single base variants.

EXAMPLE 2: Scalability

[00153] The complexity of the assay system is increased to establish scalability of the assay for use in high throughput studies. Scalability of both the spatial encoding and assay systems is demonstrated by carrying out a 24-plex x 24-site assay using a microarray model system.

[00154] The amount of biological target, here a DNA target sequence, at each assay location is systematically varied on microarray substrate. For example, in a microarray with 50 micron spot size (center to center), a $1mm^2$ area contains ~400 spots. The region around each site is optionally occupied by a region that is devoid of these spots to allow individual resolvability of the target sequences. Alternatively, the spots may be clustered, with two or more directly adjacent spots surrounded by or adjacent to a region that is devoid of target sequences.

[00155] In order to demonstrate that spatial encoding is accurate, the sites comprise different target compositions to show that the assay readout matches the expected composition of each site. With 24 target sequences, a simple digital pattern is made with each site having a different set of 12 targets present and 12 targets absent, to make a binary code (0 = absent, 1 = present). The assay readout is then determined to show that the detected regions match the

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expected signal after spatial decoding. In this particular example, the code space is large enough (2^{24}) so that even a few errors would not result in different codes being mixed up. Moreover, this design allows identification of errors and allows an estimation not only of accuracy of spatial encoding but also of accuracy calling the presence or absence of target sequences.

[00156] In an exemplary aspect, a 4 x 4 arrangement of 16 sequences is used for the array configuration. A white square indicates that the sequence is absent and a black square that it is present, i.e. 8 of the 16 possible sequences are present in this sample. In a different sample, a different pattern of absent and present sequences can be constructed. In this way, unique patterns are associated with spatial locations so that the accuracy of spatial encoding can be measured.

[00157] The ability to detect quantitative differences is evaluated by generating dose-response curves for each of the 24 assays that are carried out at each site in a 24-site assay. This allows estimation of the limit of detection, dynamic range, and power to detect a given fold-change across the range.

[00158] In one aspect, a latin square design is used to represent individual targets at different ratios by varying the number of features for each target. In other words, with multiple spots in a site, the number of spots allocated to each of the 24 target sequences can be varied and each of the 24 sites can have a different composition. A 1 x 3 inch microarray is sufficiently large to permit multiple replicates. This larger set of 24 sequences will require deconvolution, and this is accomplished using high throughput techniques such as next-generation sequencing technologies *(e.g.,* SOLiD™ technology (Life Technologies, Inc., Carlsbad, CA) or Genome Analyzer (Illumina, Inc., San Diego, CA)). The use of the 24-plex assay demonstrates both the accuracy of spatial encoding and decoding, and the quantitative response of the assay system.

EXAMPLE 3: Adaptation of the assay to preserved samples.

[00159] Genomic DNA is assayed as a proof of concept for assaying RNA, as it provides a way to establish a single-copy reference signal Once a working assay is developed for FFPE samples, it is adapted to an RNA assay. To this end, assay oligonucleotide concentrations are assayed to ensure compatibility with high multiplexing. Assuming a cell diameter of 10 microns, and delivery of a 10 micron diameter reagent droplet to an individual cell, the volume of the droplet will be ~500 µl and can contain ~3 x 10^{11} molecules at a 1 µM concentration. To assay 1,000 target sequences in 10,000 cells, ~2,000 targeting oligonucleotides would be required in a

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droplet. Therefore, each droplet could contain ~160 million copies of each assay oligo, a vast excess over the few thousand target sequences in a cell.

[00160] The handling of small absolute numbers of product molecules generated from very small or compromised samples are enhanced to counter the issue of low recovery efficiency; that is, elution is efficient and losses resulting from adsorption of molecules to surfaces are prevented. An approach to addressing the latter issue is to include a carrier material, such as glycogen or carrier nucleic acids.

Example 4: Adapting the Assay to a Biological Sample.

[00161] A control RNA template is immobilized to a solid support in order to create an artificial system. The assay is performed using T4 DNA ligase, which can repair nicks in DNA/RNA hybrids. Assays are carried out on matched slides, or different sections of the same slide, where in one case gDNA is assayed and in the other RNA is assayed. When assaying gDNA the slide can be pretreated with RNase, and when assaying RNA the slide is pretreated with DNase. Results of the assay are confirmed by extracting gDNA or RNA and quantitating the relative amounts by qPCR or RT-qPCR respectively.

[00162] In order make the tissue section RNA assays as informative as possible, pre-existing information on expression levels in specific tissues to target transcripts across a range of abundances are used in the assay design. Both high abundance transcripts, as well as some medium and low abundance transcripts, are targeted to enable an initial assessment of the quantitative performance characteristics of the assay.

[00163] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as

Curio EX1010-p. 1064
Curio v Prognosys

specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims. In the claims that follow, unless the term "means" is used, none of the features or elements recited therein should be construed as means-plus-function limitations pursuant to 35 U.S.C. §112, ¶6.

Curio EX1010-p. 1065
Curio v Prognosys

Electronic Acknowledgement Receipt

EFS ID:	37372609
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	SPATIALLY ENCODED BIOLOGICAL ASSAYS
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	04-OCT-2019
Filing Date:	14-FEB-2019
Time Stamp:	16:27:36
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		312_Amendment.pdf	98822 e3582a2667391b27f4b09ac18c1f98cbba3bf38d	yes	3

	Multipart Description/PDF files in .zip description				
	Document Description	**Start**	**End**		
	Amendment after Notice of Allowance (Rule 312)	1	1		
	Applicant Arguments/Remarks Made in an Amendment	2	3		
Warnings:					
Information:					
2	Specification	47706-0004003_MARKED_SPEC_.pdf	333540 8171798ef3c1ad089255c90b1858d0962100bd5f	no	46
Warnings:					
Information:					
3	Specification	47706-0004003_CLEAN_SPECIFICATION_.pdf	323498 e32f15e2dcf943c2943494b91df290e158952cd8	no	46
Warnings:					
Information:					
		Total Files Size (in bytes):	755860		

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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.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Confirmation No.	:	7944
			Notice of Allowance Date: 9/9/2019		
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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

AMENDMENT AFTER ALLOWANCE
PURSUANT TO 37 C.F.R. §1.312

Please amend the application as indicated on the following pages. This amendment is being filed concurrently with the payment of the issue fee.

Amendments to the Specification:

Please replace paragraph [00113] with the following amended paragraph:

[00113] Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system [[401]], two reagents 420, 422 that specifically bind to a biological molecule of interest are associated with coding identifiers 406, 408 that encode for a spatial location in the sample. These coding identifiers 406, 408 are optionally associated with sites that assist in their identification in the assay format, *e.g.*, universal priming sites 404, 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section 416 is encoded using the combination of the patterns 412, 414 created using the separate coding identifiers 406, 408 which provide a two dimensional code 418 that shows the location of any positive detection of the biological molecule 402 as well as quantifying the biological molecule 402 at each location assayed in the tissue.

REMARKS

Paragraph [00113] of the specification has been amended to delete reference number 401, since Figure 4 does not indicate a reference number 401. No new matter has been added.

Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: October 7, 2019

/Cameron Luitjens/

Cameron M. Luitjens, Ph.D., J.D.
Reg. No. 58,674

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Telephone: (612) 335-5070
Facsimile: (877) 769-7945

62121394.doc

Electronic Acknowledgement Receipt

EFS ID:	37383298
Application Number:	16276235
International Application Number:	
Confirmation Number:	7944
Title of Invention:	SPATIALLY ENCODED BIOLOGICAL ASSAYS
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004003
Receipt Date:	07-OCT-2019
Filing Date:	14-FEB-2019
Time Stamp:	15:02:22
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		312_Amendment.pdf	99634 575ed28197ce21de71354c0a783c70c1ba4024cf	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment after Notice of Allowance (Rule 312)	1	1
	Specification	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3
Warnings:			
Information:			
	Total Files Size (in bytes):	99634	

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.

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UNITED STATES PATENT AND TRADEMARK OFFICE

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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	02/14/2019	Mark S. CHEE	47706-0004003	7944

159367 7590 10/11/2019

FISH & RICHARDSON P.C.
10x Genomics
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
MARTINELL, JAMES	

ART UNIT	PAPER NUMBER
1634	

NOTIFICATION DATE	DELIVERY MODE
10/11/2019	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):

PATDOCTC@fr.com
ipadmin@10xgenomics.com

Response to Rule 312 Communication	Application No.	Applicant(s)	
	16/276,235	CHEE, Mark S.	
	Examiner	Art Unit	AIA (FITF) Status
	JAMES MARTINELL	1634	No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

1. ☑ The amendment filed on 04 October 2019 under 37 CFR 1.312 has been considered, and has been:

a) ☑ entered.

b) ☐ entered as directed to matters of form not affecting the scope of the invention.

c) ☐ disapproved because the amendment was filed after the payment of the issue fee.

Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

d) ☐ disapproved. See explanation below.

e) ☐ entered in part. See explanation below.

Addendum:

The amendment filed 07 October 2019 under 37 CFR 1.312 has been considered, and has been entered.

Because of the complexity involved in the entering of amendments filed 05/15/2019 and 06/11/2019, applicant was asked (10/03/2019) to file a substitute specification, which was done on 10/04/2019.

In addition, due to a minor error in the amendment filed 10/04/2109, applicant has filed an amendment on 10/07/2019 to paragraph 00113, which amendment has been entered.

/GARY BENZION/ Supervisory Patent Examiner, Art Unit 1637	/JAMES MARTINELL/ Primary Examiner, Art Unit 1634

OK TO ENTER: /J.M/

10/09/2019

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
Filed	:	February 14, 2019	Confirmation No.	:	7944
			Notice of Allowance Date: 9/9/2019		
Title	:	SPATIALLY ENCODED BIOLOGICAL ASSAYS			

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Alexandria, VA 22313-1450

AMENDMENT AFTER ALLOWANCE
PURSUANT TO 37 C.F.R. §1.312

Please amend the application as indicated on the following pages. This amendment is being filed concurrently with the payment of the issue fee.

OK TO ENTER: /J.M/

10/09/2019

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Mark S. Chee	Art Unit	:	1634
Application No.	:	16/276,235	Examiner	:	James Martinell
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PURSUANT TO 37 C.F.R. §1.312

Please amend the application as indicated on the following pages.

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/276,235	11/19/2019	10480022	47706-0004003	7944

159367 7590 10/30/2019

FISH & RICHARDSON P.C.
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MINNEAPOLIS, MN 55440-1022

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 Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Mark S. CHEE, San Diego, CA;
Prognosys Biosciences, Inc., San Diego, CA;

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.