



August 7, 2020

Attorney Docket No.: 47706-0004026

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

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Boston, MA 02210-1878

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Presented for filing is a new continuation patent application for prioritized examination of:

Inventor(s): MARK S. CHEE

Title: SPATIALLY ENCODED BIOLOGICAL ASSAYS

Enclosed are the following papers, including those required to receive a filing date under 37 CFR 1.53(b):

	<u>Pages</u>
Specification	44
Claims	1
Abstract	1
Declaration	1
Drawing(s)	5

Enclosures:

- Application Data Sheet, 9 pages.
- Power of Attorney, 2 pages.
- Certification and Request for Prioritized Examination (Track I), 2 pages

Basic Filing Fee	\$300
Search Fee	\$660
Examination Fee	\$760

Publication fee	\$0
Track I processing fee	\$140
Track I prioritized examination fee	\$4000

Total Claims 1	over 20	0 x \$100	\$0
Independent Claims 1	over 3	0 x \$460	\$0



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Page 2

Fee for Multiple Dependent claims	\$0
Application size fee for each 50 pages over 100	
Total Sheets: 51 x .75 - 100/50 = 0x	\$0
Total Filing fee	\$5860

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

I hereby authorize the USPTO to communicate in response to email communications from the undersigned and practitioners under Customer No. 159367 in accordance with 37 CFR 1.33 and 37 CFR 1.34 concerning any subject matter of this application via email. I understand that a copy of these communications will be made of record in the application file. (MPEP 502.03).

If this application is considered to be incomplete, or if a telephone conference would otherwise be helpful, call the undersigned at (617) 956-5901.

Send all correspondence to:

159367
Customer Number

Respectfully submitted,

/Peter R. Poulin Reg. No. 74,643/

Peter Poulin
Registration No. 74,643

Enclosures

PRP/smg
62393290.doc

**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):	
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 /Peter R. Poulin Reg. No. 74,643/	Date August 7, 2020
Name (Print/Typed) Peter Poulin	Practitioner Registration Number 74,643
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.	

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.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004026
		Application Number	
Title of Invention	Spatially Encoded Biological Assays		
The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76. This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.			

Secrecy Order 37 CFR 5.2:

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

Inventor	1				Remove	
Legal Name						
Prefix	Given Name	Middle Name	Family Name	Suffix		
	Mark	S.	CHEE			
Residence Information (Select One) • US Residency Non US Residency Active US Military Service						
City	San Diego	State/Province	CA	Country of Residence	US	
Mailing Address of Inventor:						
Address 1	11125 Flintkote Avenue					
Address 2	Suite A					
City	San Diego	State/Province	CA			
Postal Code	92121	Country	US			
All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button. Add						

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).	
☐ An Address is being provided for the correspondence information of this application.	
Customer Number	159367
Email Address	apsi@fr.com
Add Email Remove Email	

Application Information:

Title of the Invention	Spatially Encoded Biological Assays		
Attorney Docket Number	47706-0004026	Small Entity Status Claimed	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	5	Suggested Figure for Publication (if any)	

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004026
		Application Number	
Title of Invention	Spatially Encoded Biological Assays		

Filing By Reference:

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

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

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

Publication Information:

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

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

Representative Information:

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

Please Select One:	☒ Customer Number	☐ US Patent Practitioner	☐ Limited Recognition (37 CFR 11.9)
Customer Number	159367		

Domestic Benefit/National Stage Information:

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

When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending	Remove	
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	16414213	2019-05-16

Application Data Sheet 37 CFR 1.76		Attorney Docket Number		47706-0004026	
		Application Number			
Title of Invention		Spatially Encoded Biological Assays			

Prior Application Status	Pending	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
16414213	Continuation of	16402098	2019-05-02		
Prior Application Status	Pending	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
16402098	Continuation of	16276235	2019-02-14		
Prior Application Status	Patented	Remove			
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
16276235	Continuation of	15187661	2016-06-20	10308982	2019-06-04
Prior Application Status	Patented	Remove			
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
15187661	Continuation of	13080616	2011-04-05	9371598	2016-06-21
Prior Application Status	Expired	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
13080616	Claims benefit of provisional	61321124	2010-04-05		
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button. Add					

Foreign Priority Information:

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

			Remove
Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ⁱ (if applicable)
Additional Foreign Priority Data may be generated within this form by selecting the Add button. Add			

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004026
		Application Number	
Title of Invention	Spatially Encoded Biological Assays		

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

This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013. ☐ NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.	
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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004026
		Application Number	
Title of Invention	Spatially Encoded Biological Assays		

Authorization or Opt-Out of Authorization to Permit Access:

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

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

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

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

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

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

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

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

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

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

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

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004026
		Application Number	
Title of Invention	Spatially Encoded Biological Assays		

Applicant Information:

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

Applicant	1	Remove
If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section. Clear		
☒ Assignee	Legal Representative under 35 U.S.C. 117	Joint Inventor
Person to whom the inventor is obligated to assign.		Person who shows sufficient proprietary interest
If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:		
Name of the Deceased or Legally Incapacitated Inventor:		
If the Applicant is an Organization check here. ☒		
Organization Name	Prognosys Biosciences, Inc.	
Mailing Address Information For Applicant:		
Address 1	11125 Flintkote Avenue	
Address 2	Suite A	
City	San Diego	State/Province CA
Country	US	Postal Code 92121
Phone Number		Fax Number
Email Address		
Additional Applicant Data may be generated within this form by selecting the Add button. Add		

Assignee Information including Non-Applicant Assignee Information:

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

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004026
		Application Number	
Title of Invention	Spatially Encoded Biological Assays		

Assignee	1
Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.	
Remove	
If the Assignee or Non-Applicant Assignee is an Organization check here. ☒	
Organization Name	
Mailing Address Information For Assignee including Non-Applicant Assignee:	
Address 1	
Address 2	
City	State/Province
Country ⁱ	Postal Code
Phone Number	Fax Number
Email Address	
Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add	

Signature:

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

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

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

Signature	/Peter R. Poulin Reg. No. 74,643/		Date (YYYY-MM-DD)	2020-08-07
First Name	Peter	Last Name	Poulin	Registration Number
				74,643
Additional Signature may be generated within this form by selecting the Add button.				Add

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004026
		Application Number	
Title of Invention	Spatially Encoded Biological Assays		

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

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.

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- 2 A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
- 3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
- 4 A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
- 5 A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
- 6 A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
- 7 A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
- 8 A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
- 9 A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

SPATIALLY ENCODED BIOLOGICAL ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Patent Application No. 16/414,213, filed May 16, 2019, which is a continuation of U.S. Patent Application No. 16/402,098, filed May 2, 2019, which is a continuation of U.S. Patent Application No. 16/276,235 filed February 14, 2019, which is a continuation application of U.S. Patent Application No. 15/187,661 filed June 20, 2016, now U.S. Patent 10,308,982, which is a continuation of 13/080,616 filed April 5, 2011, now U.S. Patent 9,371,598, which 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 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 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.

[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 methylphosphoroamidite 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, $T_m = 81.5 + 0.41 (\% \text{ G+C})$, 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.

[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 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.

[0047] 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.

[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.

[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 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

[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 (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.

[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); (Illumina, 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 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, 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.

[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 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.

[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 a1, 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 a1 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.

[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 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. 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.

[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 2×100 target-specific oligonucleotides and 2×100 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, $(n \times X \text{ positional codes}) + (n \times Y \text{ positional codes})$ 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, portions 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 handler, which can be 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 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, electroendosmosis, 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 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 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 be 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 Analyzer

(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

[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 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 be 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 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.

[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.

[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.

[00109] The Invention in General

[00110] 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.

[00111] 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.

[00112] 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.

[00113] 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.

[00114] Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system, 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.

[00115] 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.

[00116] 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.

[00117] 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.

[00118] 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

[00119] 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.

[00120] 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 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.

[00121] 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.

[00122] 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.

[00123] 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.

[00124] 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).

[00125] 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 2×100 targeting oligonucleotides and 2×100 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, $(n \times X \text{ positional codes}) + (n \times Y \text{ positional codes})$ would be needed, or in the above example, 20,000 oligonucleotides, not counting universal primer sequences.

[00126] 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 10^9 reads for 10^6 datapoints.

Peptide Detection Systems

[00127] 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.

[00128] 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.

[00129] 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

[00130] 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.

[00131] 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.

[00132] 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.

[00133] 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.

[00134] 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.

[00135] 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, electroosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[00136] 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.

[00137] 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.

[00138] 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.

[00139] 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.

[00140] 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

[00141] 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.

[00142] 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.

[00143] 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

[00144] 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.

[00145] 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.

[00146] 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.

[00147] 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.

[00148] 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

[00149] 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.

[00150] 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

[00151] 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.

[00152] 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.

[00153] 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

[00154] 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.

[00155] 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 mm² 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.

[00156] 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.

[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 μ l and can contain $\sim 3 \times 10^{11}$ molecules at a 1 μ M 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.

[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 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.

Claims:

1. An assay system to determine spatial patterns of abundance or activity or both of multiple biological targets at multiple sites in a sample, wherein 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, wherein 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.

SPATIALLY ENCODED BIOLOGICAL ASSAYS

Abstract of the Disclosure

The present invention provides assays and assay systems for use in spatially encoded biological assays. The invention provides an assay system comprising an assay capable of high levels of multiplexing where reagents 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.

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100

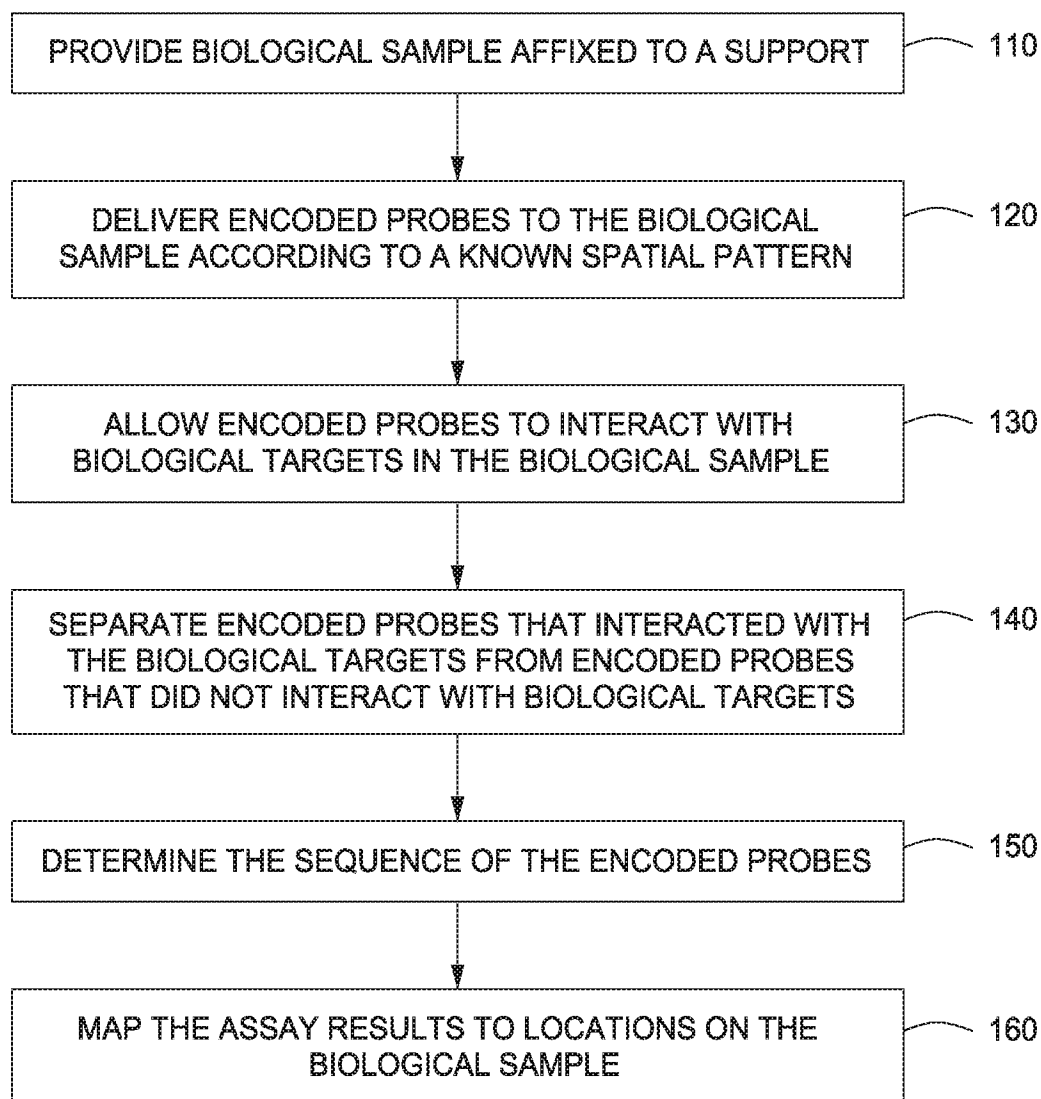


FIG. 1

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200

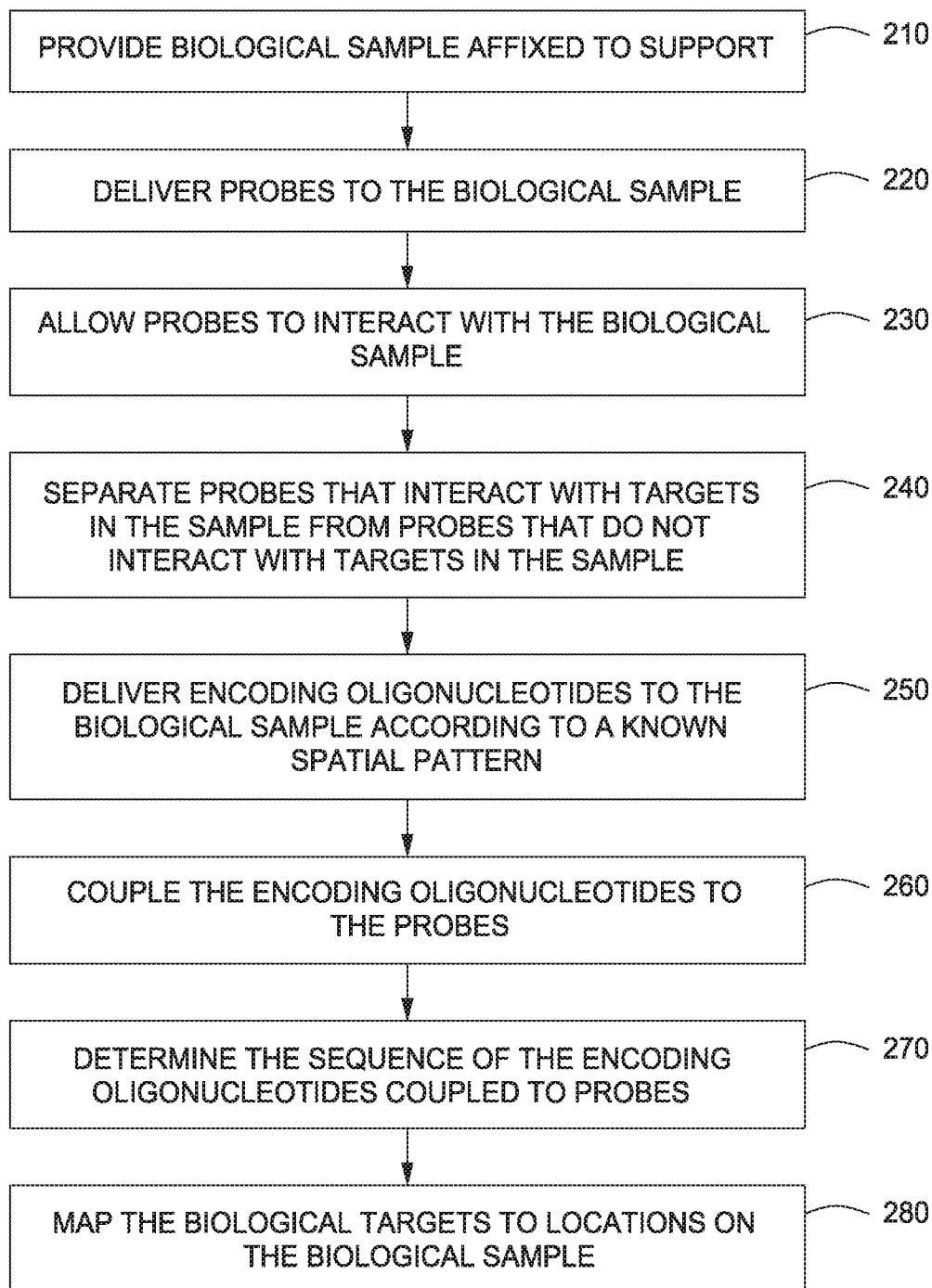


FIG. 2

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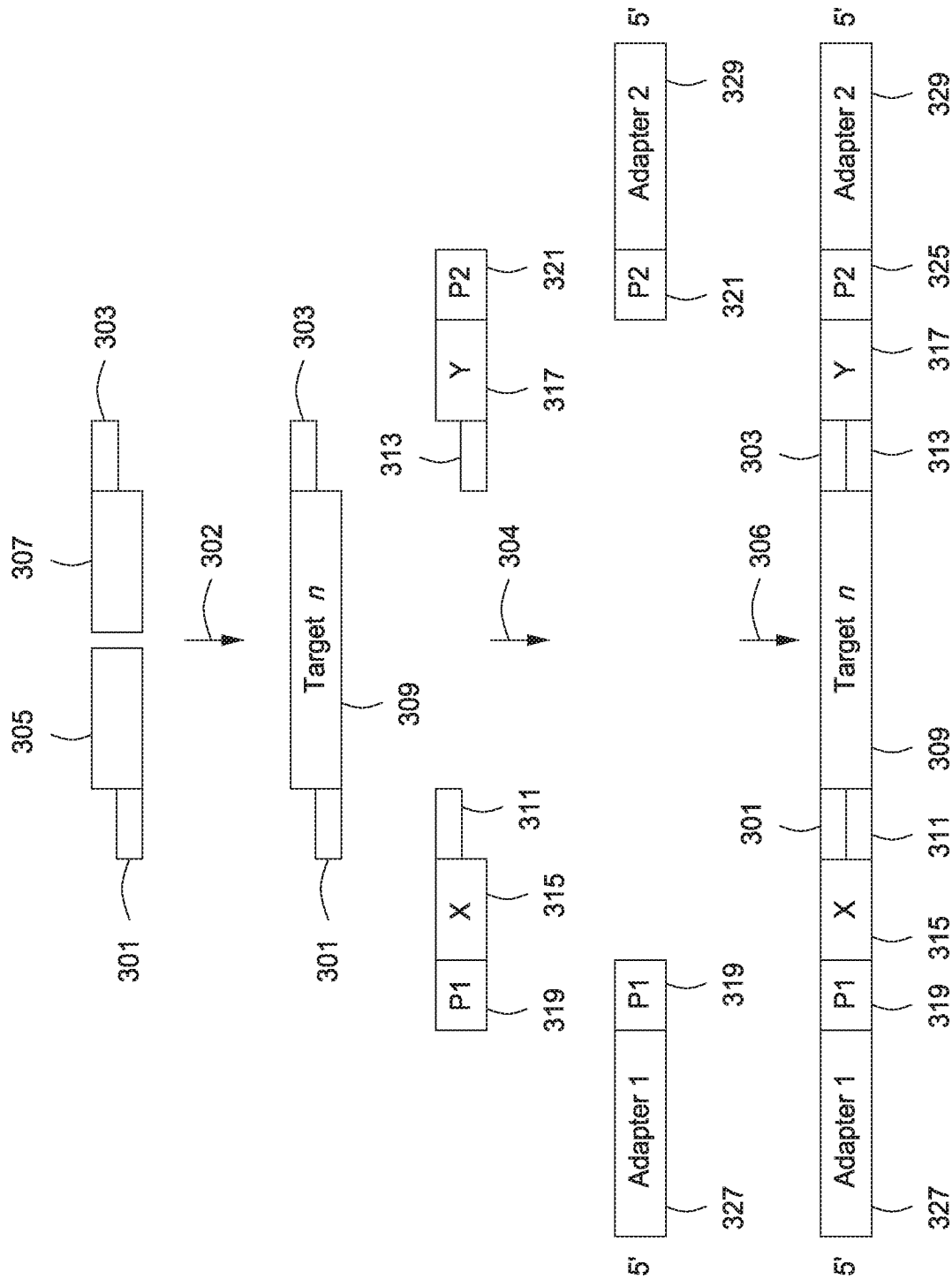


FIG. 3

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FIG. 4A

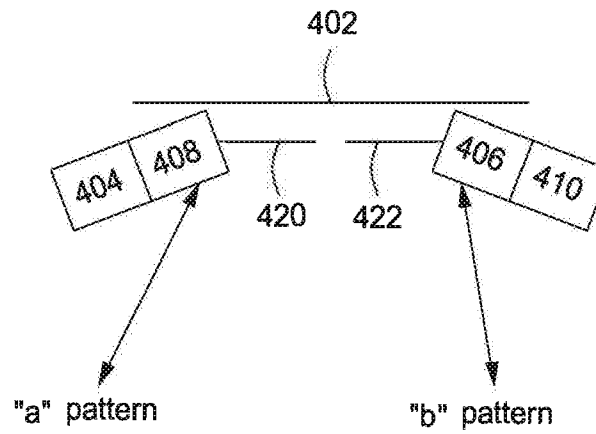


FIG. 4B

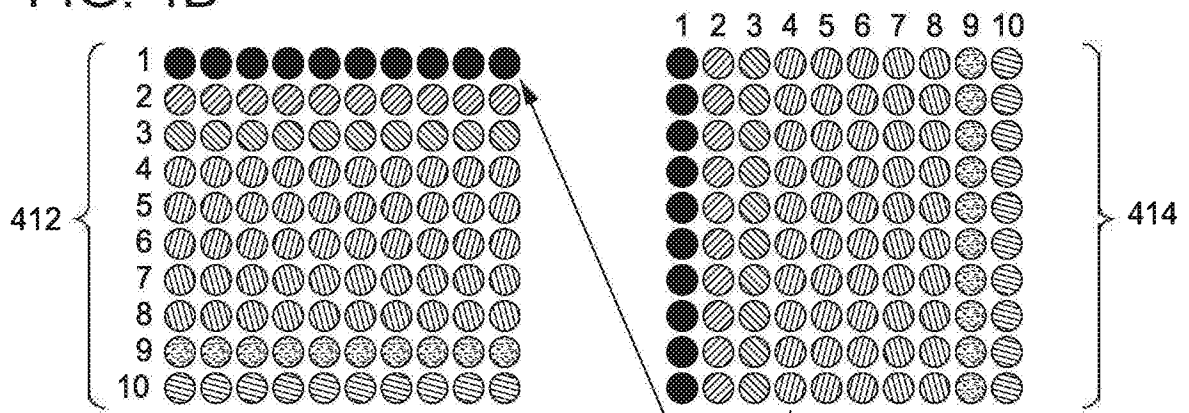
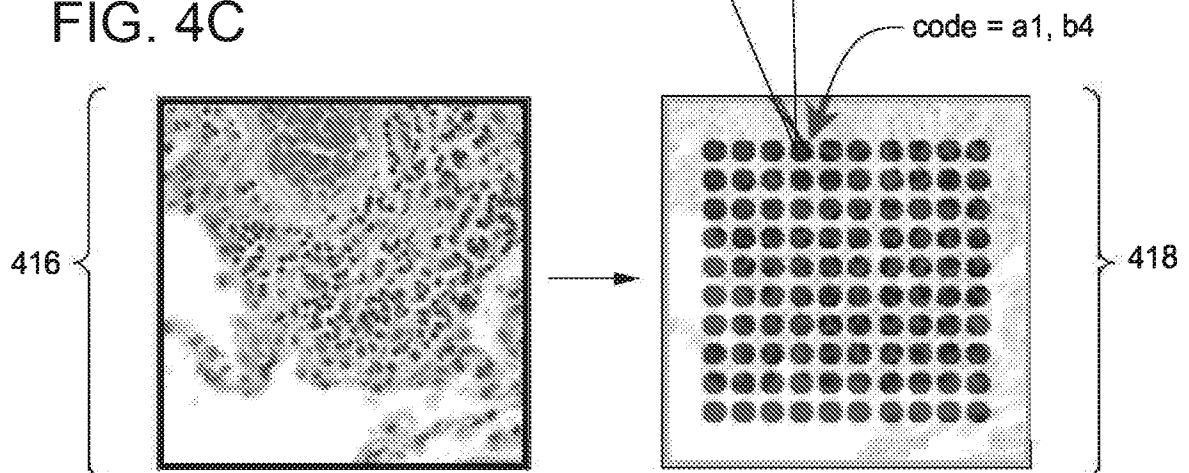


FIG. 4C



5/5

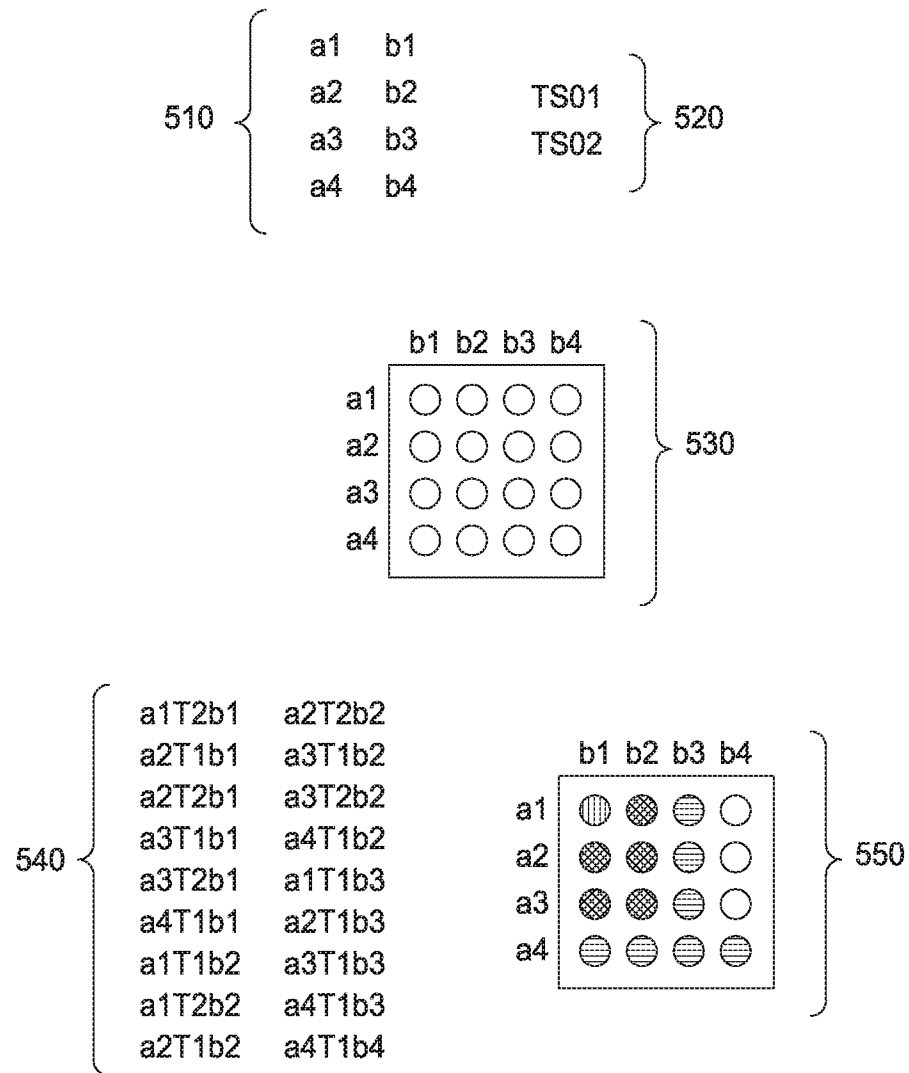


FIG. 5

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

Application Number	To be assigned
Filing Date	Herewith
First Named Inventor	Mark S. Chee
Title	SPATIALLY ENCODED BIOLOGICAL ASSAYS
Art Unit	
Examiner Name	
Attorney Docket Number	47706-0004026

SIGNATURE of Applicant or Patent Practitioner

Signature	/Peter R. Poulin Reg. No. 74,643/	Date (Optional)	August 7, 2020
Name	Peter Poulin	Registration Number	74,643
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.

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			

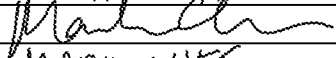
I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

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.

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

**Title of
Invention**

SPATIALLY ENCODED BIOLOGICAL ASSAYS

As the below named inventor, I hereby declare that:

This declaration ☐ The attached application, or
is directed to: ☒ United States application or PCT international application number 15/187,661
filed on 06/20/2016.

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

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

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

WARNING:

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

LEGAL NAME OF INVENTOR

Inventor: Mark S. CHEE Date (Optional) : _____

Signature: 

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

699932000201

sd-683655

Electronic Patent Application Fee Transmittal

Application Number:				
Filing Date:				
Title of Invention:	Spatially Encoded Biological Assays			
First Named Inventor/Applicant Name:	Mark S. Chee			
Filer:	Peter Poulin/Shannon Stadnick			
Attorney Docket Number:	47706-0004026			
Filed as Large Entity				
Filing Fees for Track I Prioritized Examination - Nonprovisional Application under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
REQUEST FOR PRIORITIZED EXAMINATION	1817	1	4000	4000
Pages:				
Claims:				
Miscellaneous-Filing:				
PROCESSING FEE, EXCEPT PROV. APPLS.	1830	1	140	140

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				5860

Electronic Acknowledgement Receipt

EFS ID:	40229151
Application Number:	16988284
International Application Number:	
Confirmation Number:	1697
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. Chee
Customer Number:	159367
Filer:	Peter Poulin/Shannon Stadnick
Filer Authorized By:	Peter Poulin
Attorney Docket Number:	47706-0004026
Receipt Date:	07-AUG-2020
Filing Date:	
Time Stamp:	17:23:37
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$5860
RAM confirmation Number	E202087H23568855
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	Transmittal of New Application	Transmittal.pdf	105408	no	2
			783a85b8d3fe9fa3a7a49fe56fa67b9cb87afbbe		
Warnings:					
Information:					
2	TrackOne Request	Track1.pdf	146015	no	2
			56960cc70c214ec606db7b12425231a7f5fd707e		
Warnings:					
Information:					
3	Application Data Sheet	ADS.pdf	1256418	no	9
			31c4197flee092b81947a9322c37c5c34fc68347		
Warnings:					
Information:					
4		Application.pdf	596835	yes	46
			c152461ebbfdc1880d8f9cecc3a4a9c57fec1b61		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	44	
	Claims		45	45	
	Abstract		46	46	
Warnings:					
Information:					
5	Drawings-only black and white line drawings	Figures.pdf	2027123	no	5
			df2e101676057db13e5fd94e9c9d22e6ec2ae300		

Warnings:					
Information:					
6	Power of Attorney	POA.pdf	204237	no	2
			6dafcd2d927584c17c87e28a0f1df32970d0b398		
Warnings:					
Information:					
7	Oath or Declaration filed	Declaration.pdf	49134	no	1
			8a78ea1badfc8d530956fa8eca52e1072981fe4e		
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	38462	no	2
			ea3de76ab5227260207474f352b422b58aea2917		
Warnings:					
Information:					
Total Files Size (in bytes):			4423632		

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.

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 47706-0004026	Application No. 16/988,284
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Prognosis Biosciences, Inc.	
		Filing Date August 7, 2020	Group Art Unit
(37 CFR §1.98(b))			

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Filing Date If Appropriate	Class	Subclass
	1.	4,683,195	7/28/1987	Mullis			
	2.	4,883,867	11/28/1989	Lee			
	3.	5,002,882	3/26/1991	Lunnen			
	4.	5,308,751	5/3/1994	Ohkawa			
	5.	5,455,166	10/3/1995	Walker			
	6.	5,494,810	2/27/1996	Barany et al.			
	7.	5,503,980	4/2/1996	Cantor			
	8.	5,512,439	4/30/1996	Hornes			
	9.	5,512,462	4/30/1996	Cheng			
	10.	5,559,032	9/24/1996	Pomeroy			
	11.	5,589,173	12/31/1996	O'Brien			
	12.	5,599,675	2/4/1997	Brenner			
	13.	5,641,658	6/24/1997	Adams			
	14.	5,716,825	2/10/1998	Hancock et al			
	15.	5,750,341	5/12/1998	Macevicz			
	16.	5,763,175	6/1/1998	Brenner			
	17.	5,830,711	11/3/1998	Barany et al.			
	18.	5,863,753	1/26/1999	Haugland			
	19.	5,912,148	6/15/1999	Eggerding			
	20.	6,013,440	1/11/2000	Lipshutz			
	21.	6,027,889	2/22/2000	Barany et al.			
	22.	6,060,240	5/9/2000	Kamb et al.			
	23.	6,130,073	10/10/2000	Eggerding			
	24.	6,143,496	11/7/2000	Brown			
	25.	6,153,389	11/28/2000	Haarer			
	26.	6,210,891	4/3/2001	Nyren			
	27.	6,210,894	4/3/2001	Brennan			

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.

Substitute Disclosure Form

Substitute Disclosure Form Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 47706-0004026	Application No. 16/988,284
	Applicant Prognosis Biosciences, Inc.		
	Filing Date August 7, 2020	Group Art Unit	

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Filing Date If Appropriate	Class	Subclass
	28.	6,214,587	4/10/2001	Dattagupta			
	29.	6,221,591	4/24/2001	Aerts			
	30.	6,258,558	7/10/2001	Szostak			
	31.	6,258,568	7/10/2001	Nyren			
	32.	6,266,459	7/24/2001	Walt			
	33.	6,281,804	8/28/2001	Haller			
	34.	6,309,824	10/30/2001	Drmanac			
	35.	6,344,316	2/5/2002	Lockhart			
	36.	6,355,431	3/12/2002	Chee			
	37.	6,368,801	4/9/2002	Faruqi			
	38.	6,401,267	6/11/2002	Drmanac			
	39.	6,404,907	6/11/2002	Gilchrist			
	40.	6,416,950	7/9/2002	Lohse			
	41.	6,432,360	8/13/2002	Church et al.			
	42.	6,485,982	11/26/2002	Charlton			
	43.	6,544,732	4/8/2003	Chee			
	44.	6,579,695	6/17/2003	Lambalot			
	45.	6,620,584	9/16/2003	Chee			
	46.	6,632,641	10/14/2003	Brennan			
	47.	6,673,620	1/6/2004	Loeffler			
	48.	6,699,710	3/2/2004	Kononen			
	49.	6,770,441	8/3/2004	Dickinson			
	50.	6,773,886	8/10/2004	Kaufman			
	51.	6,787,308	9/7/2004	Balasubramanian			
	52.	6,800,453	10/5/2004	Labaer			
	53.	6,812,005	11/2/2004	Fan et al.			
	54.	6,828,100	12/7/2004	Ronaghi			

Examiner Signature	Date Considered
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	Applicant Prognosis Biosciences, Inc.	
	Filing Date August 7, 2020	Group Art Unit

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Filing Date If Appropriate	Class	Subclass
	55.	6,833,246	12/21/2004	Balasubramanian			
	56.	6,878,515	4/12/2005	Landegren			
	57.	6,890,741	5/10/2005	Fan et al.			
	58.	6,897,023	5/24/2005	Fu			
	59.	6,911,345	6/28/2005	Quake			
	60.	6,913,921	7/5/2005	Fischer			
	61.	6,969,488	11/29/2005	Bridgham			
	62.	6,969,589	11/29/2005	Patil			
	63.	6,977,033	12/20/2005	Becker			
	64.	7,052,244	5/30/2006	Fouillet			
	65.	7,057,026	6/6/2006	Barnes			
	66.	7,115,400	10/3/2006	Adessi			
	67.	7,118,883	10/10/2006	Inoue			
	68.	7,163,612	1/16/2007	Sterling			
	69.	7,211,414	5/1/2007	Hardin			
	70.	7,229,769	6/12/2007	Kozlov			
	71.	7,255,994	8/14/2007	Lao			
	72.	7,264,929	9/4/2007	Rothberg			
	73.	7,297,518	11/20/2007	Quake			
	74.	7,315,019	1/1/2008	Turner			
	75.	7,329,492	2/12/2008	Hardin			
	76.	7,361,488	4/22/2008	Fan et al.			
	77.	7,378,242	5/27/2008	Hurt			
	78.	7,393,665	7/1/2008	Brenner			
	79.	7,405,281	7/29/2008	Xu			
	80.	7,407,757	8/5/2008	Brenner			
	81.	7,537,897	5/26/2009	Brenner			

Examiner Signature	Date Considered
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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.

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	Applicant Prognosis Biosciences, Inc.		
	Filing Date August 7, 2020	Group Art Unit	

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Filing Date If Appropriate	Class	Subclass
	82.	7,544,473	6/9/2009	Brennan			
	83.	7,579,153	8/25/2009	Brenner			
	84.	7,595,883	9/29/2009	El Gamal			
	85.	7,601,492	10/13/2009	Fu et al.			
	86.	7,601,498	10/13/2009	Mao			
	87.	7,611,869	11/3/2009	Fan			
	88.	7,635,566	12/22/2009	Brenner			
	89.	7,666,612	2/23/2010	Johnsson			
	90.	7,674,752	3/9/2010	He			
	91.	7,700,286	4/20/2010	Stroun et al.			
	92.	7,754,429	7/13/2010	Rigatti			
	93.	7,858,321	12/28/2010	Glezer			
	94.	7,892,747	2/22/2011	Barany et al.			
	95.	7,955,794	6/7/2011	Shen et al.			
	96.	8,003,354	8/23/2011	Shen et al.			
	97.	8,076,063	12/13/2011	Fan			
	98.	8,092,784	1/10/2012	Mao			
	99.	8,148,068	4/3/2012	Brenner			
	100.	8,148,518	4/3/2012	Buchanan			
	101.	8,288,103	10/16/2012	Oliphant			
	102.	8,337,851	12/25/2012	Aukerman			
	103.	8,343,500	1/1/2013	Wraith			
	104.	8,383,338	2/26/2013	Kitzman			
	105.	8,460,865	6/11/2013	Chee			
	106.	8,481,257	7/9/2013	Van Eijk			
	107.	8,481,292	7/9/2013	Casbon			
	108.	8,586,310	11/19/2013	Mitra			

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.

Substitute Disclosure Form

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 47706-0004026	Application No. 16/988,284
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Prognosis Biosciences, Inc.	
		Filing Date August 7, 2020	Group Art Unit
(37 CFR §1.98(b))			

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Filing Date If Appropriate	Class	Subclass
	109.	8,614,073	12/24/2013	Van Eijk			
	110.	8,685,889	4/1/2014	Van Eijk			
	111.	8,741,606	6/3/2014	Casbon			
	112.	8,778,849	7/15/2014	Bowen			
	113.	8,785,353	7/22/2014	Van Eijk			
	114.	8,815,512	8/26/2014	Van Eijk			
	115.	8,835,358	9/16/2014	Fodor			
	116.	8,865,410	10/21/2014	Shendure			
	117.	8,895,249	11/25/2014	Shen			
	118.	8,911,945	12/16/2014	Van Eijk			
	119.	8,936,912	1/20/2015	Mitra			
	120.	8,951,781	2/10/2015	Reed			
	121.	9,023,768	5/5/2015	Van Eijk			
	122.	9,062,348	6/23/2015	Van Eijk			
	123.	9,080,210	7/14/2015	Van Eijk			
	124.	9,085,798	7/21/2015	Chee			
	125.	9,121,069	9/1/2015	Lo			
	126.	9,194,001	11/24/2015	Brenner			
	127.	9,290,808	3/22/2016	Fodor			
	128.	9,290,809	3/22/2016	Fodor			
	129.	9,328,383	5/3/2016	Van Eijk			
	130.	9,334,536	5/10/2016	Van Eijk			
	131.	9,340,830	5/17/2016	Lipson			
	132.	9,371,598	6/21/2016	Chee			
	133.	9,376,716	6/28/2016	Van Eijk			
	134.	9,376,719	6/28/2016	Van Eijk			
	135.	9,404,156	8/2/2016	Hicks			

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.

Substitute Disclosure Form

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 47706-0004026	Application No. 16/988,284
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Prognosis Biosciences, Inc.	
		Filing Date August 7, 2020	Group Art Unit
(37 CFR §1.98(b))			

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Filing Date If Appropriate	Class	Subclass
	136.	9,447,459	9/20/2016	Van Eijk			
	137.	9,453,256	9/27/2016	Van Eijk			
	138.	9,493,820	11/15/2016	Van Eijk			
	139.	9,506,061	11/29/2016	Brown			
	140.	9,574,230	2/21/2017	Van Eijk			
	141.	9,593,365	3/14/2017	Frissen et al.			
	142.	9,657,335	5/23/2017	Van Eijk			
	143.	9,670,542	6/6/2017	Van Eijk			
	144.	9,702,004	7/11/2017	Van Eijk			
	145.	9,745,627	8/29/2017	Van Eijk			
	146.	9,777,324	10/3/2017	Van Eijk			
	147.	9,850,536	12/26/2017	Oliphant et al			
	148.	9,896,721	2/20/2018	Van Eijk			
	149.	9,898,576	2/20/2018	Van Eijk			
	150.	9,898,577	2/20/2018	Van Eijk			
	151.	10,023,907	7/17/2018	Van Eijk			
	152.	10,030,261	7/24/2018	Frissen et al.			
	153.	10,095,832	10/9/2018	Van Eijk			
	154.	2001/0055764	12/1/2001	Empendocles et al			
	155.	2002/0040275	4/4/2002	Cravatt			
	156.	2002/0048766	4/25/2002	Doyle et al.			
	157.	2002/0064779	5/30/2002	Landegren			
	158.	2002/0137031	9/26/2002	Wolber			
	159.	2002/0150909	10/17/2002	Stuelpnagel			
	160.	2002/0164611	11/7/2002	Bamdad			
	161.	2002/0168645	11/14/2002	Taylor			
	162.	2003/0040035	2/27/2003	Slamon			
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.							

Substitute Disclosure Form

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Attorney Docket No. 47706-0004026	Application No. 16/988,284
	Applicant Prognosis Biosciences, Inc.	
	Filing Date August 7, 2020	Group Art Unit

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Filing Date If Appropriate	Class	Subclass
	163.	2003/0064398	4/3/2003	Barnes			
	164.	2003/0087232	5/8/2003	Christians			
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(37 CFR §1.98(b))			

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		Filing Date August 7, 2020	Group Art Unit
(37 CFR §1.98(b))			

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

Under 35 U.S.C. § 120, this application relies on the earlier filing date of application 16/414,213, filed on 5/16/2019, application number 16/402,098, filed on 5/2/2019, application number 16/276,235, filed on 2/14/2019, application number 15/187,661, filed on 6/20/2016 and application number 13/080,616, filed on 4/5/2011. The documents corresponding to Desig. ID Nos. 154, 253, 255, 273, 317, and 360 have not been submitted to and/or cited by the Office in the prior application. All other listed documents were submitted to and/or cited by the Office in the prior application, and so copies of those documents are not provided in this application.

This statement is being filed within three months of the filing date of the application or before the receipt of a first action on the merits.

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.

Respectfully submitted,

Date: August 13, 2020

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(54) **Tissue cassette for transporting**

(57) A cassette (100') for transporting tissue including a first flat reference porous structure (116') for supporting the tissue, and a second porous structure (128') having a compression resistance. The first flat reference

porous structure (116') and the second porous structure (128') can be positioned into an abutting, contacting or non-contacting position for securing the tissue therebetween.

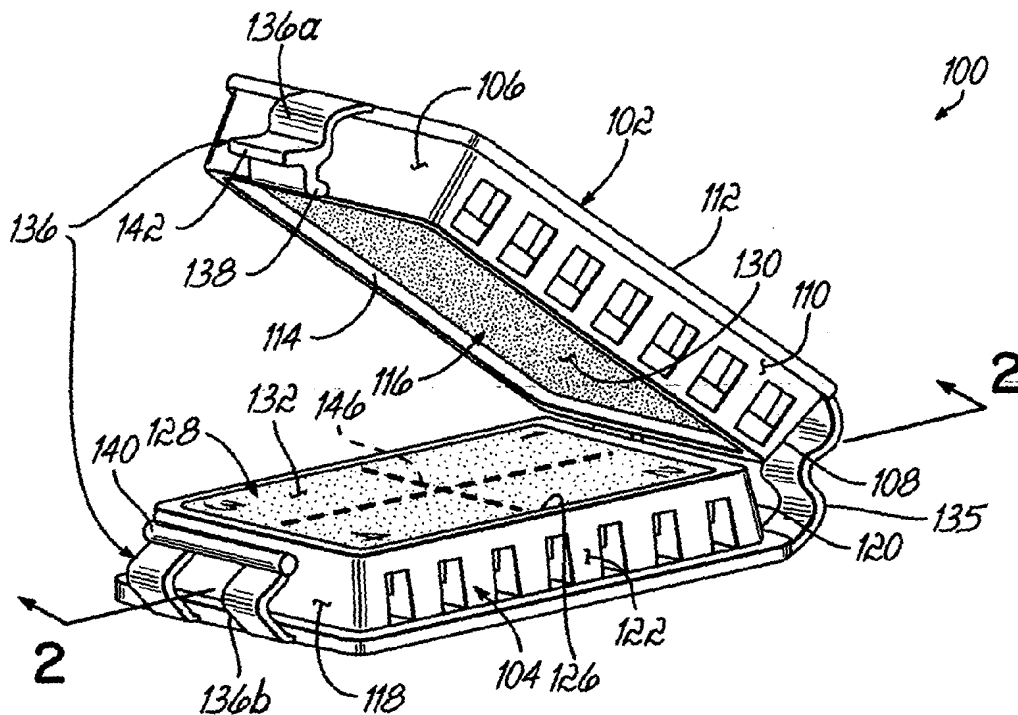


FIG. 1

EP 1 782 737 A1

Description

[0001] The present invention relates to devices for the transport of tissue from a harvesting site to a pathology lab, more particularly, but not exclusively, to a tissue cassette for transporting tissue from a harvesting site to a pathology lab.

[0002] Screening tissue samples for disease is an extremely common practice in modern medicine. Otherwise known as a biopsy, a patient has tissue samples harvested from their body by a physician or other medical professional and then transported to a pathology lab in a container filled with tissue preservative for slide preparation, review, and diagnosis. When tissue is deposited unrestrained into tissue preservative solution it can curl and contort as it hardens. Some examples of tissue types that are prone to curl are colon tissue and skin tissue, however, other types of tissue curl or distort as well.

[0003] More specifically, after the physician or other medical professional has harvested the tissue and obtained the biopsy sample, the biopsy sample is then placed into what is known as a "fixing solution" preservative solution. The fixing or preservative solution is commonly a solution of buffered formaldehyde known as formalin. For example, a biopsy sample is commonly harvested by using a sharp, hollow needle to gather tissue inside the lumen of the needle. Accordingly, the biopsy samples are commonly long and skinny and rather snake-like. The preservative solution will kill the pathogens to protect the safety of the pathology lab workers. The tissue can curl up into a ball or take on other three dimensional contortions. Therefore, by the time the biopsy samples have arrived at the pathology lab, they may have hardened or semi-hardened into contorted shapes.

[0004] The tissue samples must be embedded in a paraffin block for sectioning and subsequent diagnosis. The biopsy samples must be reconfigured to be perfectly flat in the paraffin mold. Any contorted or curled biopsy sample must be straightened before embedding in the paraffin. Without straightening the tissue sample, a misdiagnosis could occur for reasons to be discussed below. Straightening these biopsy samples is difficult because a high level of precision is necessary and the size of the sample is often extremely small.

[0005] After the sample has been embedded, a microtome is used to slice very thin sections of the biopsy sample and paraffin combination. The average section is usually 3 μ m to 5 μ m thick. Usually, the technologist will take no more than about thirty slices into the biopsy and paraffin combination. The total depth into the paraffin of all of these combined sections is around 0.001 inch. Therefore, if the biopsy sample is not correctly repositioned to be perfectly flat before it is embedded in the paraffin, it is quite possible that a portion of the biopsy sample will never be sectioned and thus excluded from the pathologist's subsequent examination.

[0006] Many times, landmark indicators are used when taking biopsy samples. Landmark indicators indicate to

the medical professional the location of harvest relative to the patient's body. The landmark indicators ensure that a follow-up can be accurately planned during a subsequent surgery, in staging of the tumor, etc. Sutures have been secured to the biopsy sample to provide one type of landmark indicator. However, sutures can be difficult and time consuming to apply. Currently, there is a need for a less time-consuming and more accurate manner to identify the orientation of the sample in relation to the patient's anatomy.

[0007] One aspect of the invention is a cassette for holding tissue. The cassette includes a first flat reference porous structure for supporting the tissue. The cassette also includes a second porous structure having a compression resistance. The first flat reference porous structure and the second porous structure can be positioned into an abutting position for securing the tissue therebetween.

[0008] Another aspect of the invention is a method for improving the quality of sections of a biopsy sample. The method includes placing the biopsy sample into a tissue cassette and moving at least a portion of the tissue cassette to apply a compressive force to flatten the biopsy sample against a flat reference surface of the tissue cassette.

[0009] The invention will now be described by way of example with reference to the accompanying drawings in which:

FIG. 1 is a perspective view of a tissue cassette in an open position according to one embodiment.

FIG. 2 is a cross-sectional view of the tissue cassette taken generally along line 2-2 in FIG. 1 but shown in a closed or latched position.

FIG. 3 is a perspective cross-sectional view of the tissue cassette also taken generally along line 2-2 of FIG. 1 and illustrating the cassette in an open position.

FIGS. 4A-C are top plan views of a landmark indication system in different uses with the tissue cassette of FIG. 1.

FIGS. 5A and 5B are illustrations of respective first and second microscope slides comparing biopsy samples that had been previously held in a conventional biopsy container (FIG. 5A) and samples held with the tissue cassette of FIG. 1 (FIG. 5B).

FIGS. 6A and 6B are further illustrations of respective first and second microscope slides comparing biopsy samples that had been previously held in a conventional biopsy container with formalin (FIG. 6A) and samples held with the tissue cassette of FIG. 1 (FIG. 6B).

FIG. 7A illustrates a tissue sample obtained from a conventional tissue transporting or biopsy container, with the tissue positioned inside of a paraffin mold prior to embedding and sectioning.

FIG. 7B illustrates a section of the tissue sample of FIG. 7A positioned upon a slide for diagnosis by a medical professional.

FIG. 8A illustrates a tissue sample obtained from the tissue cassette of FIG. 1, with the tissue positioned inside of a paraffin mold prior to embedding and sectioning.

FIG. 8B illustrates a section of the tissue sample of FIG. 8A positioned upon a slide for diagnosis by a medical professional.

FIG. 9 is a perspective view of a tissue cassette according to another embodiment and shown in a closed position.

FIG. 10 is a perspective view of the tissue cassette shown in FIG. 9, but illustrated prior to the securement of respective porous membranes.

FIG. 11 is a cross sectional view taken along line 11-11 of FIG. 9.

FIG. 11A is an enlarged view of encircled portion 11A shown in FIG. 11.

[0010] The present application is directed to a cassette for transporting tissue after a biopsy has been performed. Generally, the cassette includes two opposing porous surfaces for fixing or holding tissue samples therebetween.

[0011] Referring now to the drawings, FIG. 1 illustrates a tissue cassette 100. The tissue cassette 100 is constructed and arranged to hold biopsy tissue samples after they have been removed from the patient and before the pathologist processes them for inspection. The tissue cassette 100 is therefore designed to be placed in another container (not shown) adapted to hold a tissue preservative solution, such as a formalin solution, such that the solution can fully contact the tissue cassette 100 and any tissue sample(s) therein. The tissue cassette 100 includes a first perforated structure or frame 102 and a second perforated structure or frame 104. The terms "perforated" and "porous" are used herein as analogous or synonymous terms meant to convey the fact that fluid solution can reach the tissue through the pores or perforations of the structure. As illustrated in FIG. 1, the first perforated structure 102 and the second perforated structure 104 are generally of the same size and are positioned opposite from one another. This arrangement allows the first perforated structure 102 and the second perforated structure 104 to easily be brought together to apply a

compressive force to the biopsy sample after it has been inserted therein. The first perforated structure 102 includes a forward surface 106, a rearward surface 108, and two side surfaces 110. It also includes an outer surface 112. The rearward surface 108, and any other surface, can have multiple minor surfaces that taken together comprise the surface. These five surfaces 106, 108, 110, and 112 combine together to define an interior area 114 that contains a first porous structure 116. In some embodiments, the porous structure 116 is a foam pad, however, in other embodiments other types of materials may be used. Some types of materials for the foam pad are polyester or polyurethane with an open cell reticulated structure. Furthermore, special foams with hydrophilic properties could be used to assure wetting of intricate samples. In addition, while one porous structure 116 is illustrated inside the interior area 114 in FIG. 1, two or more porous structures can be used in other embodiments. In this illustrated embodiment, the interior area 114 is substantially box shaped, however, in other embodiments the first perforated structure 102 may have an interior area 114 that is different in configuration. For example, the interior area 114 could be an oval shape, a cylindrical shape, a rectangular shape, a square shape, or any other area readily apparent to those skilled in this art. Accordingly, the interior area 114 may be sized to receive a variety of biopsy sample sizes.

[0012] Similarly, the second perforated structure or frame 104 includes a forward surface 118, a rearward surface 120, side surfaces 122, and an outer surface 124. The combination of all these surfaces 118, 120, 122, and 124 defines an interior area 126 that contains a second porous structure 128 that may be constructed out of foam or other materials. The perforated structures 102, 104 can be constructed out of a variety of materials. In the illustrated embodiment, the perforated structures 102, 104 are constructed out of a plastic material, such as a high-density polyethylene (HDPE) or Acetel. In other embodiments, however, other materials may be used. In addition, the porous structures 116, 128 may include porous membranes 130 and 132. These porous membranes 130, 132 are constructed and arranged to avoid damage to the tissue sample(s) and to minimize or prevent artifacts in the tissue sample(s) so extremely small biopsy samples, such as those less than 0.5 mm in size, can be effectively secured in the tissue cassette 100. In the illustrated embodiment, the porous membranes 130, 132 may be formed out of lens paper or filter paper, however, those skilled in the art will recognize that other materials can be used in other embodiments. For example, the porous membranes 130, 132 can be a porous material such as a thermoplastic porous film or netting, a woven or non-woven material made from cotton, or other natural or synthetic fiber materials or other suitable materials. One suitable material is sold by Delstar Technologies, Inc., Middletown, Delaware, under the name Delnet® and is an apertured film or netting formed from high density polyethylene, having a flat surface facing the tis-

sue samples. One or both membranes 130, 132 may be eliminated as long as undesirable artifacts are not formed on the biopsy sample(s) by the porous structures 116, 128. The membranes 130, 132 may be about 0.001 inch thick to allow unimpeded fixing fluid access and wicking action to the tissue surface. In addition, the membranes 130, 132 have a porosity of about 100 μm to about 400 μm some having porosity closer to about 200 μm . The membranes 130, 132 should remain taut and should remain temperature, moisture, and reagent stable. In addition, the membranes 130, 132 should not degrade when placed in the reagent solution, such as a formalin solution, and may be designed or formulated so as not to degrade when exposed to the chemicals used in the tissue processing. Furthermore, the membranes 130, 132 may be heat staked to the porous structures 116, 128 or the perforated structures 102, 104 or may be fixed in place by any other suitable method. The membranes 130, 132 need not be the same material. For instance, one of the membranes 130, 132 can be thinner and more compliant and conform to undulations in the biopsy sample. In addition, one of the membranes 130, 132 and the corresponding porous structure 116, 128 may be simply trapped in its perforated structure 102, 104 thereby allowing a free-floating configuration particularly adapted to conform to thick and thin tissue if necessary. In sum, these artifact inhibiting or minimizing porous membranes 130, 132 are sufficiently compliant to avoid damaging the biopsy sample, but sufficiently firm for flattening the biopsy sample before embedding in a material, such as paraffin.

[0013] The porous structures 116, 128 may have differing levels of compression resistance. Some embodiments, however, have porous structures 116, 128 that have the same level of compression resistance. Typically, the compression resistance ranges between about 0.5 lbs/in² to about 4 lbs/in² at about 50% compression. One porous structure 116 or 128 may be more compliant and have a compression resistance of about 0.5 lbs/in². The other porous structure 116 or 128 may be less compliant with a compression resistance of about 2-4 lbs/in². The porous structure 116 or 128 having the higher compression resistance can also be known as the reference structure and its corresponding porous membrane 130, 132 can be known as the reference surface. The typical porosity of the porous structures 116, 128 is around 0.020" to 0.025" open cell pores. In addition, in some embodiments, polyurethane foam with a hydrophilic-formulation can be used without a porous membrane if the wetting is great enough and the pore size is small enough. Moreover, the porous structures 116, 128 should be formed out of a material that does not degrade in the formalin or, in some cases, the chemicals used in the tissue processing. The smaller pore size would trap the biopsy sample in the tissue cassette 100 and the enhanced wetting properties would overcome the potential disadvantage of the smaller pore size.

[0014] In the illustrated embodiment, the second po-

rous structure 128 has more compression resistance than the first porous structure 116. Accordingly, when a biopsy sample is introduced into the tissue cassette 100, the porous structure 128 having higher compression resistance will provide resistance to deformation upon closing of the tissue cassette 100. Therefore, the flat surface of the biopsy sample will be created along the surface of the biopsy sample that is in contact with the porous membrane 132 and the second porous structure 128.

[0015] The tissue cassette 100 also includes a connector system made up of a compliant hinge 135 and a clasp 136. Those skilled in the art, however, recognize that other connector systems can be used in other embodiments. For example, in one alternative embodiment, the connector system could be two clasps that lock together on either side of the tissue cassette 100. Moreover, in another embodiment, the connector system 134 could be elastic bands that wrap around the periphery of the perforated structures 102, 104. Accordingly, any structure that can be used to urge the porous structures 116, 128 together is contemplated. In addition, not all tissue cassettes require a connector because the resilient porous structures 116, 128 could apply sufficient force in some other manner not requiring a connector.

[0016] The compliant hinge 135 is coupled to the first perforated structure 102 at the rearward surface 108. The compliant hinge 135 is also coupled to the second perforated structure 104 at the rearward surface 120. This arrangement provides a "clam shell" like design that allows the first perforated structure 102 and the second perforated structure 104 to be easily separated from one another and to easily clamp down. Moreover, this arrangement allows for a maximum separation between the forward end surfaces 106 and 118 of the first and second perforated structures 102 and 104. This arrangement enables a user to easily place the biopsy sample inside the interior areas 114 and 126 using the harvesting instrument, and, if necessary, a portion of the medical professional's hand.

[0017] The clasp 136 illustrated in FIG. 1 is one structure that can assist in coupling the perforated structures 102, 104, however, those skilled in the art recognize that other mechanisms can be used so long as they provide for a compressive force to be applied to the biopsy sample upon closure. The clasp 136 of the illustrated embodiment has a top portion 136a and a bottom portion 136b. The top portion 136a provides a latch 138 at the end of the top portion 136a. This latch 138 slides over a bar 140 located on the bottom portion 136b, and then retracts around the bar 140 to firmly lock into place. In addition, the top portion 136a includes a tab 142 which allows the medical professional to easily open the tissue cassette 100 when needed by simply pressing their thumb or finger to the underside of the tab 142 in order to retract the latch 138 from underneath the bar 140. The clasp 136 forces the opposing porous surfaces 116, 128 together so as to apply a compressive force to the biopsy sample that is placed therein. The clasp 136 also includes the latch

138 and bar 140 to maintain that compressive force constantly and uniformly until the tissue cassette 100 is opened and the biopsy sample is ready to be embedded in a material, such as paraffin.

[0018] FIGS. 2 and 3 respectively illustrate the biopsy cassette 100 in closed and open positions. Outer surface 112 is shown to have a plurality of pores 144. These pores 144 allow for introduction of the fixing solution, such as formalin. The porous structures 116, 128 allow the fluid to reach the porous membranes 130, 132 so that the biopsy sample can become fixed in its flattened and undamaged state. Therefore, the tissue cassette 100 prepares the biopsy sample for inspection.

[0019] In use, the tissue cassette 100 operates to properly flatten and leave undamaged a biopsy sample placed therein. Initially, a medical professional performs a biopsy on a patient to obtain a biopsy sample. This is usually done using a needle or other hollow instrument in order to obtain a biopsy sample inside of the lumen defined in the needle or other instrument. The biopsy sample is then taken directly to the tissue cassette 100 where it is placed on one of the porous membranes 130, 132. At this point, the biopsy sample may not be flat. It could be coiled or contorted in any number of configurations.

[0020] The tissue cassette 100 is then closed and the porous membranes 130, 132 may apply uniform and constant pressure to the biopsy sample. The differences in compression resistance between the porous structures 116, 128 result in one of the sides of the biopsy sample becoming flattened. The second porous structure 128 having a higher compression resistance will not compress substantially and the first porous structure 116 will compress so as not to damage the biopsy sample and introduce artifact therein. The first porous structure 116 may surround all sides of the tissue sample except the side which is against the second porous structure 128. Flattening of one side of the tissue sample can occur due to one of the porous membranes 130 or 132 being taut. In addition, the other porous membrane 130 or 132 can be free-floating inside of its perforated structure 102, 104, either by not being heat staked and simply resting upon a porous structure 116 or 128 or by being fixed only to the porous structure 116 or 128 and not fixed to the perforated structure 102, 104. Likewise, the porous structure 116 and/or 128 may or may not be fixed to the associated perforated structure 102, 104. Accordingly, the biopsy sample may be flattened and fixed to prevent curling or distortion of the biopsy sample and a loss of visible margins.

[0021] Tissue cassette 100 may be immersed in a container filled with a fixing solution. The biopsy sample can then be hardened with one side flat and without visible artifact. Having a flat surface can be especially important for skin biopsy samples. The tissue cassette 100 can thereafter be stored until it is ready to be opened by a medical professional. Use of tissue cassettes 100 may allow a biopsy sample to be held flat, for example, within less than a 0.0025 inch variance. This level of precision

can be important for skin tissue biopsies because the samples do not curl up and distort the margins for excision of malignant tissue. Therefore, when the technologist introduces the biopsy sample into the paraffin and then makes slices using a microtome, the possibility that an incorrect diagnosis due to curling or distortion of the biopsy sample is reduced because the margin intended by the surgeon or other medical professional taking the sample is properly preserved throughout the histological process. The pathologist has the assurance that the margin that will be delivered back to the harvesting medical professional will be as the medical professional intended.

[0022] Referring now to FIGS. 4A-C, a landmark indication system 146 may be used on, for example, the porous membrane 132 associated with the porous structure 128 of greater compression resistance. The medical professional that harvests the biopsy sample places the biopsy sample on the porous membrane 132 and uses the landmark indication system 146 to communicate the anatomic position of the harvested tissue or other information concerning the biopsy sample. The landmark indication system 146 includes four quadrants 148 that are identified using labels 150. Illustrative examples are provided in FIGS. 4A-C discussed below. FIGS. 4A-C illustrate some uses of the landmark indication system 146, however, those skilled in this art recognize that the landmark indication system 146 can be used in other manners.

[0023] Referring now to FIG. 4A, the landmark indication system 146 is used with biopsy samples 152 and 154 taken from a prostate gland. The biopsy samples 152 and 154 represent prostate cores harvested by a medical professional during a biopsy. Biopsy samples 152 illustrate cores harvested from the left side of the prostate gland. Accordingly, the "Left" label 150 is circled to make this indication. Similarly, the biopsy samples 154 are taken from the right side of the prostate gland and the "Right" label 150 is accordingly circled. Thus, the histotechnician or other medical profession will be able to label the paraffin sections to accurately reflect the area of the prostate where the cores were harvested.

[0024] FIG. 4B illustrates another use of the landmark indication system 146. In the center of the landmark indication system 146 is a skin tissue sample 156 taken from a finger. The skin tissue sample 156 includes a lesion 158. The orientation of the skin tissue sample 156 is identified by circling the "Proximal" and "Distal" labels 150 as illustrated in FIG. 4B. Again, this system enables the medical professional harvesting the skin tissue sample 156 to easily communicate the orientation of the skin sample, relative to the proximal and distal ends of the patient's finger. Thus, the likelihood of error decreases.

[0025] FIG. 4C illustrates the landmark indication system 146 for use with taking a multitude of biopsy samples 160. The tissue cassette 100 will keep the biopsy samples 160 in place once it is closed. Thus, the medical professional, such as a surgeon, can create a surgical report and make notes under the heading of quadrant

"1" corresponding to the label 150 of "1" that is circled. Such notes can describe characteristics of those samples. For instance, assume that the samples 160 in the quadrant 148 having the "1" label 150 are all from the pancreas. In addition, assume a biopsy was also taken from the gall bladder, kidney, and liver. The samples 160 can be organized in the quadrants 148 and then the labels 150 corresponding to the numbers can be circled. The surgical report can be written by the surgeon to provide information noting that the samples 160 in the quadrant 148 with the label 150 having "1" were taken from the pancreas, those under "2" from the gall bladder, and so forth. Thus, the landmark indication system 146 can provide information to the pathologist or histotechnician in many different manners.

[0026] The pathologist and the histotechnician can use information from labels 150 communicated by the medical professional when subsequently preparing the gross description. The gross description is prepared by the histotechnician or the pathologist opening the tissue cassette 100 and observing the number, placement, size, and/or anatomic orientation indicated by the medical professional who harvested the tissue. Subsequently, the tissue cassette 100 can be closed for further tissue processing without the need for additional manipulation of the biopsy sample before embedding. The tissue cassette 100 completely preserves the orientation of the biopsy sample throughout the entire tissue fixing process.

[0027] To perform an embedding process the histotechnician or the pathologist removes the biopsy sample from the tissue cassette 100. The landmark indication system 146 makes the anatomic harvest position easily identifiable and able to be oriented and processed into standard embedding molds. Alternatively, when using a system having a sectionable cassette, the tissue cassette 100 keeps the biopsy sample flat and indicates the harvested orientation of the biopsy samples using the landmark indicator system 146. The tissue is prefixed and substantially hardened before removing it from the tissue cassette 100 and placing it into a sectionable cassette, other support, embedding medium mold, etc.

[0028] Referring now to FIGS. 5A and 5B, and FIGS. 6A and 6B, first slides 162, 164 shown respectively in FIGS. 5A and 6A illustrate biopsy samples taken from a conventional tissue preservative container and freely submerged in the preservative solution. The second slides 166, 168 shown respectively in FIGS. 5B and 6B illustrate biopsy samples taken from the tissue cassette 100 which was then submerged in the preservative solution. The second slides 166, 168 illustrate samples that are defined and have full width. The robustness of the sections of the slide is improved. Accordingly, the tissue cassette 100 greatly reduces the margin of error during embedding a biopsy sample for later diagnosis.

[0029] FIG. 7A illustrates a mold 170 for holding a biopsy sample 172 during embedding with a material such as paraffin wax. The mold 170 receives the biopsy sample 172 after sample 172 has hardened into the config-

uration illustrated. The biopsy sample 172 is convoluted and includes high points 174 and low points 176. After embedding the biopsy sample 172 in paraffin wax, a plurality of sections can be taken through the biopsy sample 172 using a microtome. The line 178 illustrates the most common area that a section will be taken through the biopsy sample 172. The biopsy sample 172 includes a lesion 180, such as a group of cancerous cells. The line 178 is below the lesion 180. Referring to FIG. 7B, the section taken through line 178 is illustrated as being placed upon a slide 182. Three sections of the tissue sample 172 are located on the slide 182. The first section 172a corresponds to the length of the biopsy sample 172 between points A and B illustrated in FIGS. 7A and 7B. The second section 172b corresponds to the length of the biopsy sample 172 between points C and D. The third section 172c corresponds to the length of the biopsy sample between points E and F. The lesion 180 does not appear because it is between points D and E close to a high point 174. Accordingly, the diagnosis will be inaccurate.

[0030] Referring now to FIG. 8A, a biopsy sample 172' has been taken from the tissue cassette 100 of FIG. 1 and placed into a mold 170. After placing the biopsy sample 172' into the mold 170, the biopsy sample 172' is embedded in a material such as paraffin wax. Sections are then taken with a microtome, such as through line 178. The tissue cassette 100 of FIG. 1 has formed the biopsy sample 172' flat so the line 178 passes through the lesion 180'. FIG. 8B illustrates that the medical professional places the section taken through line 178 upon the slide 182 in preparation for diagnosis. The biopsy sample 172' has a flat section 172a' that is visible along the entire length of the biopsy sample 172'. Accordingly, the section of the lesion 180a' appears inside of the flat section 172a' and will be discovered by the pathologist or other medical professional. Thus, the tissue cassette 100 helps ensure that the proper diagnosis of a biopsy sample 172 is performed.

[0031] Referring now to FIGS. 9-11 and 11A, a tissue cassette 100' is shown according to a second illustrative embodiment. The tissue cassette 100' may include any of the features discussed above with respect to the first embodiment. Certain differences exist between the first and second embodiments as will be apparent from the following description and a review of the respective drawing figures. Like reference numerals with prime marks (') are used to illustrate corresponding elements in the first and second embodiments with structural and/or functional differences being apparent from a review of the respective drawing figures, the written description, or both. The tissue cassette 100' comprises first and second perforated structures, including a lid 102' and a base 104' which may be connected by a hinge 135' at one end and a clasp structure 136' at the opposite end. Apertures 144' are provided to allow fluid flow into the cassette 100'. The clasp structure may comprise a projection 136a' on the lid 102' received within a recess 136b' on the base 104'.

The lid 102' also includes a second clasp structure 236 adjacent to the hinge 135'. This clasp structure 236 comprises a projection 236a and a recess 236b as best shown in FIG. 10 in the open position and FIG. 11A in the closed position. As the lid 102' is closed, the hinge, which is frangible, breaks and the clasp structure 236 engages to hold the lid 102' to the base 104' in the closed position along with clasp structure 136' as shown in FIGS. 11 and 11A. Sidewalls 240, 242 of the lid 102' are received within complementary receiving areas 246, 248 of the base 104' (FIG. 10). Therefore, in the closed position shown in FIG. 9, tissue samples are prevented from escaping from the sides of the cassette 100' as well as the ends of the cassette 100' having the respective clasp structures 136', 236. A tab 200 on the lid 102' may be used to apply upward force on the lid 102' to decouple the clasp 136'.

[0032] As shown in FIGS. 11 and 11A, porous membranes 130', 132' may be heat staked to the perforated structures 102', 104' through the use of projections 202. These projections 202 are shown in FIG. 10 prior to the heat staking operation as small cylindrical elements. After heat staking, the cylindrical elements 202 form mushroom-shaped heads which retain the edges of the membranes 130', 132' against the upper surfaces of the porous structures or foam 116', 128'. As further shown in FIGS. 11 and 11A, a fluid path may be formed completely between the first and second perforated structures 102', 104' such that fluid (such as formalin) may travel between the lid 102' and base 104' in the direction of the arrow 203 shown in FIG. 11A. The fluid path extends between additional portions 102a', 102b', 104a', 104b' of the perforated structures 102', 104' and between the "mushroomed" projections 202. Projections 202 may be staggered relative to each other when the lid 102' is closed instead of aligned as shown in FIG. 11A. This fluid path allows full saturation of the tissue sample(s). A spacing 204 is also shown between the upper and lower porous membranes 130', 132'. The spacing 204 may or may not be present depending on the needs of a particular application. For example, larger tissue samples may require a spacing 204 of any suitable dimension that will still allow the sample to be properly held, while smaller specimens may require no spacing 204.

[0033] It should further be noted that a nonstick coating may be applied to one or both of the porous membranes 130', 132' such that the tissue sample or samples may be easily removed from the surface 130' or 132' having a nonstick coating. Alternatively, the membrane material itself may comprise a nonstick-type material such as PTFE. As another alternative, one or more tissue samples may be adhesively secured on one of the porous membranes 130' or 132' so as to retain the sample(s) on the membrane 130' or 132'. After fixing with a fluid such as formalin, the membrane having the adhesively secured tissue sample or samples may be cut out of the cassette 100' and placed in the bottom of an embedding mold or sectionable cassette for embedding in a material such as paraffin. Whether the tissue sample or samples

are adhesively secured to one of the membranes or not, the lid 102' may be entirely removed from the base 104' and discarded either before or after the biopsy sample or samples are retrieved. The biopsy sample or samples may be placed in the bottom of a conventional paraffin mold and the mold may be filled with molten liquid paraffin. While the paraffin is still molten, the base 104' may be placed into contact with the paraffin. The paraffin then cools and hardens. The base 104' may then be used as a fixture for retention in a microtome chuck. A microtome operation may then be performed on the hardened paraffin and slide preparation and analysis may take place. **[0034]** While these embodiments have been described in considerable detail, additional advantages and modifications will readily appear to those skilled in the art.

Claims

1. A cassette for holding tissue comprising a first flat reference porous structure for supporting the tissue, a second porous structure having a compression resistance, wherein the first flat reference porous structure and the second porous structure can be positioned into an abutting position for securing the tissue therebetween.
2. The cassette of claim 1 further comprising a second perforated structure defining an interior area containing the second porous structure therebetween.
3. The cassette of claim 2 wherein a connector connects said first flat reference porous structure and said second perforated structure and compresses said structures together to firmly trap the tissue therebetween.
4. The cassette of claim 3 wherein said connector connects said first flat reference porous structure and said second perforated structure using a clasp and a hinge.
5. The cassette of any one of claims 2 to 4 wherein the first flat reference porous structure and said second perforated structure are arranged to form a clam shell type configuration.
6. The cassette of any preceding claim further comprising a first perforated structure defining an interior area containing the first flat reference porous structure having a second compression resistance.
7. The cassette of any preceding claim wherein said second porous structure includes foam to provide a predetermined compression resistance.
8. The cassette of any preceding claim wherein said second porous structure include a non-artifact induc-

ing porous membrane.

9. The cassette of any preceding claim wherein the compression resistance of the first flat reference porous structure is greater than the compression resistance of the second porous structure. 5
10. The cassette of any preceding claim wherein one of the porous structures has a landmark indication system inscribed thereon. 10
11. A method for holding a tissue sample, comprising placing the biopsy sample into a tissue cassette, and moving at least a portion of the tissue cassette to apply a compressive force to flatten the biopsy sample against a flat reference surface of the tissue cassette. 15
12. The method of claim 11 wherein the tissue sample is placed into the tissue cassette on a landmark indication system corresponding to a location of harvest of the tissue sample. 20
13. The method of claim 12 further including marking a label on the landmark indication system corresponding to the location or orientation of harvest of the tissue sample from the patient. 25
14. The method of any one of claims 11 to 13 wherein moving the tissue cassette occurs by rotating a portion of the tissue cassette around a pivot. 30
15. The method of any one of claims 11 to 14 further comprising flattening the tissue sample between the flat reference surface and a compressive, porous structure. 35

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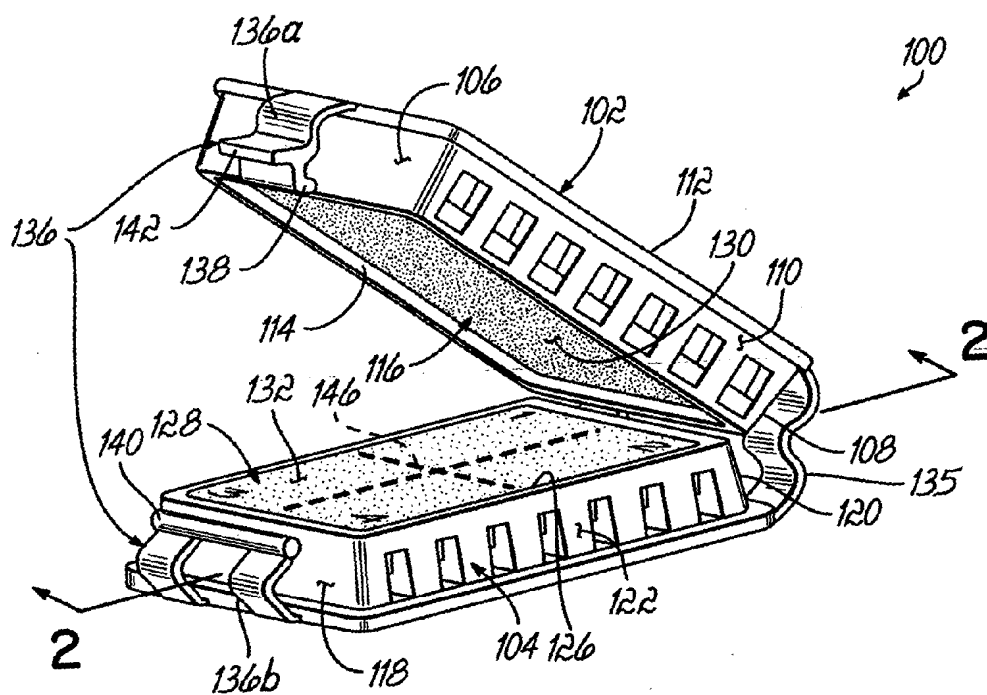


FIG. 1

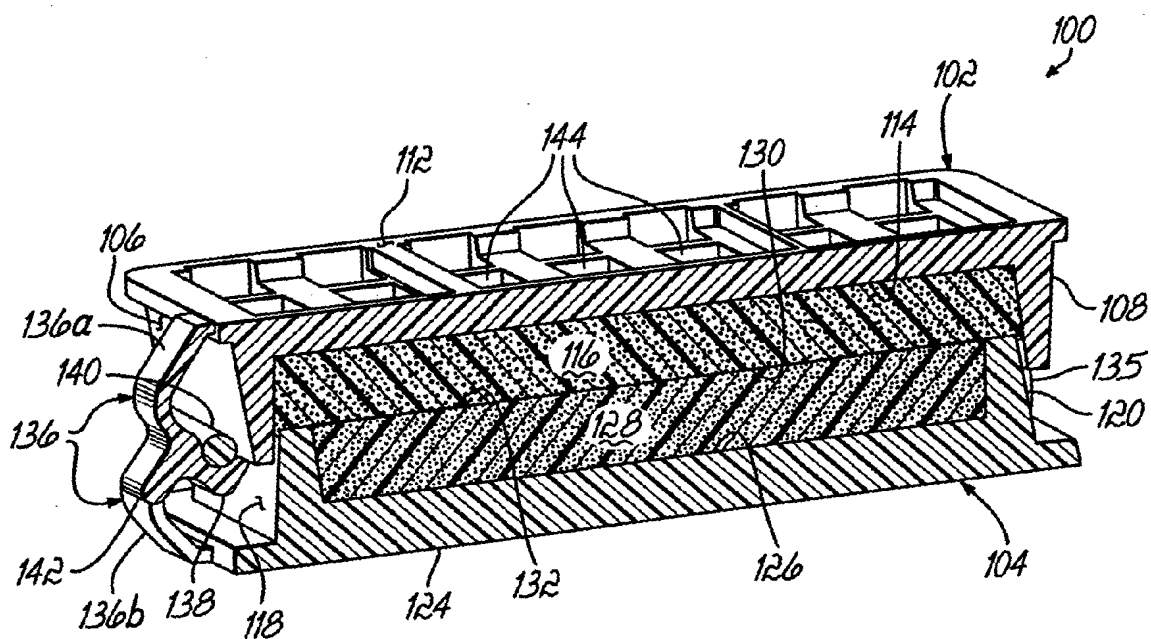


FIG. 2

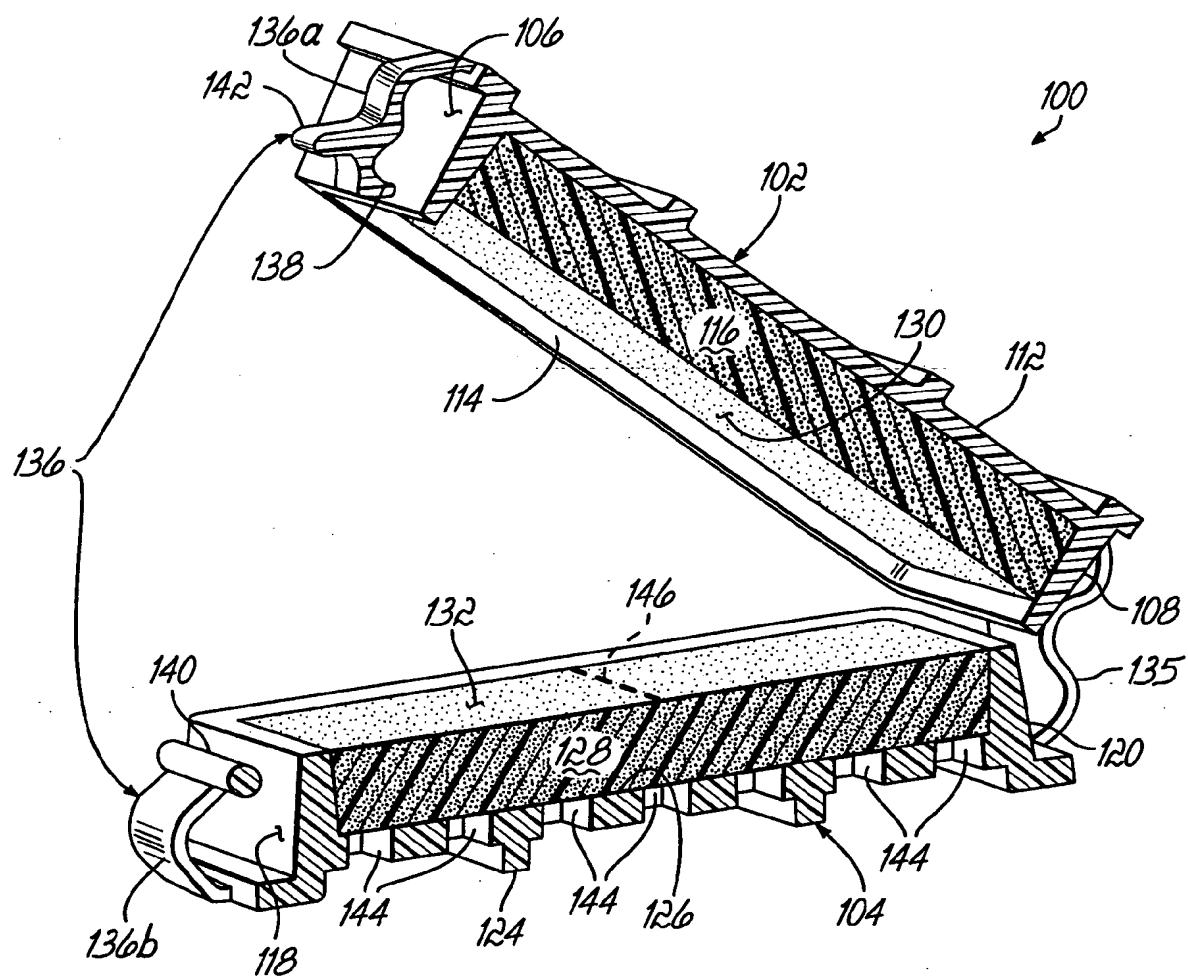


FIG. 3

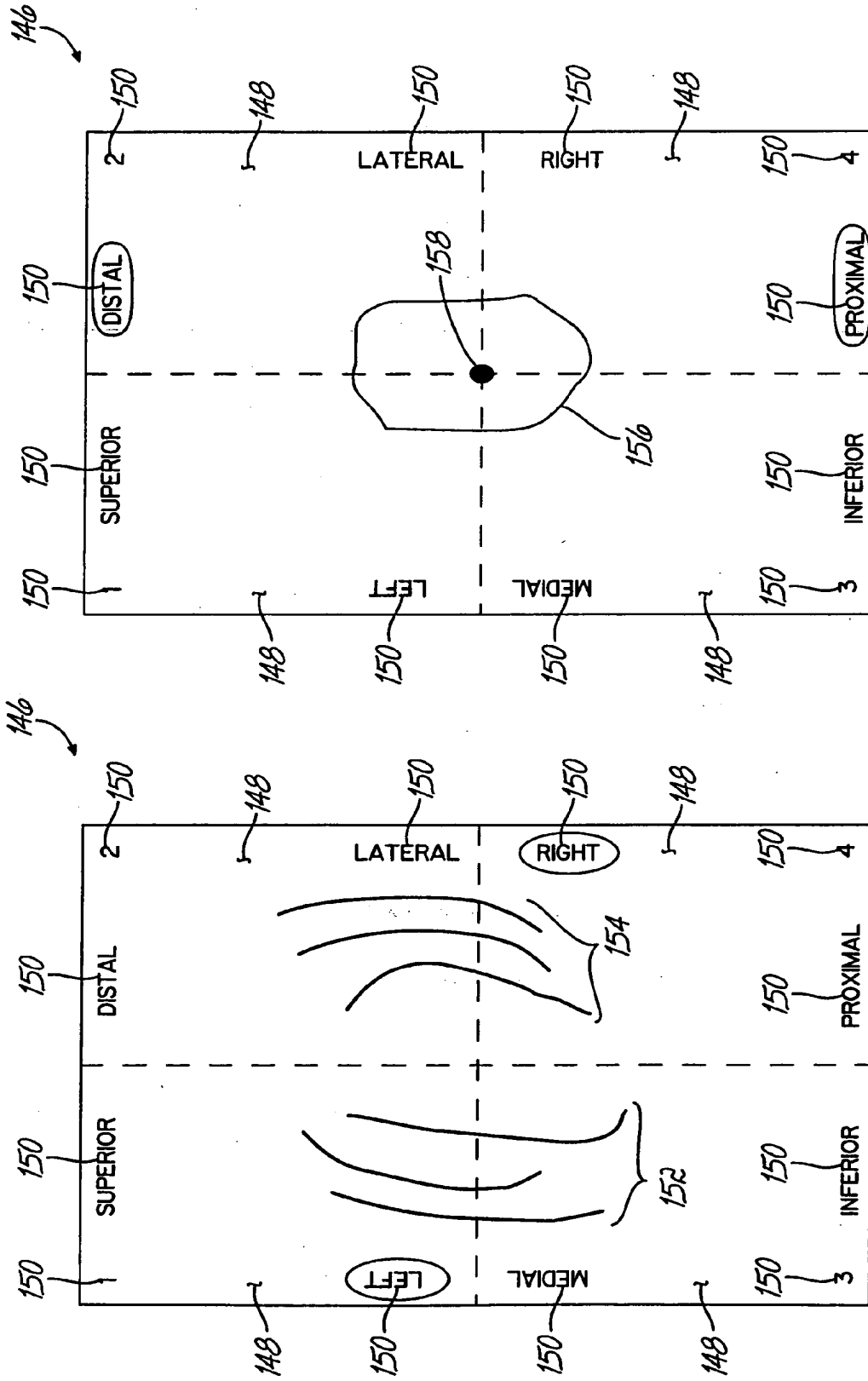


FIG. 4B

FIG. 4A

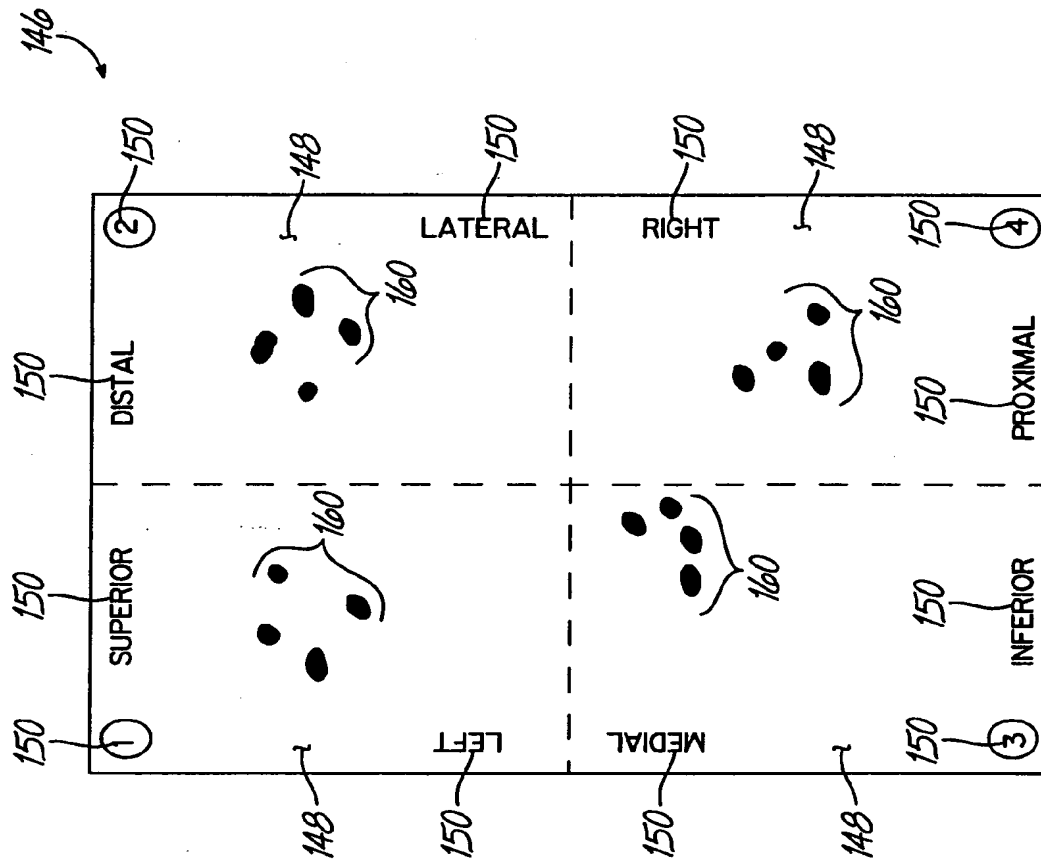


FIG. 4C

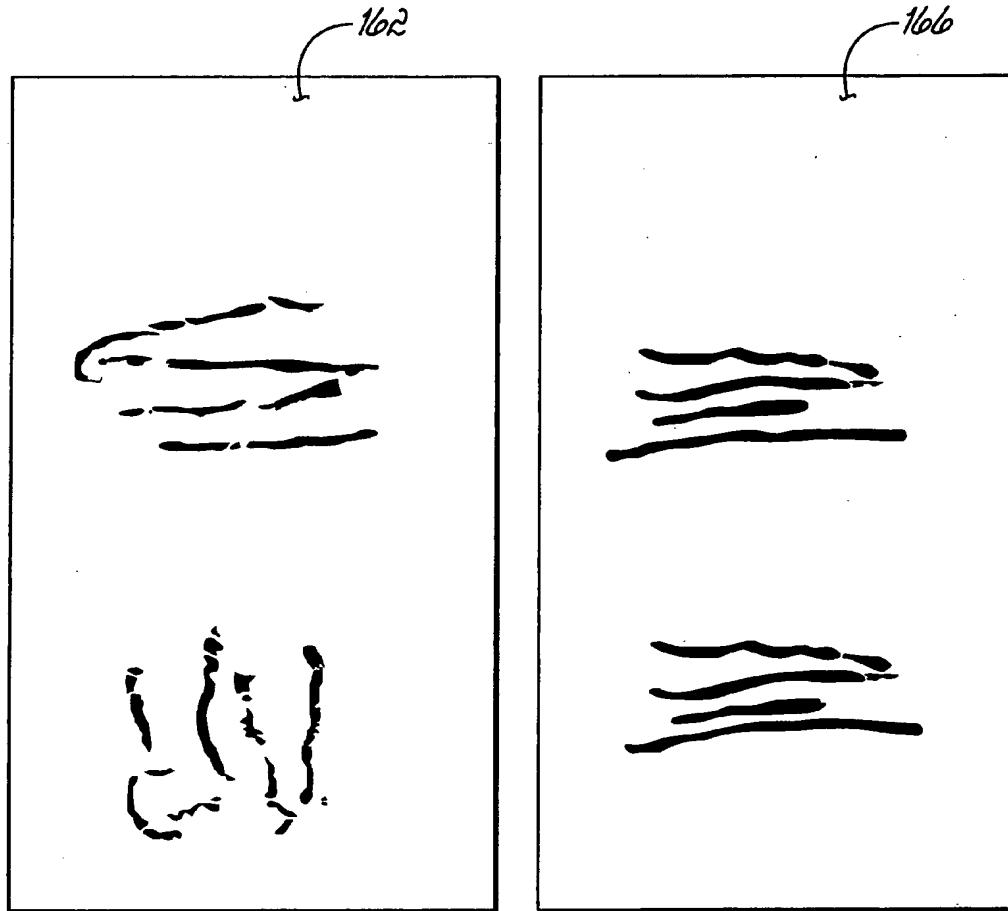


FIG. 5A

FIG. 5B

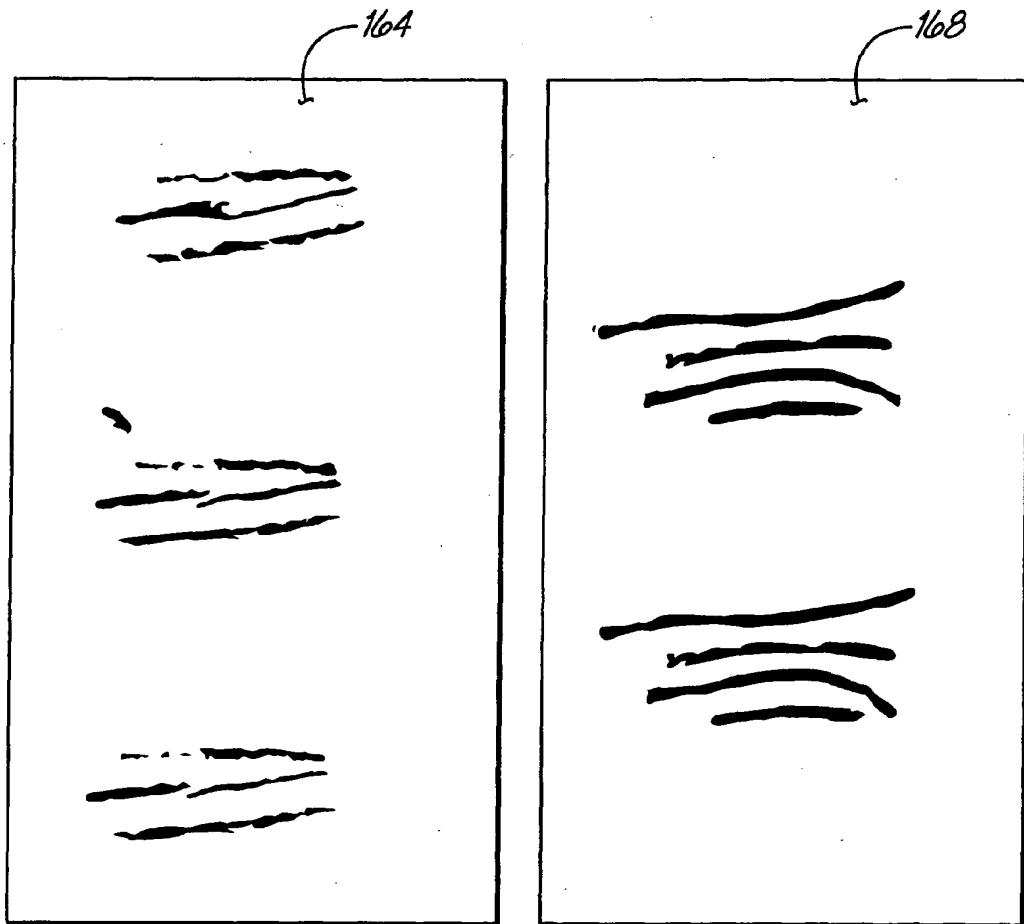
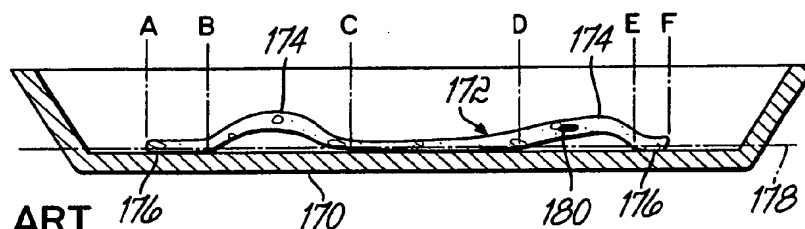
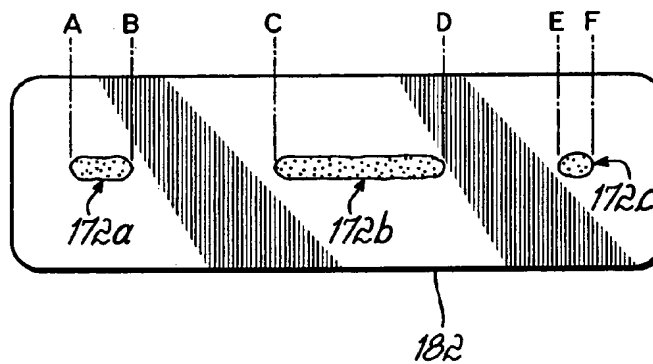


FIG. 6A

FIG. 6B



PRIOR ART
FIG. 7A



PRIOR ART
FIG. 7B

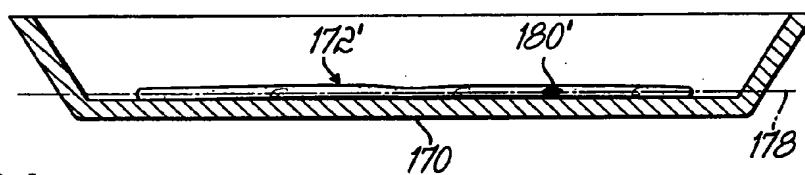


FIG. 8A

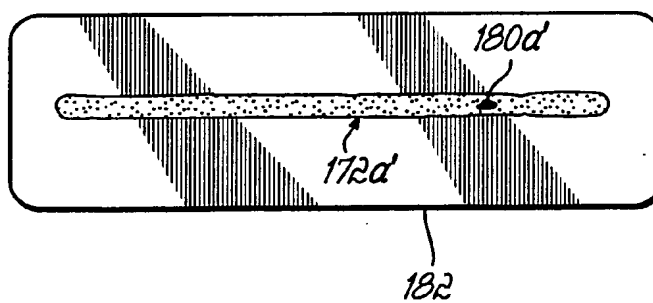


FIG. 8B

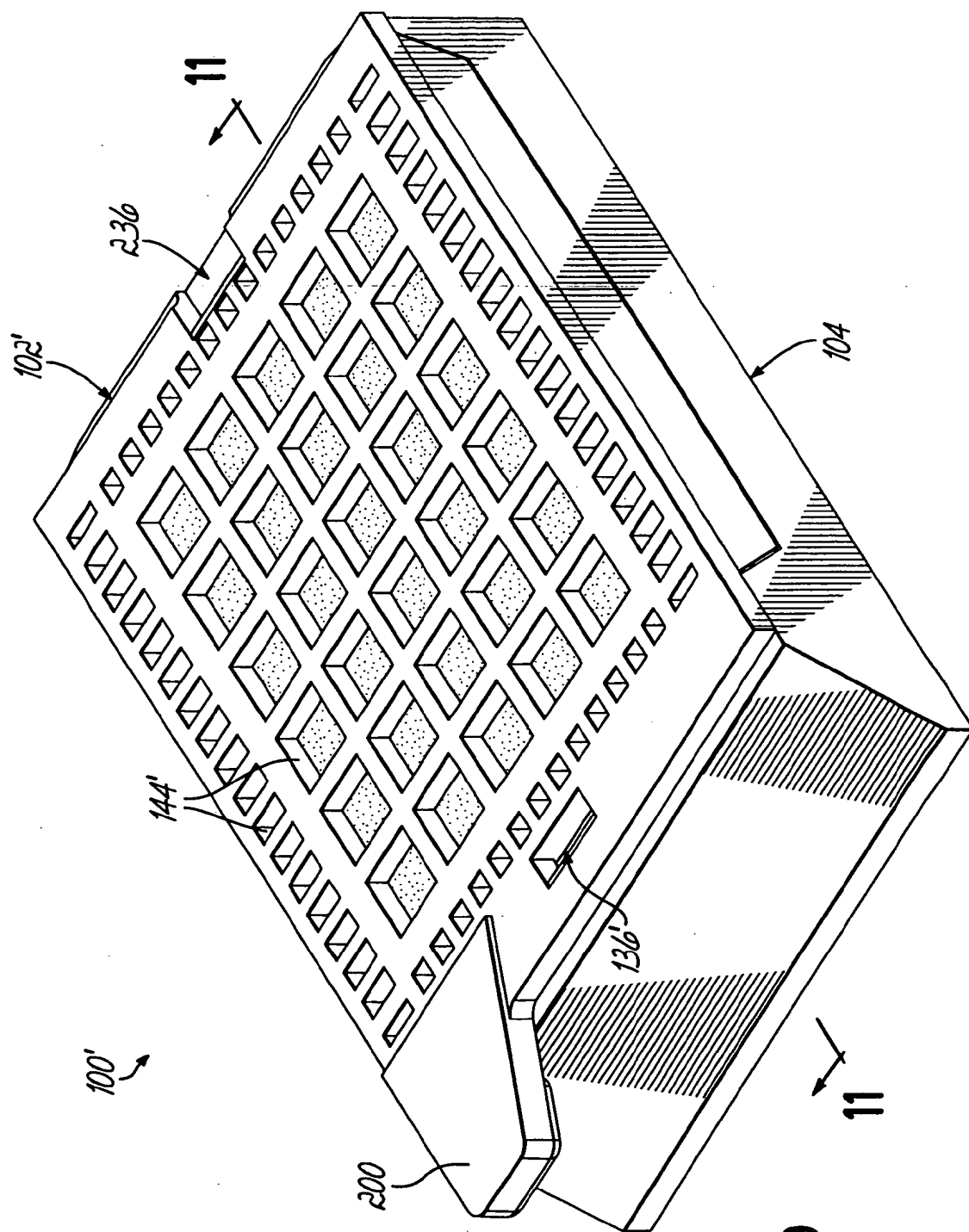
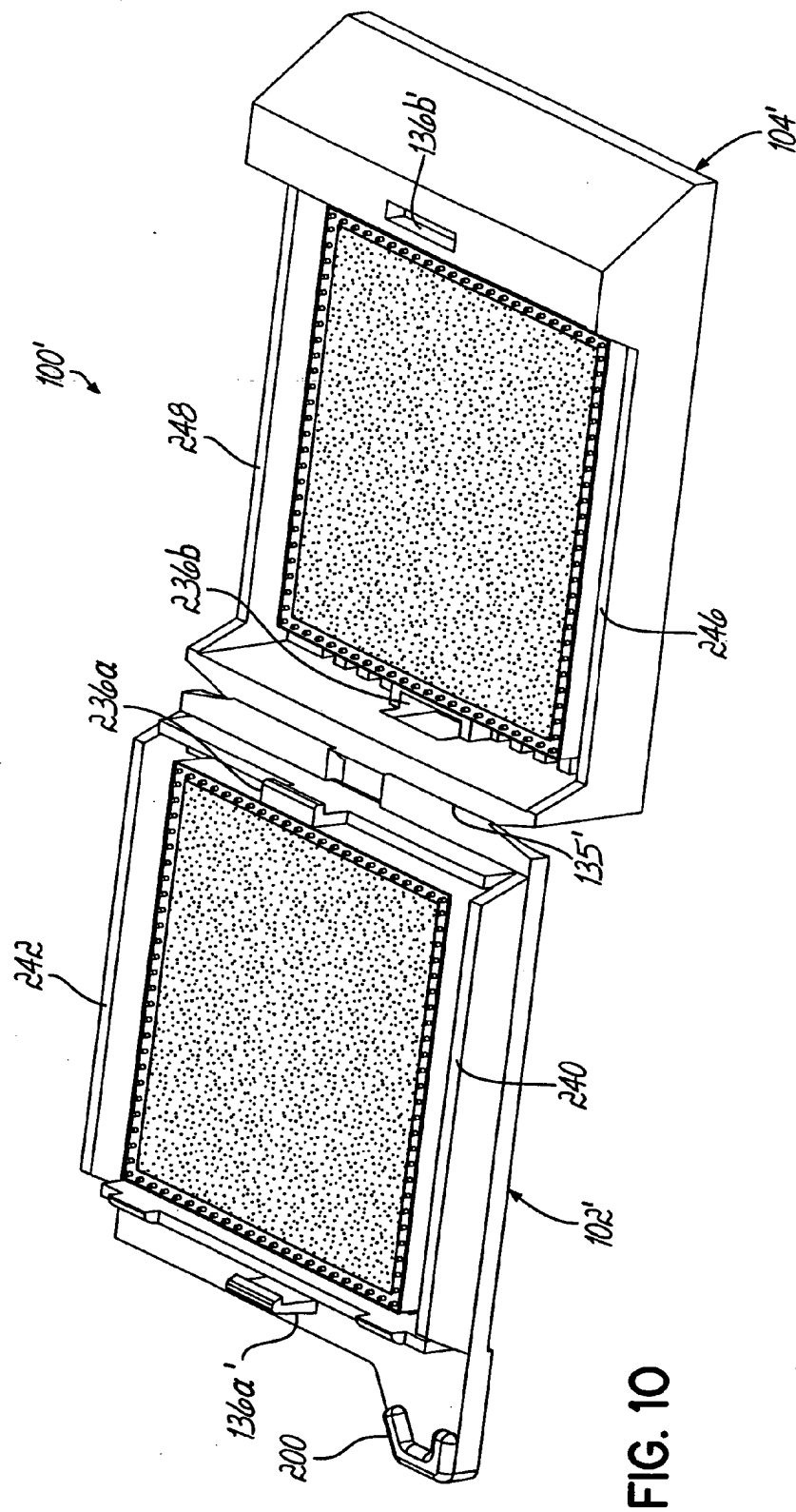


FIG. 9



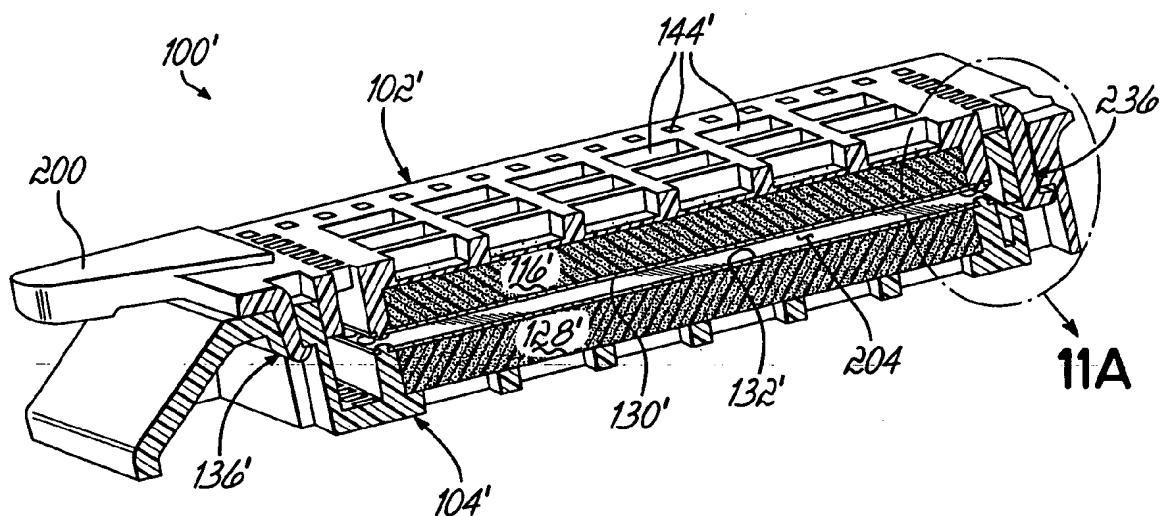


FIG. 11

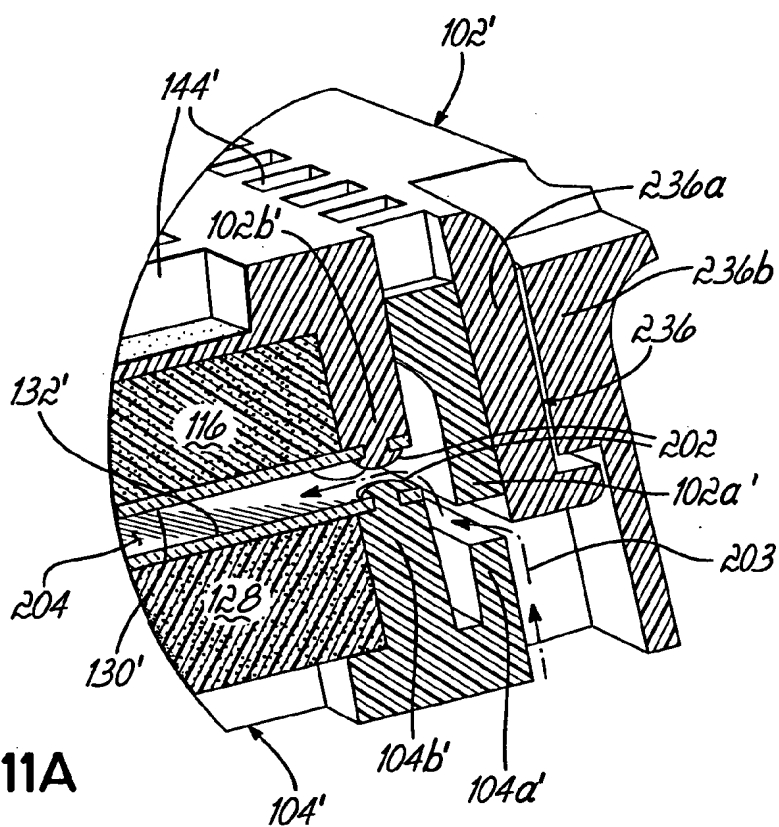


FIG. 11A



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 06 07 6973

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Place of search Munich		Date of completion of the search 2 February 2007	Examiner Skowronski, Maik
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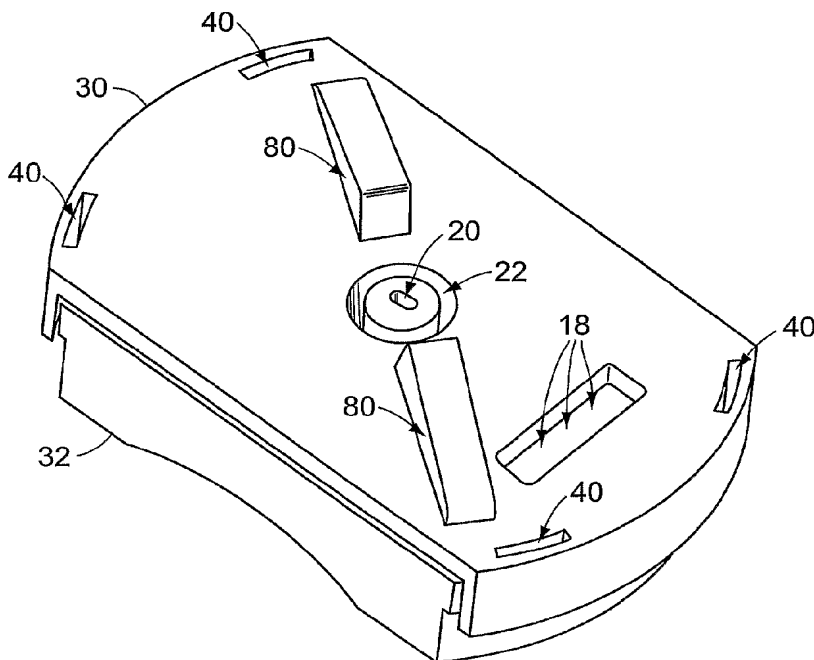
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10/448,856 30 May 2003 (30.05.2003) US</p> <p>(71) Applicant (for all designated States except US): APPLERA CORPORATION [US/US]; 850 Lincoln Centre Drive, Foster City, CA 94404 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): WOJTOWICZ, Janusz [PL/US]; 220 Ventura Avenue, Palo Alto, CA 94306 (US). HAGA, Douwe [US/US]; 21 West Summit Drive, Redwood City, CA 94062 (US). OLDHAM, Mark [US/US]; 16500 Soda Springs Road, Los Gatos, CA 95033</p> | <p>(US). COX, Steven [AU/US]; 39 Sandpoint Drive, Richmond, CA 94804 (US). RECKNOR, Michael [US/US]; 6153 Castle Drive, Oakland, CA 94611 (US). SCHROTH, Gary [US/US]; 100 Monasterio Court, San Ramon, CA 94583 (US). FEREA, Tracy [US/US]; 148 Farley Street, Mountain View, CA 94043 (US).</p> <p>(74) Agents: MAKROGIANNIS, Phil, N. et al.; 850 Lincoln Centre Drive, Foster City, CA 94404 (US).</p> <p>(81) Designated States (unless otherwise indicated, for every kind of national protection available): 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.</p> <p>(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,</p> |
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[Continued on next page]

(54) Title: APPARATUS AND METHOD FOR HYBRIDIZATION AND SPR DETECTION



(57) Abstract: An apparatus and method for hybridization providing a hybridization chamber. The hybridization chamber has a hybridization frame and detection frame. The hybridization chamber can be contained in housing that is releasably mated to enclose the hybridization chamber.



ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

APPARATUS AND METHOD FOR HYBRIDIZATION AND SPR DETECTION**FIELD**

[001] The present teachings can be related to apparatuses and methods for conducting reactions on a solid surface with biological samples, including hybridization assays. The apparatus improves the process of hybridization and detection.

BACKGROUND

[002] In the biological field, reactions on a solid surface can be used for hybridization assays. A known member of a binding pair on the solid surface can hybridize with a target member of the binding pair from the biological sample to form a duplex in the hybridization fluid. A pattern of duplexed binding pairs on the solid surface provides information about the biological sample. The pattern on the solid surface can be detected to map the information relative to the known members of the binding pairs on the solid surface. It is desirable to control the volume over the solid surface during hybridization and to provide access to the solid surface during detection.

[003] It can be desirable to have a sealed hybridization chamber to reduce leaks or evaporation of the hybridization fluid during processing and to maintain precise control over reaction temperature, duration, mixing, and other hybridization parameters. Hybridization parameters can include elevated temperatures, such as 55°C, for 18-24 hours with 400 rpm agitation. It can be desirable that a housing sealing the hybridization chamber can provide the container for shipping the hybridization chamber that can be sealed from the environment.

SUMMARY

[004] According to various embodiments, an apparatus for hybridization can include a substrate and a hybridization chamber including a detection frame, a hybridization frame, and a portion of the substrate, wherein the detection frame is positioned on the substrate, and the hybridization frame is positioned proximate to the detection frame to form the hybridization chamber. According to various embodiments, an apparatus for hybridization can include a housing including a base and a cover, a substrate including a microarray, wherein the substrate is positioned within the base, and a hybridization chamber including a frame and a portion of the substrate, wherein the cover releasably mates with the base to seal the hybridization chamber, and wherein the cover includes an inlet port to prevent a filling device from contacting the microarray.

[005] According to various embodiments, a method for hybridizing can include providing a housing including a base and a cover, providing a substrate including a microarray, providing a hybridization chamber including a frame and a portion of the substrate, and releasably mating the cover to the base to seal the hybridization chamber and compress the frame.

[006] According to various embodiments, an apparatus for hybridization can include a housing including a base and a cover, wherein the base and the cover are releasably mated, a substrate including a microarray, wherein the housing is configured to house the substrate, a detection frame, wherein the detection frame is positioned on the substrate, and the detection frame and the substrate form a detection volume, and a hybridization frame, wherein the hybridization frame, the substrate and the cover form a hybridization volume, wherein the hybridization volume and the detection volume are in fluid contact with the microarray. According

to various embodiments, an apparatus for hybridization can include a substrate including a microarray, wherein the substrate includes a binding layer and a support, and a hybridization chamber including a frame and a portion of the substrate, wherein the frame is positioned on the support through the binding layer, wherein the binding layer includes a blackening agent for absorbance.

[007] According to various embodiments, an apparatus for hybridization can include a substrate, and a hybridization chamber including a detection frame, a cover, and a portion of the substrate, wherein the cover includes a cavity to disperse a hybridization fluid over the portion of the substrate, wherein the detection frame is positioned on the substrate, and the substrate is configured for SPR detection.

According to various embodiments, an apparatus for hybridization can include a housing including a cover, a substrate including a microarray, and a hybridization chamber including a frame and a portion of the substrate and wherein the cover includes a cavity for dispersing a hybridization fluid over the microarray, wherein the cover is mated with the frame to seal the hybridization chamber, wherein the cover includes an inlet port, and wherein the substrate is configured for SPR detection.

[008] According to various embodiments, a method for hybridizing can include providing a housing including a cover, providing a substrate including a microarray, providing a hybridization chamber including a frame and a portion of the substrate, dispersing a hybridization fluid over the microarray, and detecting hybridization by SPR.

[009] It is to be understood that both the foregoing general description and the following description of various embodiments are various and explanatory only and are not restrictive.

BRIEF DESCRIPTION OF THE DRAWINGS

[010] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate various embodiments. In the drawings,

[011] Figs. 1A-1C illustrate various embodiments of proximate detection and hybridization frames;

[012] Figs. 2A-2C illustrate various embodiments of the cover;

[013] Figs. 3-4 illustrate various embodiments of the apparatus assembled and disassembled;

[014] Figs. 5A-5D illustrate various embodiments of the inlet port and elevated portion;

[015] Figs. 6-11 illustrate various embodiments of the apparatus assembled and disassembled;

[016] Fig. 12 illustrates various embodiment of the substrate and the detection frame with cover slip for detection;

[017] Fig. 13 illustrates various embodiment of the substrate and the detection frame with a plurality of barriers forming an array of chambers and microarrays;

[018] Fig. 14 illustrates various embodiment of the substrate and the detection frame with cover for SPR detection;

[019] Figs. 15 illustrates various embodiment of the cover with an inlet port and an outlet port for hybridization fluid flow-through;

[020] Fig. 16 illustrates various embodiment of the cover with an inlet port and an outlet port;

[021] Figs. 17A-17F illustrate various embodiments of the substrate magnified to show attachment of the detection frame to the support, adhesive layer, binding layer and the micro-array;

[022] Fig. 18 illustrates various embodiments of the cap; and

[023] Fig. 19 illustrates various embodiments of coupling for SPR.

DESCRIPTION OF VARIOUS EMBODIMENTS

[024] Reference will now be made to various embodiments, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers are used in the drawings and the description to refer to the same or like parts.

[025] The term “hybridization” as used herein refers to the process of forming a duplex between two members of specific binding pair. The specific binding pair is frequently complementary or partially complementary strands of a polynucleotide. It will be understood by those skilled in the art of molecular biology that the term “polynucleotide” as used herein includes analogs of naturally occurring polynucleotides and does not convey any limitation of the length of the polynucleotide. One of the polynucleotide strands may be immobilized on a solid substrate.

[026] Polynucleotide strands used for hybridization can be labeled with a detectable marker to as facilitate the detection of duplexes. Examples of detectable markers can include, but are not limited to fluorescent dye, radioisotopes, enzyme, or other markers. According to various embodiments, detection can be provided by a CCD camera that detects the detectable markers. Polynucleotide strands used for hybridization can be non-labeled. Hybridization of non-labeled binding pairs can be detected by surface plasmon resonance (SPR).

[027] Hybridization can be used for a variety of purposes, including understanding the structure-activity relationship between different materials, detecting and screening single nucleotide polymorphisms (SNPs), and sequencing an unknown material. The term “specific binding pair” refers to a pair of molecules that bind to one another with a specificity that is detectable above background levels of non-specific molecular interactions. Examples of specific binding pairs can include, but are not limited to antibody-antigen (or hapten) pairs, ligand-receptor pairs, biotin-avidin pairs, polynucleotides with complementary base pairs, nucleic acid binding proteins and cognate nucleic sequences, members of multi-protein complexes, and the like. Each specific binding pair can include two members, or additional compounds can specifically bind to either member of a given specific binding pair.

[028] According to various embodiments, the apparatus for hybridization can provide a hybridization chamber with polynucleotides in the liquid phase or bound on a substrate. For illustrative purposes, the figures describe polynucleotides bound on a substrate to form a microarray. Microarrays can have densities of 4 binding sites per square millimeter or up to 10^4 binding sites per square millimeter. Binding sites can be positioned on the substrate by pin spotting, ink-jetting, photo-lithography, and other methods known in the art of high density deposition. The features described in the figures can be easily applied to an apparatus for hybridization with nucleotides in the liquid phase.

[029] According to various embodiments, “SPR” as used herein refer to a surface-sensitive, optical detection method that can be used to monitor hybridization and other protein-nucleic acid binding. SPR can be sensitive to the thickness and index of refraction of material at the interface between a free electron metal (e.g.,

gold, silver, copper, cadmium, aluminum) and a bulk medium, such as air or water. The microarray can be deposited on the metal substrate. SPR can use an evanescent wave generated by a light source that is substantially monochromatic, incoherent, near-infrared light source, for example a laser beam or light-emitting diode (LED). The wave can be linearly polarized parallel to the plane of incidence to impinge onto the metal. The metal can be coated onto a support, including glass or plastic such as polycarbonate, to form a thin film portion of the substrate. The substrate can be constructed entirely out of metal.

[030] According to various embodiments, as illustrated in Fig. 19, the substrate can be coupled to a prism (Kretschmann Prism Coupling), or the substrate can be coupled a grating (Grating Coupling). SPR can be observed as the reduction in light intensity reflected from the prism-metal interface (Kretschmann Prism Coupling) or from the grating-metal interface (Grating Coupling) when, for a correct combination of refractive indices, incident light wavelength, and angle of incidence, as indicated by θ in Fig. 19, photons from an incident light beam enter into surface plasmon modes within the metal and are absorbed. According to various embodiments, the angle of incidence for greatest photon absorption, and hence for least reflected light intensity, can be determined. Hybridization on the metal surface of the substrate can change the index of refraction, thereby shifting the angle of incidence for least reflected light intensity. The shift in the angle of incidence can be used to detect the accretion or reduction of materials on the metal surface in contact with the hybridization fluid.

[032] According to various embodiments, the binding of specific binding pairs can increase the thickness of the microarray such that the microarray can provide a shift in the angle of incidence that can indicate the presence of binding. By

monitoring either the position of the angle of incidence or the reflectivity at a fixed angle near the angle of incidence, the presence or absence of binding at a binding site can be detected. SPR is relatively fast and can be performed without labeling. Detection can be provided by a CCD camera at a fixed angle. The SPR image arises from variations in the reflected light intensity from different binding sites. The hybridization chamber with a transparent cover can be used for detection with SPR.

[033] According to various embodiments, grating-coupled SPR can be observed by a CCD camera detector from a microarray with a surface area of at least 1.0 cm by 1.0 cm. The CCD camera can provide simultaneous detection of the entire matrix of binding sites on the microarray.

[034] According to various embodiments, Figs. 1A-1C illustrate an apparatus for hybridization showing the relative position of the detection frame and hybridization frame. These embodiments can relate to a housing or an instrument. According to various embodiments, the instrument can provide a lid and a holder configured like jaws that can be held together to form the hybridization chamber. In Figs. 1A-1C, the complete housing or instrument is not shown. Detection frame 14 can be positioned on substrate 10 bounding micro-array 12. Hybridization frame 16 can be positioned proximate to said detection frame such that it can form a seal with cover/lid 18 (cover in the housing embodiment and lid in the instrument embodiment). According to various embodiments, hybridization frame 16 can be compressible to better form such seal. Compressible materials are known in the material science arts. According to various embodiments, the hybridization frame and/or the detection frame can be constructed of at least one elastomeric material chosen from Silicone Rubber, FDA approved Silicone Rubber, EPDM Rubber, Neoprene (CR) Rubber, SBR Rubber, Nitrile (NBR) Rubber, Butyl Rubber, Hypalon

(CSM) Rubber, Polyurethane (PU) Rubber, Viton Rubber, and polydimethylsiloxane (Slygard™ elastomer by Dow Corning).

[035] The term “frame” as used herein refers to gaskets, rings, or seals. The frame can be angular or circular in overall shape and/or cross-section. The frame can be constructed of compressible, elastomeric material. The frame does not include any internal means of channeling flow such as ports or means of mixing such as a reaction recess in the frame. The frame does not permit leaching of contaminants into the hybridization chamber. The frame is unitary and does not include multiple members. The frame is not separated into multiple frames such as grid plates. The term “detection frame” as used herein refers to the frame that forms the detection chamber and adheres on the substrate. The term “hybridization frame” as used herein refers to frame that forms the hybridization chamber either with the detection frame by being positioned on the detection frame or outside the detection frame by being positioned adjacent or near the detection frame, or without the detection frame.

[036] The term “proximate” as used herein refers to the location of the detection and hybridization frames, including positioned on each other such that they are one on top of the other with each one having the same width or either one being wider, adjacent to each other such that they are contact each other on the side with each one having the same center or either one being off center, or near each other such that they do not contact each other but are a short distance apart with either one surrounding the other.

[037] According to various embodiments, Fig. 1A illustrates hybridization frame 16 can be positioned on detection frame 14. According to various embodiments, detection frame 14 can be harder than hybridization frame 16 to

provide more stability during detection. Hybridization frame 16 can be compressed between detection frame 14 and cover/lid 18 to form a sealed hybridization chamber. Fig. 1B illustrates hybridization frame 16 can be positioned adjacent to detection frame 14. Hybridization frame 16 can be compressed between substrate 10 and cover/lid 18 to form a sealed hybridization chamber. Fig. 1C illustrates hybridization frame 16 can be positioned near detection frame 14. Hybridization frame 16 can be compressed between substrate 10 and cover/lid 18 to form a sealed hybridization chamber. Although the detection and hybridization frames illustrated in Figs. 1A-1C are rectilinear, detection and hybridization frames can be any shape including circular, elliptical, and combinations thereof. As described below, the hybridization chamber can include overlapping hybridization volume and detection volume.

[038] According to various embodiments, the detection frame and hybridization frame can be replaced with one frame that forms both the hybridization chamber and the detection chamber. In the SPR detection embodiment, the hybridization chamber can also be the detection chamber. It is clear to one skilled in the art that the features described in the two-frame embodiments can similarly benefit one-frame embodiments.

[039] According to various embodiments, the apparatus for hybridization can include a housing or an instrument. As illustrated in Figs. 1A-1C, the apparatus for hybridization can relate to either a housing or instrument without a housing. In a cartridge, the cover and base of the housing can operate in a similar fashion as the lid and holder of an instrument. According to various embodiments, Figs. 2-11 and Figs. 14-16 can relate to a housing. According to various embodiments, a housing can be positioned within an instrument for processing (including filling, binding reactions, washing, drying, etc.) and benefit from the features illustrated in Figs. 2-11

and Figs. 14-16. The housing can be removed from the instrument for detection or detection can take place in the instrument.

[040] According to various embodiments, Figs. 2A-2C illustrate an apparatus for hybridization using a housing having different covers. Figs. 2A-2C illustrate detection frame 14 can be positioned on substrate 10 bounding microarray 12 and hybridization frame 16 can be positioned on detection frame 14. Hybridization frame 16 can be compressible so that it forms a seal with cover 18 to enclose the hybridization chamber. According to various embodiments, the detection frame and hybridization frame can be proximate to each other, as described above. Figs. 2A-2C illustrate that cover 18 can have an elevated portion 22 and an inlet port 20. As illustrated in Fig. 2A, elevated portion 22 can project from the upper surface of cover 18 such that cover 18 is substantially parallel to microarray 12 on substrate 10. As illustrated in Figs. 2B and 2C, elevated portion 22 can be a portion of said cover and can be domed or vaulted such that cover 18 is not substantially parallel to microarray 12 on substrate 10. Fig. 2B illustrates an elevated portion 22 which can have a hemispherical dome. Fig. 2C illustrates an elevated portion 22 which can have a rectilinear or angled vault. Any other variation or combination of a dome or a vault can be used to increase the hybridization volume of the hybridization chamber. As illustrated in Fig. 2C, port 20 can be moved to the side of the elevated portion 22. The port does not have to be centered and can be anywhere on the elevated portion or other portion of the cover. The elevated portion can be a separate component other than the cover that attaches to the cover.

[041] According to various embodiments, the hybridization chamber provides a hybridization volume less than 300 microliters. The amount of hybridization fluid can be 1.0 to 50 microliters.

[042] According to various embodiments, Figs. 2-11 and Figs. 14-16 illustrate an apparatus for hybridization including a housing with cover and base. According to various embodiments, a housing can be assembled in a locked position and disassembled in an unlocked position. The housing can include a cover which itself can include a top and a compression plate. The compression plate can include an elevated portion, inlet port, and an exhaust port to allow the gas displaced by the hybridization liquid to escape to the atmosphere. The inlet port and exhaust port can be blocked to prevent hybridization fluid from escaping from the hybridization chamber by a cap. According to various embodiments, the cap can be a sealing tape on the top of inlet port, can enclose the elevated portion (e.g. Figs. 3 and 4), or can plug the inlet port (e.g. Fig. 5D). The cover and base in a locked position can form a window that exposes a portion of the underlying substrate. A bar code can be positioned on the to substrate and can be accessed and/or read through the window. The top can provide an opening to allow the elevated portion of the compression plate to protrude. The top and base can include a releasably mating mechanism. The compression plate can have a raised portion on the opposite side to elevated portion to engage hybridization frame. Splitting the cover into a top and compression plate can provide a releasably mating mechanism that can be moved in a locked position by pressing down, sliding, rotating, and/or flipping the top. Pressing down can engage the compression plate on hybridization frame. Sliding, rotating, and/or flipping positions the top to the locked position without moving the compression plate. The top can be released by sliding, rotating, and/or flipping it in the reverse direction. According to various embodiments, methods for hybridization include providing the apparatus and releasably mating the cover and base according these actions.

[043] According to various embodiments, a bar code can be positioned on the substrate. The term "bar code" refers to any marking that can identify the substrate, including identifying the microarray and/or hybridization. The bar code can be one-dimensional (e.g. bars), two-dimensional (e.g. dot matrices), or three-dimensional (e.g. holograms). Positioning a bar code on the substrate can include positioning an adhesive label with a bar code printed on the label, positioning multi-layered opaque coatings of contrasting colors, or marking the substrate directly, as is described below. According to various embodiments, a bar code can be positioned on the housing or frames.

[044] According to various embodiments, a bar code can be positioned directly on a metal substrate by marking. A bar code can be marked on the metal substrate with a light beam from a laser or LED. The bar code can be marked to a depth within the substrate so that it can be read with a reader apparatus which can read refracted laser light from the substrate after the substrate is exposed to a light beam from the reader apparatus. The bar code can be marked onto the substrate to a depth into the substrate such that incident light refracted from the substrate can show the bar code as being black on a white background or white on a black background. According to various embodiments, the bar code can be positioned in one step or a plurality of steps. The substrate surface can be modified by ablating metal residue on the surface. Ablating can provide a uniform surface for positioning the bar code and can establish a consistent angle of refraction of the returned light pattern. The substrate surface can be marked with a bar code. The marked bar code can be read by exposing the bar code to a light beam from a reader apparatus. The reader can be positioned to determine whether the marked bar code provides sufficient contrast to its background to permit its being read by the reader apparatus.

According to various embodiments, the bar code can be marked on the substrate to a depth between 1.27×10^{-5} meters and 2.54×10^{-5} meters.

[045] According to various embodiments, the housing, including the cover and base can be manufactured from injection molding plastic. Injection molding can reduce the cost of producing the housing and can provide a disposable container that can be discarded after hybridization processing. The housing can be composed of acrylonitrile-butadiene-styrene plastic, polyurethane, polyvinylchloride, polycarbonate, polyethylene, TEFLON™, polystyrene, KALREZ™, or other materials known in the art of consumables manufacturing.

[046] The term “releasably mated” or “releasably mating” as used herein refers to mating the cover and base such that they can be in a locked position during hybridization and then released to provide access to the substrate for detection. The base and cover are releasably mated by the releasably mating mechanism. Various embodiments of the releasably mating mechanism are described herein. According to various embodiments, the locked position can be subjected to several types agitation under which the cover and base remain mated. These types of agitation include tilting the housing, rolling the housing, moving the housing back and forth, moving the housing in a circular pattern, and rotating the housing about an axis.

[047] Fig. 3 illustrates an embodiment of a housing assembled and in a locked position. Fig. 4 illustrates the housing of Fig. 3 disassembled. The housing includes cover 18 and base 32 held in the locked position by releasably mating mechanism 40. The releasably mating mechanism 40 can move the cover 18 to the locked position by pressing down and clamping. The cover 18 can be released by unclamping releasably mating mechanism 40 and pulling up. An indentation in cover 18 can form window 38. A bar code (not shown) positioned on substrate 10 can be

read through window 38. Cap 36 can block hybridization fluid from escaping from the hybridization chamber. Fig. 6 illustrates the housing disassembled. Cap 36 can be connected to hybridization frame 16 via tether 60. Both the cap 36 and hybridization frame 16 can be removed to provide access to the substrate 10 for detection.

[048] According to various embodiments, Fig. 6 illustrates a housing assembled and in a locked position. Fig. 7A illustrates the housing of Fig. 6 disassembled. The housing can include base 32 and cover 18. Cover 18 can include top 30 and compression plate 34. Top 30 and base 32 can include releasably mating mechanism 40. Top 30 can include handle 80 for manually handling top 30, and window 38 for accessing the bar code (not shown) positioned on substrate 10. Top 30 can have an opening to allow the elevated portion 22 of compression plate 34 to protrude. Releasably mating mechanism 40 can be moved to a locked position by rotating top 30. Splitting the cover 18 into top 30 and compression plate 34 can provide for a releasably mating mechanism 40 that can be moved in a locked position by rotating the top without rotating the compression plate 34. Rotating top 30 can push compression plate 34 down to engage hybridization frame 16. Top 34 can be released by rotating in the reverse direction and pulling up. The underside of base 32 can include an indentation that provides stacking of one housing on top of another. According to various embodiments, Fig. 7C illustrates that handles 80, elevated portion 22, and cap 36 can fit into indentation 90 to permit stacking of two or more housings. Fig. 7B illustrates the housings stacked on top of each other. According to various embodiments, compression plate 34 can have a non-uniform thickness to provide compression of hybridization frame 16. Fig. 7D illustrates compression plate 34' that has non-uniform thickness.

[049] According to various embodiments, Fig. 8 illustrates a housing that is assembled but not in the locked position. Compression plate 34 can be positioned in base 32. Top 30 can be hinged on base 32. Top 30 can be divided into two portions that are hinged on opposite sides of base 32. According to various embodiments, the top 30 can be hinged on one side of base 32 or the top can be divided into more than two portions. Top 30 can include ribs 100 to compress compression plate 34 when the cover is moved to the locked position. The two portions of top 30 can form an opening so that the elevated portion 22 of compression plate 34 can protrude through top 30 when the cover is in a locked position. Top 30 and base 32 can include releasably mating mechanism 40. Releasably mating mechanism 40 can clamp top 30 to base 32 when the top 30 is flipped along the hinged portion into the locked position. Clamping top 30 to base 32 can force ribs 100 to push compression plate 34 down to engage the hybridization frame (not shown). Top 30 can be released by unclamping releasably mating mechanism 40.

[050] According to various embodiments, Figs. 9A-9B illustrate a housing that is unassembled and assembled, respectively. Fig. 9A illustrates the housing unassembled with cover 18 including top 30 and compression plate 34. Compression plate 34 can include elevated portion 22 and inlet port 20. Top 30 can include five sides similar to a "match-box" with a closed end. Base 32 can include handle 80 for pushing and pulling base 32 into and out of top 30. Top 30 can include ribs 100. Compression plate 34 can include sloped ribs to compress compression plate 34 when it is positioned in base 32 and base 32 is pushed into top 30 to the locked position. Fig. 9B illustrates a side view of the assembled housing. The broken lines illustrate the interior of the housing, including base 32, compression plate 34, and ribs 100. Top 30 can include an opening that allows elevated portion

22 to protrude above top 30. Handle 80 can protrude from the open end of top 30. Ribs 100 and the sloped ribs of compression plate 34 can provide the releasably mating mechanism and compress compression plate 34. Pushing base 32 into top 30 can force ribs 100 to push compression plate 34 down to engage the hybridization frame (not shown). Base 34 can be released by pulling handle 80 horizontally away from top 30.

[051] According to various embodiments, Fig. 10 illustrates a housing that is unassembled and assembled. Fig. 10 illustrates the housing unassembled with cover 18 which can include top 30 and compression plate 34. Top 30 can include handles 80 for rotating top 30 to the locked position. Cap 36 can block hybridization fluid from escaping from the hybridization chamber through inlet port 20 in elevated portion 22 of compression plate 34. Top 30 can include an opening to allow elevated portion 22 of compression plate 34 to protrude above top 30. Base 32 and top 30 can include releasably mating mechanism 40. Releasably mating mechanism 40 can include prongs that move along a guide when the top 30 is rotated to move the top 30 to a locked position. Splitting the cover 18 into a top 30 and compression plate 34 can provide for releasably mating mechanism 40 that is moved in a locked position by rotating top 30 without rotating compression plate 34. Rotating top 30 can push compression plate 34 down to engage hybridization frame 16. Top 34 can be released by rotating in the reverse direction and pulling up. Base 32 can include window 120 that provides access to the underside of substrate 10. A bar code can be positioned to the underside of substrate 10 can be accessed through window 120.

[052] According to various embodiments, Fig. 11 illustrates a bottom view of an assembled housing similar to the housing illustrated in Fig. 6, but with a different releasably mating mechanism. Base 32 can include openings that provide visual

access to substrate 10. Releasably mating mechanism 40 can maintain top 30 and base 32 in the locked position. Fig. 10 illustrates releasably mating mechanism 40 that can include prongs that can move along a guide when top 30 is rotated to move the top 30 in a locked position. Fig. 11 illustrates releasably mating mechanism 40 that can include prongs that can move along a guide when the top 30 is rotated to move the top 30 in a locked position, where the guide can include flexible tabs which provide some flexibility between top 30 and base 32 during insertion of the prong into the guide and/or during rotation.

[053] According to various embodiments, the housing can relate to batch-processing hybridization or continuous-processing hybridization. It is understood by one skilled in the art, that features of batch-processing hybridization can be applied to continuous-processing hybridization by adding an outlet port. It is also understood by one skilled in the art, that features of continuous-processing hybridization can be applied to batch-processing hybridization by eliminating the outlet port. It is also understood by one skilled in the art, that in a combination of batch-processing hybridization and continuous-processing hybridization the features of both can be applied.

[054] According to various embodiments, Figs. 14-16 illustrate an apparatus for hybridization including a housing where the cover mates directly with the base, which is the substrate. It is understood by one skilled in the art, that features of housings where the substrate is positioned in the base can be applied to housings where the substrate is the base. It is also understood that features of housings where the substrate is the base can be applied to housings where the substrate is positioned in the base.

[055] According to various embodiments, Fig. 14 illustrates a housing including a cover 18 and a substrate 10, wherein the substrate is the base. Cover 18 can include window 150. Window 150 can be a portion of cover 18 or it can be transparent material that forms to the surface of cover 18. Cover 18 includes inlet port 20 and outlet port 160. The detection frame 14 (which is also the hybridization frame) can be positioned between cover 18 and substrate 10. Substrate 10 can be adapted for SPR. Substrate 10 can include a free electron metal. Substrate 10 can be constructed of metal or substrate 10 can include of a support coated with a thin metal film. According to various embodiments, as illustrated in Fig. 19(a), substrate 10 can be coupled to grating 190. According to various embodiments, as illustrated in Fig. 19(b), substrate 10 can be coupled to prism 192. Fig. 19 illustrates angle of incidence θ and the arrow in the grating or prism illustrates the direction of propagation for the surface plasmon. According to various embodiments, as illustrated in Fig. 14, a microarray, a grating or a prism in the hybridization chamber can be aligned with window 150 to provide access for light from a light source and toward a detector.

[056] According to various embodiments, Fig. 16 illustrates the bottom of cover 18. Cover 18 can include cavity 72 which can disperse or collect the hybridization fluid from inlet port 20 or outlet port 160, respectively. According to various embodiments, cover 18 can include an inlet port 20 with cavity 72. Cavity 72 can include a uniform expansion or graduated expanding sections. Cavity 72 can disperse the hybridization fluid over the portion of the substrate that includes the microarray. Dispersing the hybridization fluid can provide uniform contact between the hybridization fluid and the microarray. Cover 18 can be mated to detection frame

14 with an adhesive that is compatible with both detection frame 14 and cover 18. The adhesive provide either releasable mating or permanent mating.

[057] According to various embodiments, Fig. 15 illustrates a housing where the substrate can be the base. According to various embodiments, any base described above can be used with the cover shown in Fig. 15. Fig. 15 illustrates that cover 18 can include top 30 and compression plate 34. Top 30 can include two elevated portions 22. One elevated portion 22 includes inlet port 20 and the other elevated portion includes outlet port 160. Compression plate 34 compresses hybridization frame 16 on detection frame 14 which can be attached to substrate 10 when top 30 contacts substrate 10. Fig. 16 illustrates a bottom view of top 30. Top 30 can include a dispensing cavity 72 adjacent to inlet port 20. Dispensing cavity 72 can be positioned on the inside of hybridization frame 16 and can provide a telescoping width to disperse the hybridization liquid over the portion of substrate 10 within the hybridization chamber. There can be a similar structure adjacent to outlet port 160 to collect the hybridization liquid from the hybridization chamber.

[058] According to various embodiments, the releasably mating mechanism can include of spring tabs, prongs and guides, flexible tabs that move the cover to the locked position once the desired compression is achieved. In addition to these, any variety of releasable fastener known in the art of packaging can be implemented as a releasably mating mechanism.

[059] It is understood to one skilled in the art that combining or selecting features of various embodiments of the housing can apply the housing to the particular application and processing environment for the hybridization. As such many permutations of the features described above are contemplated in this description.

[060] According to various embodiments, Figs. 5A-5D illustrate the inlet port and elevated portion of the cover. Figs. 5A-5D illustrate an elevated portion similar to Fig. 2A where the elevated portion can project from the upper surface of the cover. The features described in conjunction with Figs. 5A-5D can also apply to elevated portions illustrated in Figs. 2B and 2C where the elevated portion can be a portion of the cover. The features described in conjunction with Figs. 5A-5D can apply to elevated portions that are separate components from the cover. The features described in conjunction with Figs. 5A-5D can apply to inlet ports directly in the cover without elevated portions. The Fig. 5A illustrates a cross-section of elevated portion 22 which includes inlet port 20, filling cavity 70 and dispensing cavity 72. Filling cavity 70 and dispensing cavity 72 can be in fluid contact with vertical overlap and slight horizontal overlap. The horizontal overlap can provide a stop for the filling device. The filling device can provide the hybridization fluid to the hybridization chamber but the stop can prevent the filling device from contacting the substrate below the dispensing cavity 72 and hybridization chamber.

[061] The term "filling device" as used herein refers to any device for providing hybridization fluid to the hybridization chamber. Filling devices can include, but are not limited to a pipette, a syringe, a tube, and any other device known in the art of handling laboratory or manufacturing fluids. The filling device can be automated or manually operated.

[062] Fig. 5B illustrates a cross-section of filling device 74 positioned into inlet port 20 of elevated portion 22. Inlet port 20 can be configured to restrain the filling device 74 to prevent the filling device 74 from contacting the substrate below. Inlet port 20 can be configured to provide an opening around filling device 74 to allow the gas displaced by the hybridization fluid to escape to the environment. With the

filling device 74 restrained, the hybridization fluid can be dispensed into overhead cavity 76. The hybridization fluid fills only a portion of the hybridization volume leaving overhead cavity 76 clear. Fig. 5C illustrates a cross-section of filling device 74 positioned into inlet port 20 of elevated portion 22. Elevated portion 22 can include an exhaust port 78 to allow the gas displaced by the hybridization fluid to escape to the environment. The exhaust port 78 can be configured to have a small enough width such that no substantial amount of hybridization fluid escapes during hybridization or can be covered by cap 36 along with inlet port 20. Fig. 5D illustrates a cross-section of filling device 74 positioned in inlet port 20 of elevated portion 22. Inlet port 20 can be plugged by a plug or can be plugged by a portion of cap 36. Plugged inlet port 20 is configured to restrain filling device 74. The plug and/or cap can be constructed of a gas permeable material to allow the gas displaced by the hybridization fluid to escape while retaining any evaporating hybridization fluid during the hybridization protocol. Elevated portion 22 can provide depth for insertion of filling device 74 into overhead cavity 76 to prevent the filling device 74 from contacting the substrate below. The length and/or the width of inlet port 20 can be configured to prevent filling device 74 from contacting the substrate. According to various embodiments, the other portions of the cover can include an exhaust port to allow the gas displaced by the hybridization fluid to escape while the inlet port is occupied by the filling device.

[063] According to various embodiments, the inlet port can be configured to be cylindrical or conical. The inlet port can be tapered holes to receive a filling device tip or the tapered tip of a filling device from an automated liquid delivery apparatus. The inlet port (or outlet port) can be threaded to accept liquid fittings, such as valves, Luers, barbs, o-rings, etc. for automated filling. The inlet port can be

sealed with a septum, a tapered plug, threaded plug, or similar device that can be part of or separate from the cap.

[064] According to various embodiments, adding the hybridization fluid includes taking the substrate through several wash and reagent addition steps and agitation as known in the art of hybridization. The substrate can then be removed from the housing manually or robotically. The detection chamber can be filled with overlay fluid to facilitate detection and remove any bubbles from the detection chamber that can interfere with detection. A cover slip or overlay is positioned on the detection frame to provide a flat optical surface for detection.

[065] According to various embodiments, Figs. 12 and 13 illustrate the substrate and detection chamber. Fig. 12 illustrates substrate 10 with detection frame 14 bounding microarray 12. Microarray 12 can be unitary as in Fig. 12 or divided into sections. Detection frame 14 can be made of harder material than the hybridization frame material. The detection frame 14 configured to maintain cover slip 130 substantially parallel to microarray 12 enclosing a detection volume of hybridization fluid in the detection chamber. Cover slip 130 can be transparent for detection and can be constructed of glass or plastic. In one embodiment, the detection chamber can hold 2.0 to 3.0 milliliters of detection volume. Fig. 13 illustrates substrate 10 with detection frame 14 bounding an array of microarrays 142 separated by barriers 140. Barriers 140 divide the detection chamber into an array of chambers such that positioning a cover slip on detection frame 14 provides an array of fluidically isolated chambers which can be interrogated individually by a detector.

[066] According to various embodiments, Figs. 18A-18F illustrates a cross-section of the substrate and detection frame. Figs. 18A-18F illustrate that substrate

10 can include support 180, adhesive layer 182, and binding layer 184. The microarray 12 binds to binding layer 184. Binding layer 184 can be NYLON, cellulose, nitrocellulose, gel, polymeric, or other porous membrane known in the art of polymer chemistry. Binding layer 184 can be sprayed on, laminated on, deposited on via chemical vapor deposition, or deposited on via electrostatic deposition on the substrate support 180 and adhesive layer 182. The support 180 can be non-absorbent. The support 180 can be glass, fused silica, silicon, plastic, metal, ceramic, or polymeric. The adhesive layer 182 can be hydrophobic to seal the hybridization chamber, such as a pressure sensitive acrylic adhesive. Detection frame 14 can be adhered to the microarray, any one of the layers in substrate 10, or the interface of those layers. Detection frame 14 can be adhered to each of these via its own adhesive or by using the adhesive properties of these layers. Fig. 18A illustrates detection frame 14 adhered to microarray 12. Fig. 18B illustrates detection frame 14 adhered to the interface of binding layer 184 and micro-array 12. Fig. 18C illustrates detection frame 14 adhered to binding layer 184. Fig. 18D illustrates detection frame 14 adhered to the interface of binding layer 184 and adhesive layer 182. Fig. 18E illustrates detection frame 14 adhered to adhesive layer 182. Fig. 18F illustrates detection frame 14 adhered to the interface of adhesive layer 182 and support 180. Each of these adhesion contacts provides a seal for the bottom of the hybridization chamber. According to various embodiments, the detection frame 14 can be adhered to the adhesive layer 182 through binding layer 184. Support 180 can be coated with adhesive layer 182 and binding layer 184. The outline of detection frame 14 can be removed from the binding layer 184. Detection frame 14 can be positioned on the adhesive layer 182 where the binding layer has been removed.

[067] According to various embodiments, binding layer 184 can be transparent or opaque. Binding layer 184 can be blackened to render it opaque and increase absorption of light during detection. Increasing absorption reduces light refracted by the binding layer 184 and reduces light that generates noise signals to the detector. Binding layer 184 can be blacked by adding carbon black into its composition. Binding 184 can include multiple layers of transparent or opaque quality.

[068] According to various embodiments, a method for hybridizing can include providing a housing including a cover, providing a substrate including a microarray, providing a hybridization chamber including a frame and a portion of the substrate, dispersing a hybridization fluid over the microarray, and detecting hybridization by SPR. Providing the substrate can include coupling a grating to the substrate. Providing the substrate can include coupling a prism to the substrate.

[069] According to various embodiments, a method for hybridizing can include providing a housing including a base and a cover, providing a substrate including a microarray, providing a hybridization chamber including a frame and a portion of the substrate, and releasably mating the cover to the base to seal the hybridization chamber and compress the frame. Releasably mating can include pressing down and sliding the cover to a locked position, pressing down and clamping the cover to a locked position, rotating the cover to a locked position, and sliding the base to a locked position. The cover can include a hinged portion so that releasably mating can include flipping and clamping the hinged portion to a locked position.

[070] According to various embodiments, a method of hybridization can include providing a hybridization fluid to the hybridization volume, hybridizing the

fluid with the microarray, releasing the cover and the base, removing the hybridization frame, positioning a cover slip over the detection frame to seal the detection volume, and detecting results from the hybridizing on the microarray. The method can include positioning the substrate within the base, positioning the hybridization frame proximate to the detection frame, and releasably mating the cover and the base.

[071] For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[072] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements. Moreover, all ranges disclosed herein are to be understood to encompass any and all subranges subsumed therein. For example, a range of "1 to 10" includes any and all subranges between (and including) the minimum value of 1 and the maximum value of 10, that

is, any and all subranges having a minimum value of equal to or greater than 1 and a maximum value of equal to or less than 10, e.g., 5.5 to 10.

[073] It is noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the," include plural referents unless expressly and unequivocally limited to one referent. Thus, for example, reference to "a substrate" includes two or more substrates. As used herein, the term "include" and its grammatical variants are intended to be non-limiting, such that recitation of items in a list or otherwise is not to the exclusion of other like items that can be substituted or added to the listed items.

[074] It will be apparent to those skilled in the art that various modifications and variations can be made to various embodiments described herein without departing from the spirit or scope of the present teachings. Thus, it is intended that the various embodiments described herein cover other modifications and variations within the scope of the appended claims and their equivalents.

WHAT IS CLAIMED IS:

1. An apparatus for hybridization comprising:
a substrate; and
a hybridization chamber comprising a detection frame, a hybridization frame,
and a portion of said substrate;
wherein said detection frame is positioned on said substrate, and said
hybridization frame is positioned proximate to said detection frame to form said
hybridization chamber.
2. The apparatus of claim 1, further comprising a housing for said
substrate and hybridization chamber, wherein said housing comprises a base and a
cover.
3. The apparatus of claim 2, wherein said base and said cover releasably
mate to seal said hybridization chamber.
4. The apparatus of claim 2, wherein said hybridization frame is
compressible and capable of forming a seal with said cover.
5. The apparatus of claim 4, wherein said cover comprises a compression
plate.
6. The apparatus of claim 2, wherein said cover comprises an elevated
portion.

7. The apparatus of claim 6, wherein said elevated portion comprises an inlet port.

8. The apparatus of claim 7, wherein said inlet port prevents a filling device from contacting said substrate.

9. The apparatus of claim 6, wherein said elevated portion projects from an upper surface of said cover.

10. The apparatus of claim 6, wherein said elevated portion is a portion of said cover.

11. The apparatus of claim 2, wherein said housing further comprises a window, wherein said window provides access to a bar code affixed to said substrate.

12. The apparatus of claim 2, wherein said cover comprises an inlet port and an outlet port for hybridization fluid flow-through.

13. The apparatus of claim 1, further comprising an instrument, wherein said instrument comprises a holder and a lid.

14. The apparatus of claim 13, wherein said holder and lid close to seal said hybridization chamber.

15. The apparatus of claim 1, wherein said portion of said substrate comprises said microarray.
16. The apparatus of claim 1, wherein hybridization frame is positioned on said detection frame.
17. The apparatus of claim 1, wherein said detection frame is constructed of harder elastomeric material than hybridization frame and forms a detection chamber.
18. The apparatus of claim 17, wherein said detection chamber comprises a plurality of barriers, said plurality of barriers forming a chamber array, wherein said chamber array is in fluid contact with an array of microarrays.
19. An apparatus for hybridization comprising:
a housing comprising a base and a cover;
a substrate comprising a microarray, wherein said substrate is positioned within said base; and
a hybridization chamber comprising a frame and a portion of said substrate;
wherein said cover releasably mates with said base to seal said hybridization chamber; and
wherein said cover comprises an inlet port to prevent a filling device from contacting said microarray.

20. The apparatus of claim 19, wherein said cover comprises an elevated portion positioned over said hybridization chamber.

21. The apparatus of claim 20, wherein said elevated portion comprises said inlet port.

22. The apparatus of claim 21, wherein said elevated portion comprises a filling cavity and a dispensing cavity, wherein said filling and dispensing cavities are aligned to prevent said filling device from contacting said portion of said substrate.

23. The apparatus of claim 19, wherein inlet port is configured to restrain said filling device to prevent said filling device from contacting said substrate.

24. A method for hybridizing comprising:
providing a housing comprising a base and a cover;
providing a substrate comprising a microarray;
providing a hybridization chamber comprising a frame and a portion of said substrate; and

releasably mating said cover to said base to seal said hybridization chamber and compress said frame.

25. The method of claim 24, wherein releasably mating comprises pressing down and sliding said cover to a locked position.

26. The method of claim 24, wherein releasably mating comprises pressing down and clamping said cover to a locked position.

27. The method of claim 24, wherein releasably mating comprises rotating said cover to a locked position.

28. The method of claim 24, wherein releasably mating comprises sliding said base to a locked position.

29. The method of claim 24, wherein said cover comprises a hinged portion, and wherein releasably locking comprises flipping and clamping said hinged portion to a locked position.

30. An apparatus for hybridization comprising:
a housing comprising a base and a cover, wherein said base and said cover are releasably mated;
a substrate comprising a microarray, wherein said housing is configured to house said substrate;
a detection frame, wherein said detection frame is positioned on said substrate, and said detection frame and said substrate form a detection volume; and
a hybridization frame, wherein said hybridization frame, said substrate and said cover form a hybridization volume;
wherein said hybridization volume and said detection volume are in fluid contact with said microarray.

31. A method of hybridization using the apparatus of claim 30, comprising:
providing a hybridization fluid to said hybridization volume;
hybridizing said fluid with said microarray;
releasing said cover and said base;
removing said hybridization frame;
positioning a cover slip over said detection frame to seal said detection volume; and
detecting results from said hybridizing on said microarray.

32. A method for assembling the apparatus of claim 30, comprising:
positioning said substrate within said base;
positioning said hybridization frame proximate to said detection frame; and
releasably mating said cover and said base.

33. An apparatus for hybridization comprising:
a substrate comprising a microarray, wherein said substrate comprises a binding layer and a support; and
a hybridization chamber comprising a frame and a portion of said substrate, wherein said frame is positioned on said support through said binding layer;
wherein said binding layer comprises a blackening agent for absorbance.

34. The apparatus of claim 33, further comprising a housing for said substrate and hybridization chamber, wherein said housing comprises a base and a cover.

35. The apparatus of claim 34, wherein said base and said cover releasably mate to seal said hybridization chamber.

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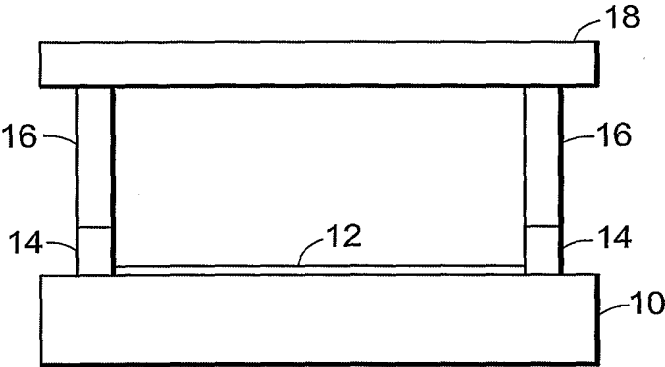


FIG. 1A

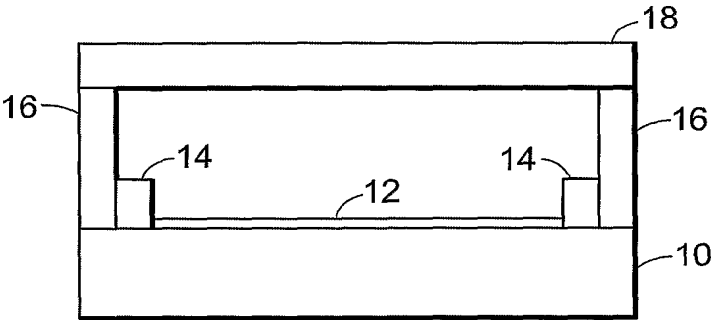


FIG. 1B

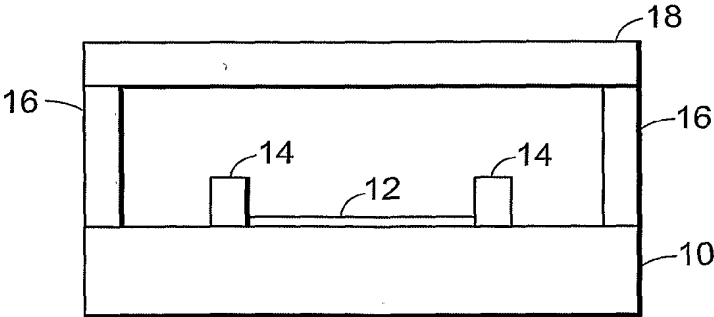


FIG. 1C

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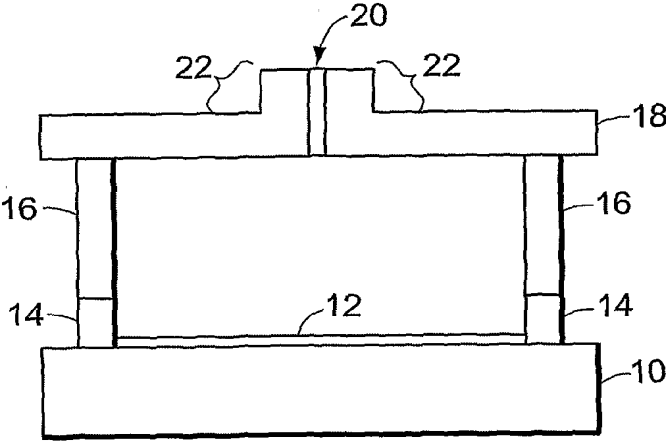


FIG. 2A

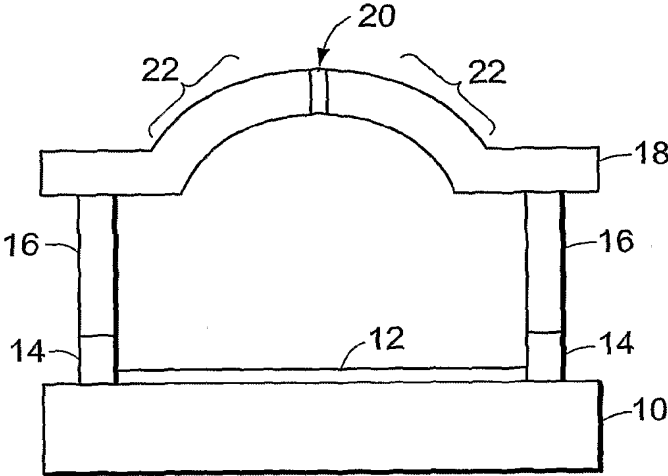


FIG. 2B

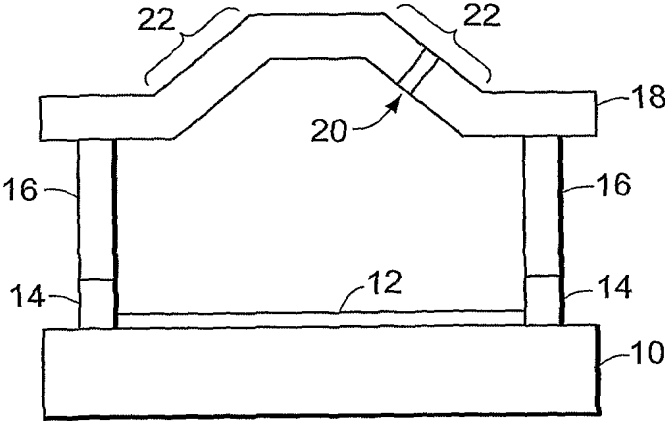


FIG. 2C

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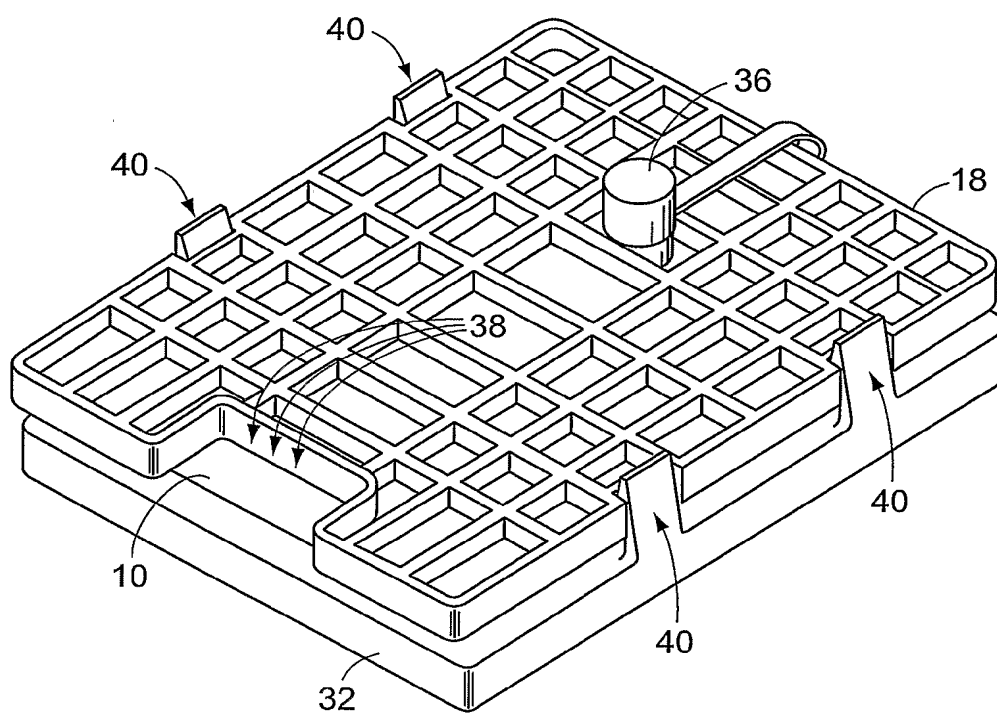


FIG. 3

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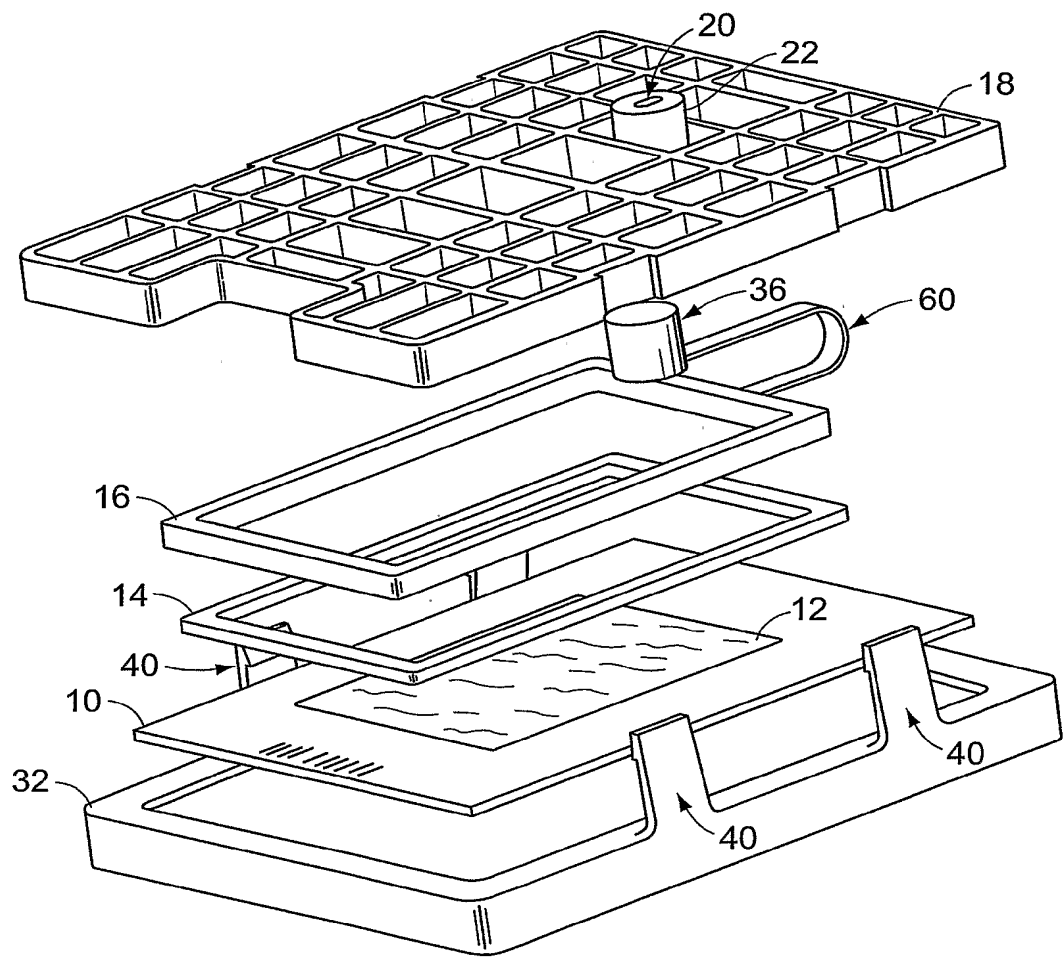


FIG. 4

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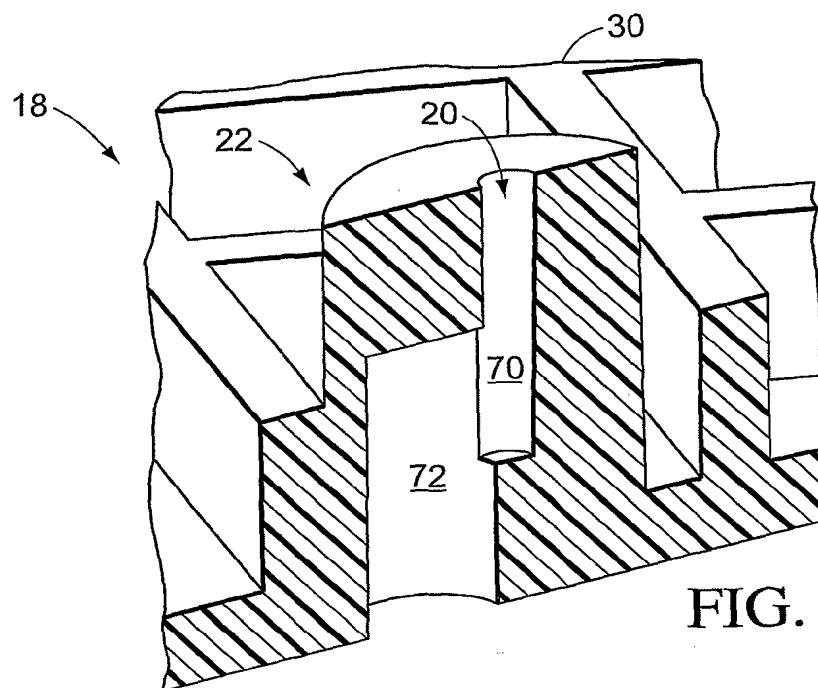


FIG. 5A

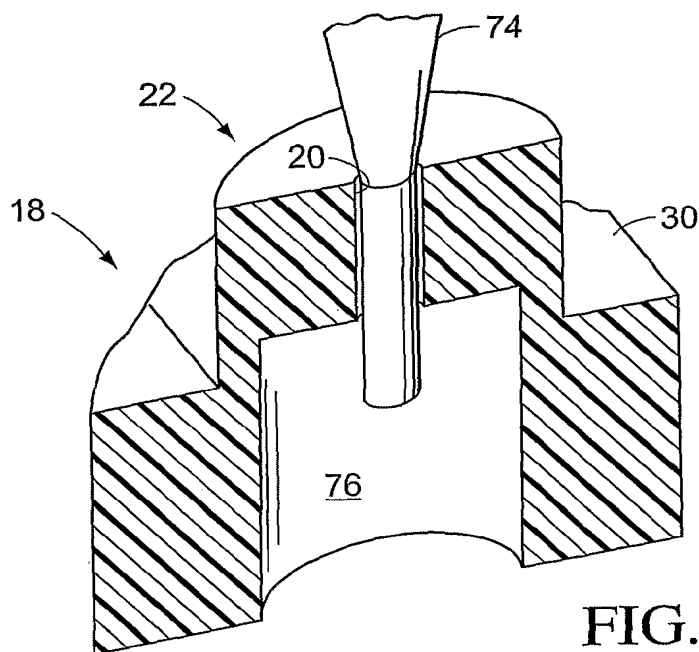


FIG. 5B

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FIG. 5C

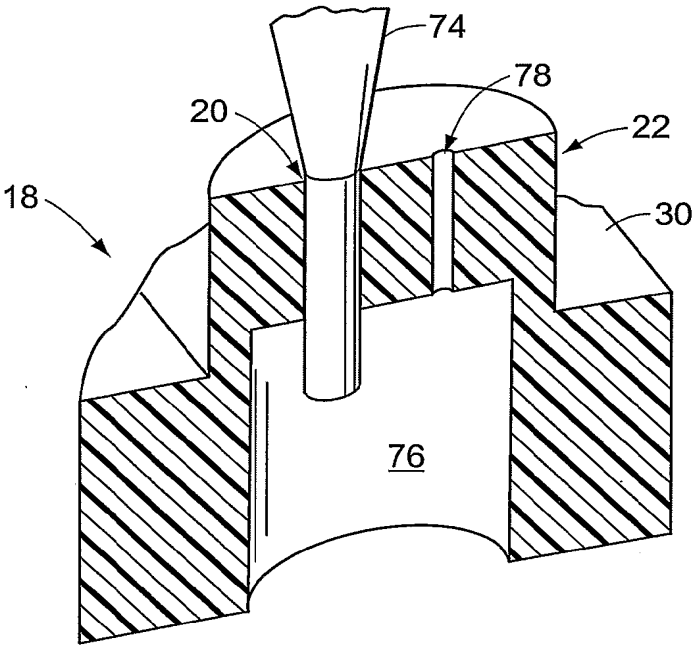
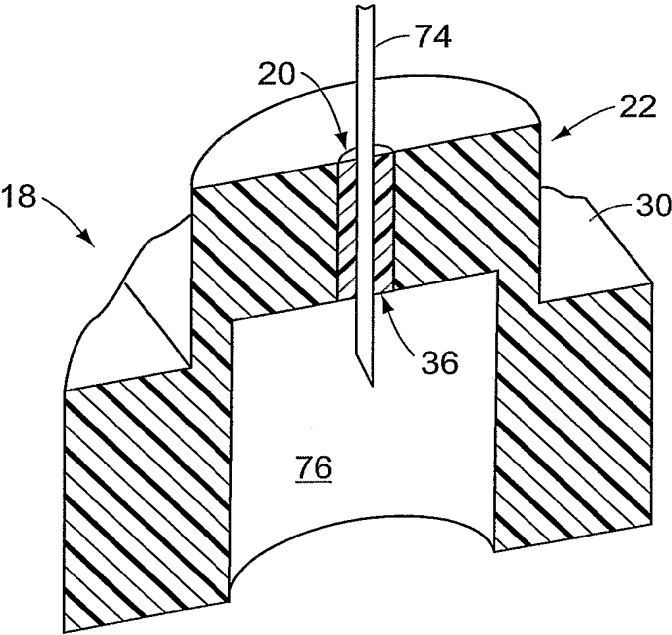


FIG. 5D



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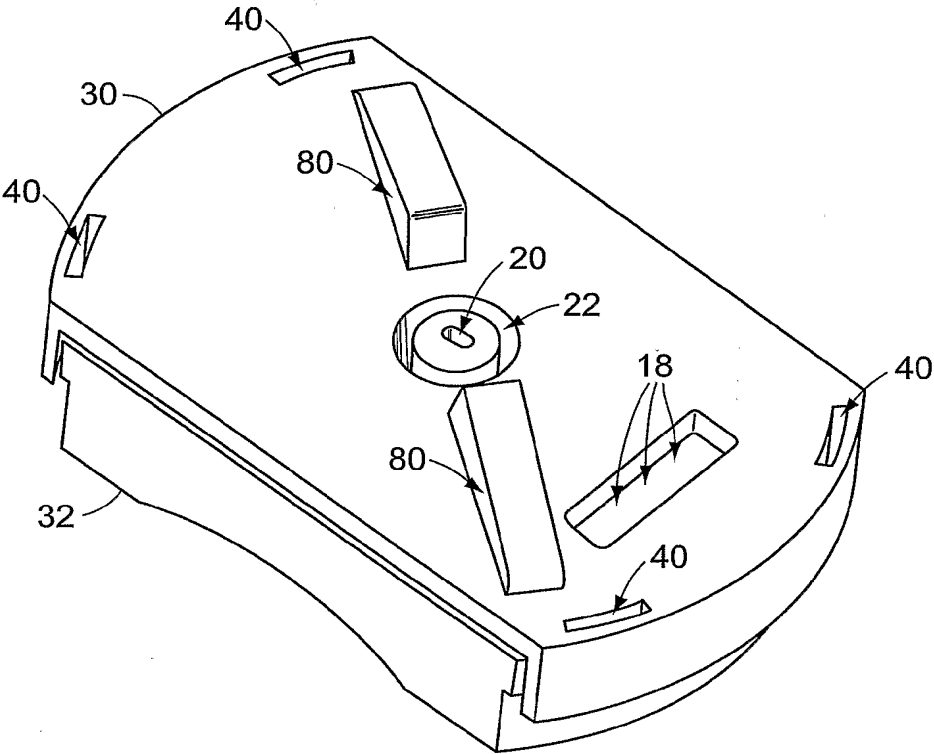


FIG. 6

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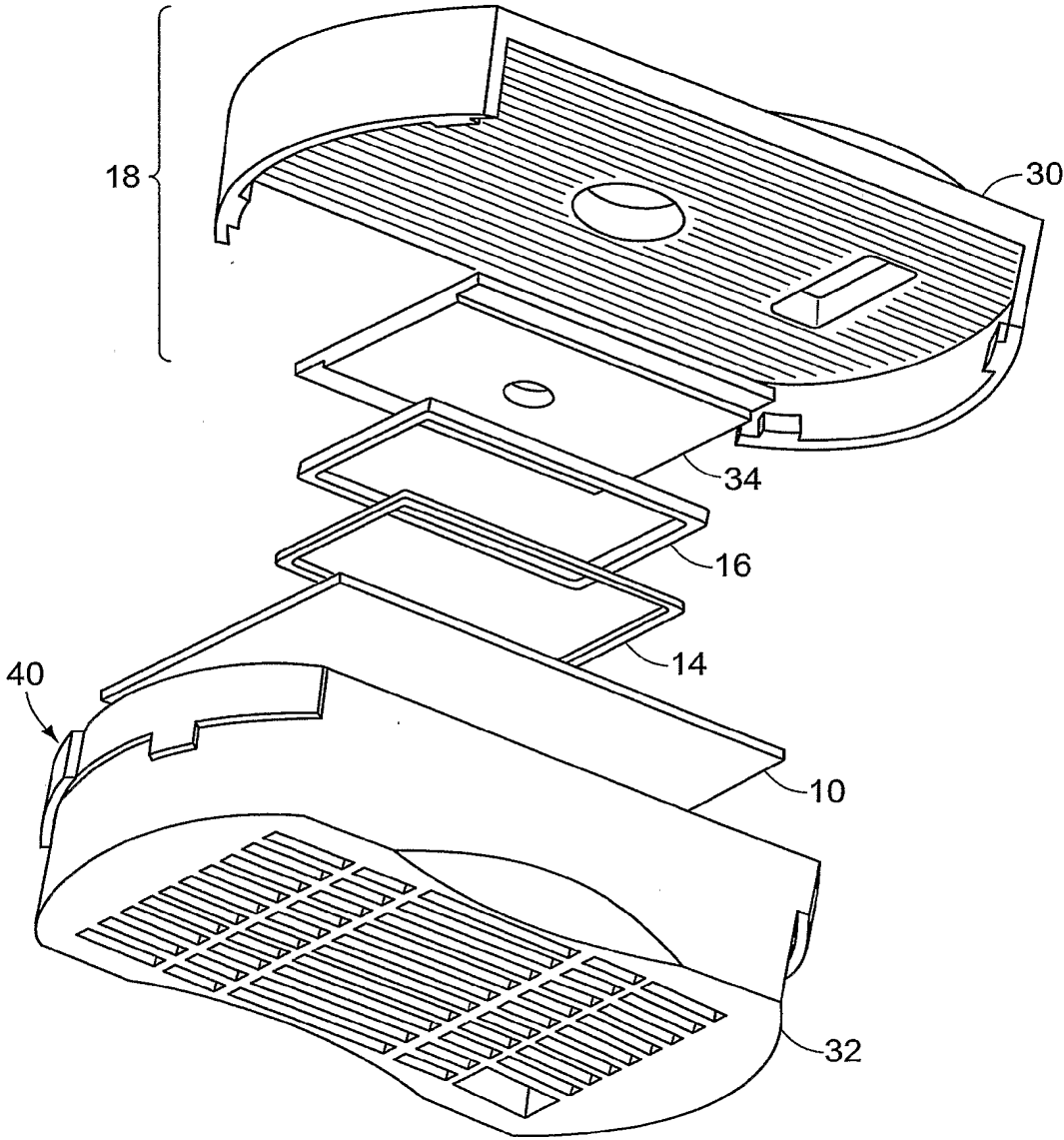


FIG. 7A

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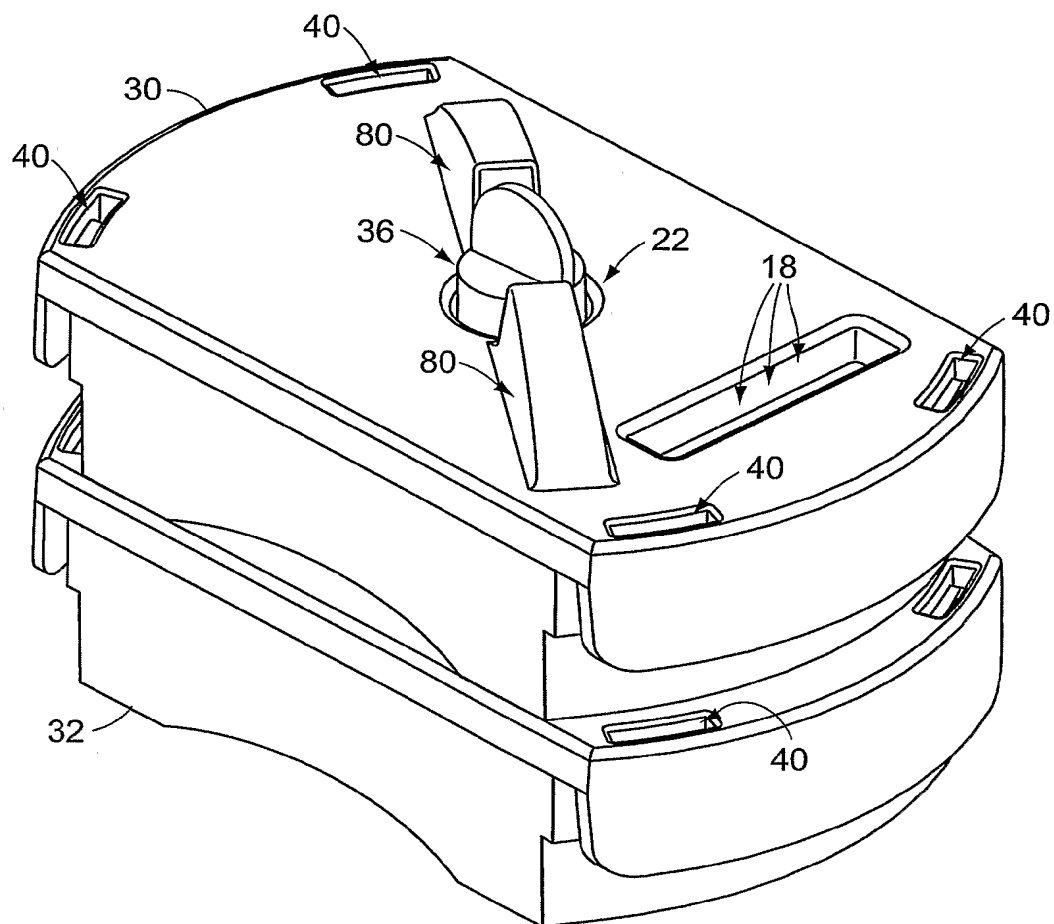


FIG. 7B

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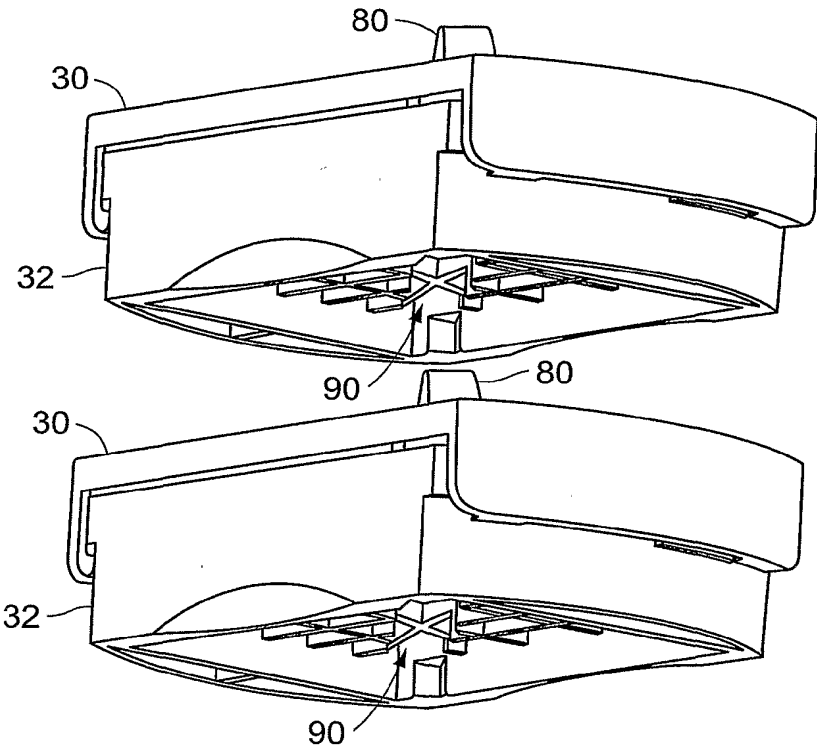


FIG. 7C

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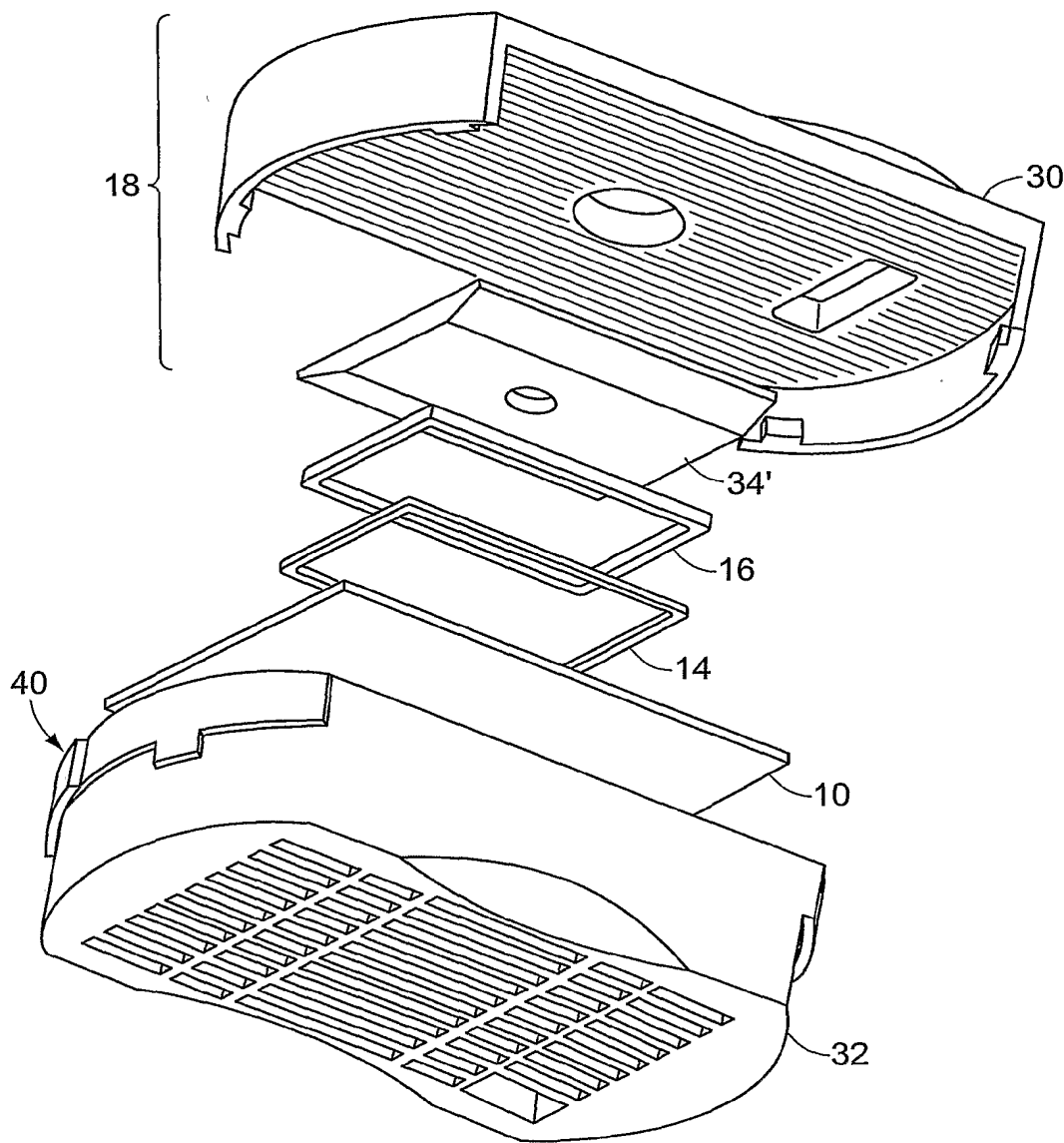
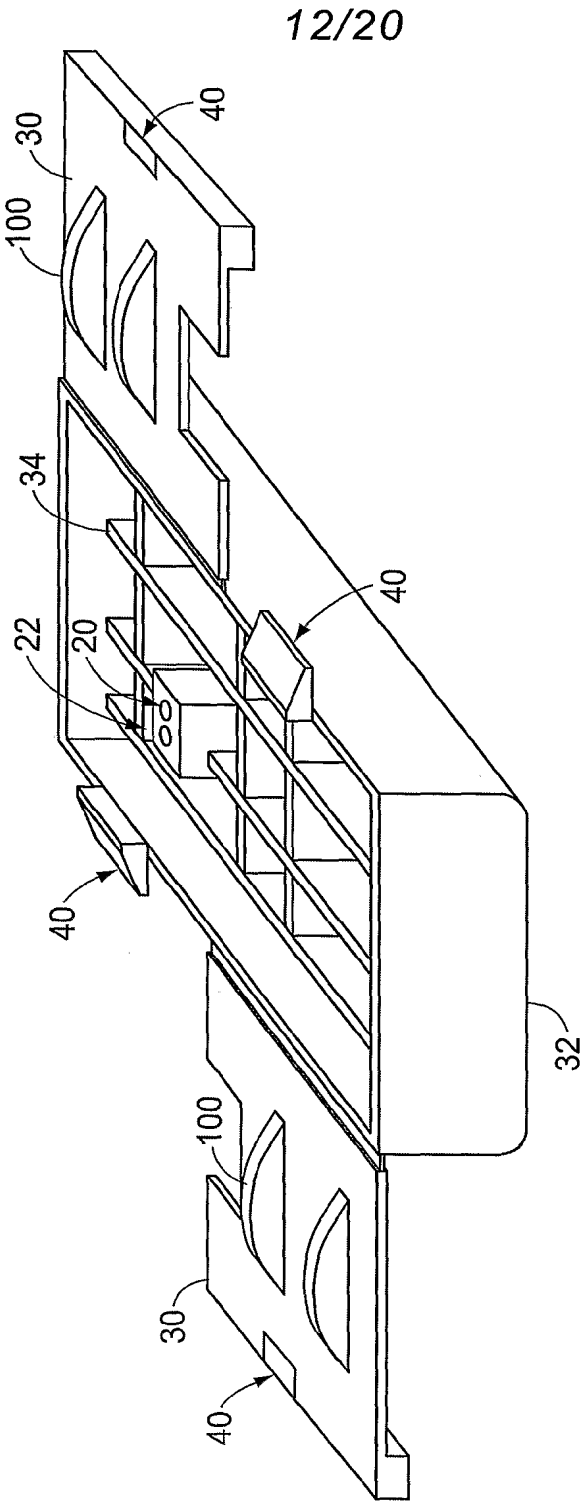
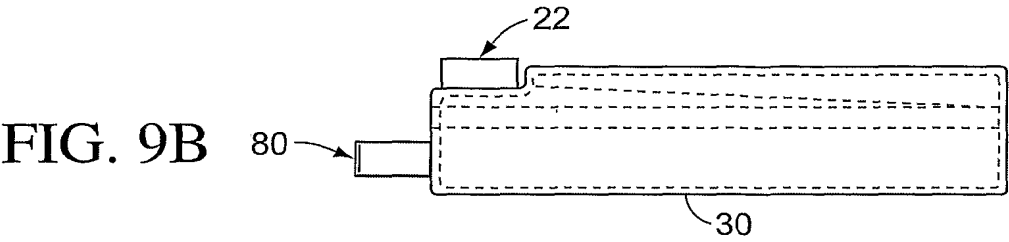
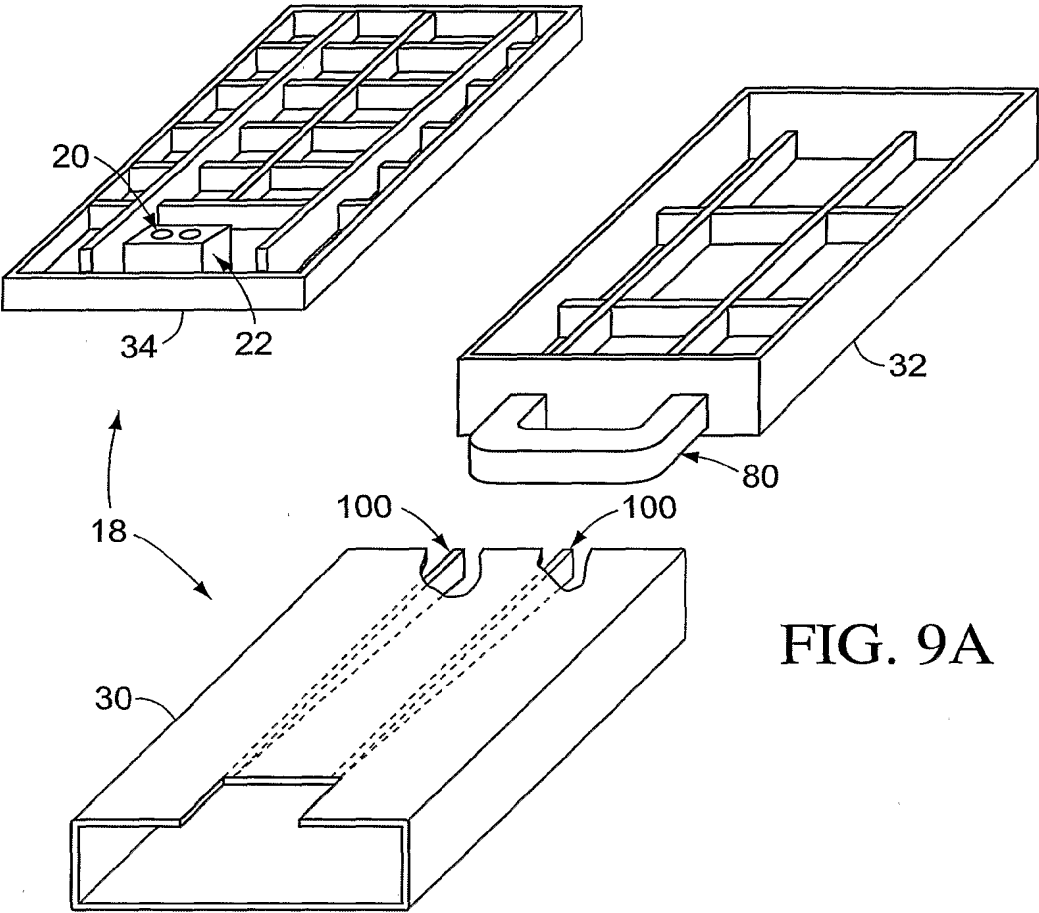


FIG. 7D



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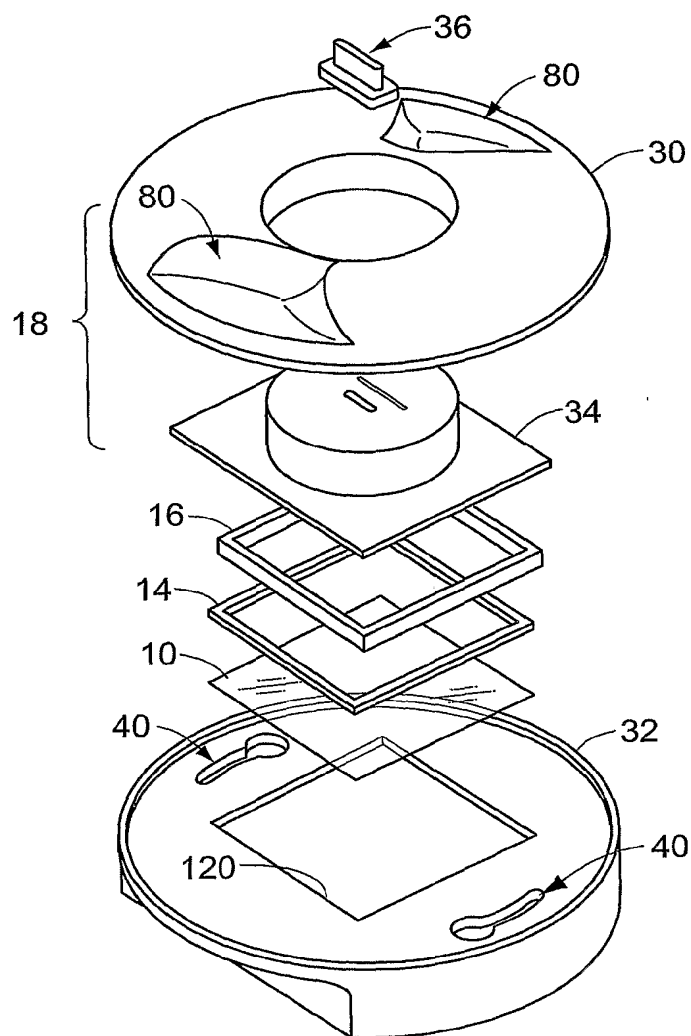


FIG. 10

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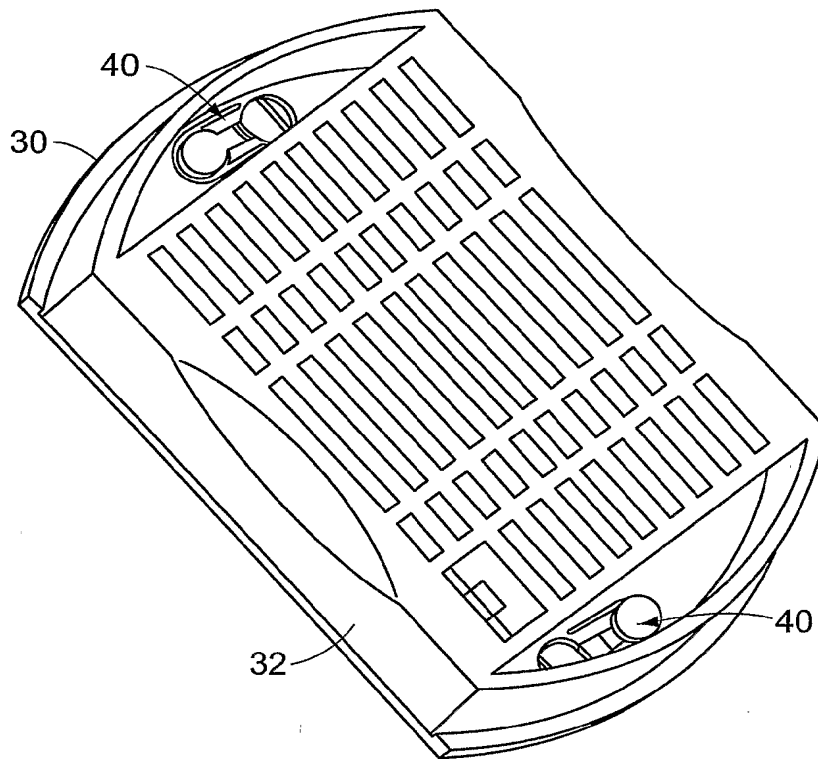


FIG. 11

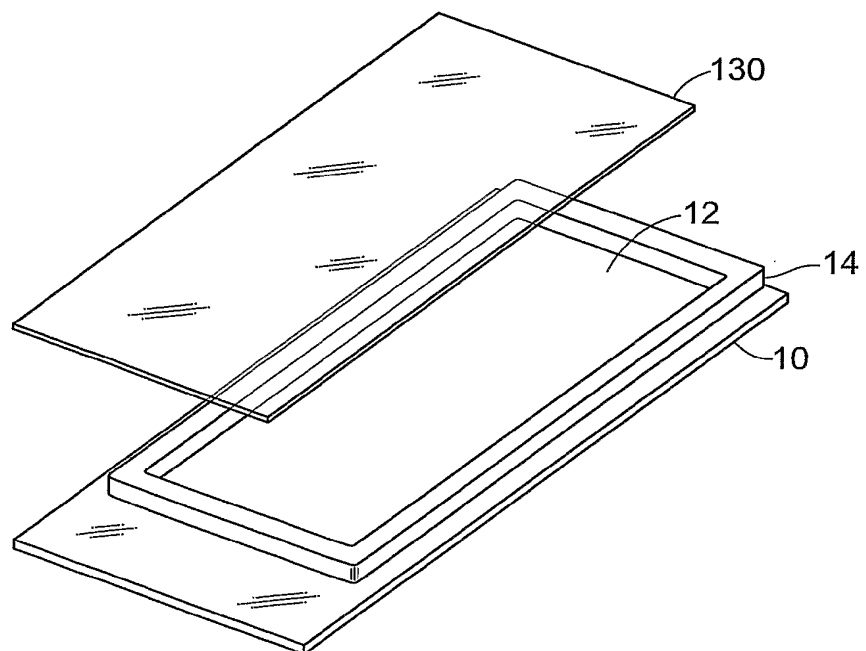


FIG. 12

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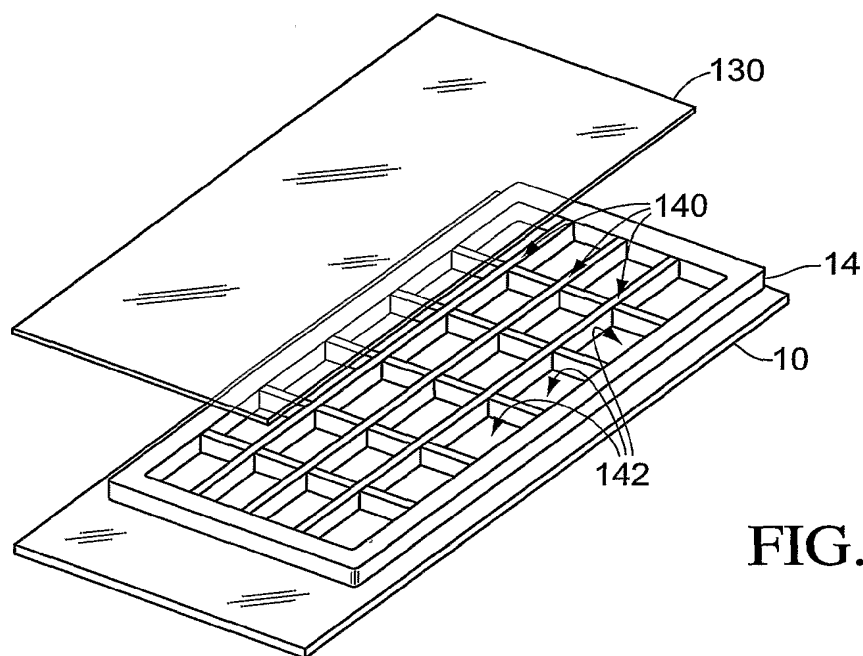


FIG. 13

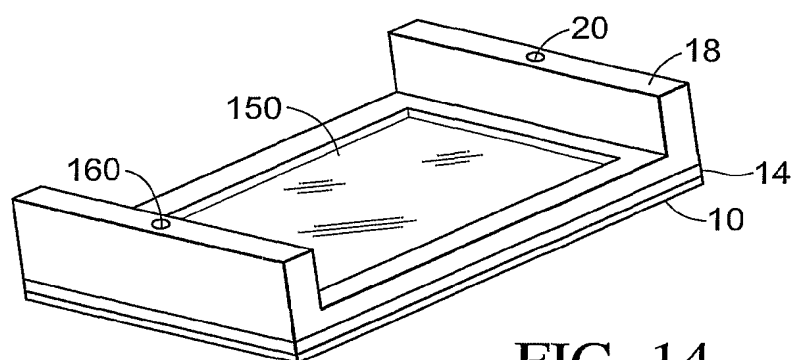


FIG. 14

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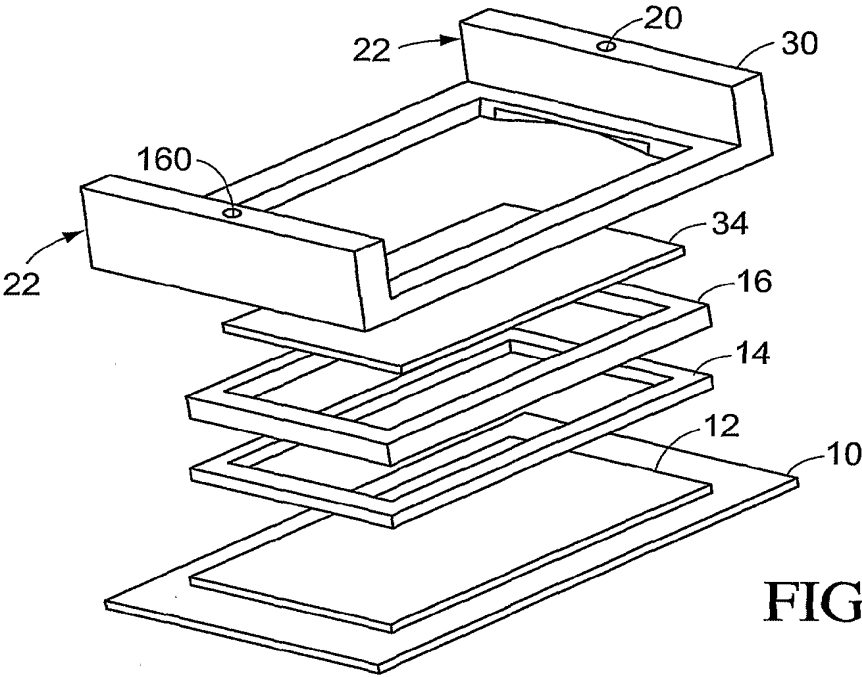


FIG. 15

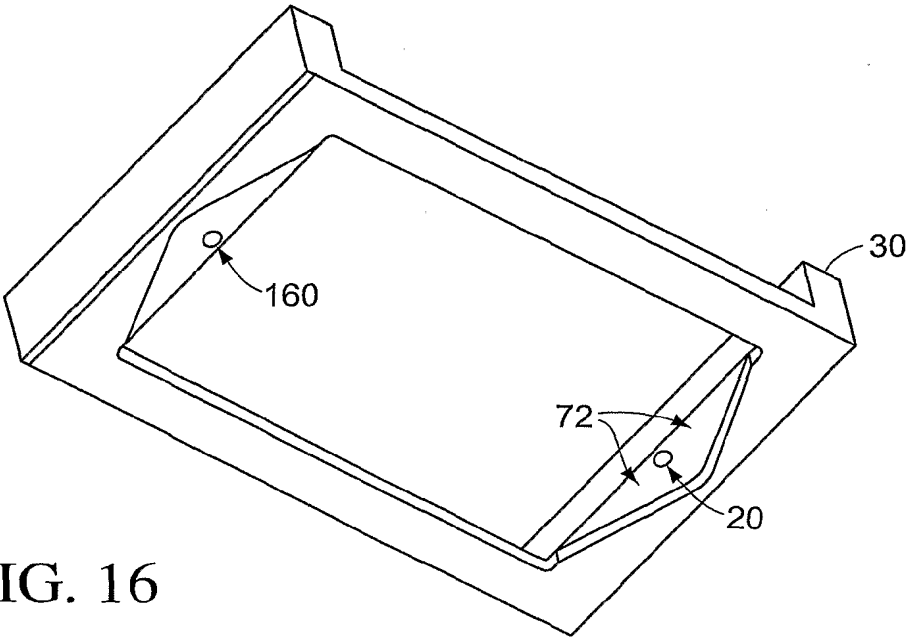


FIG. 16

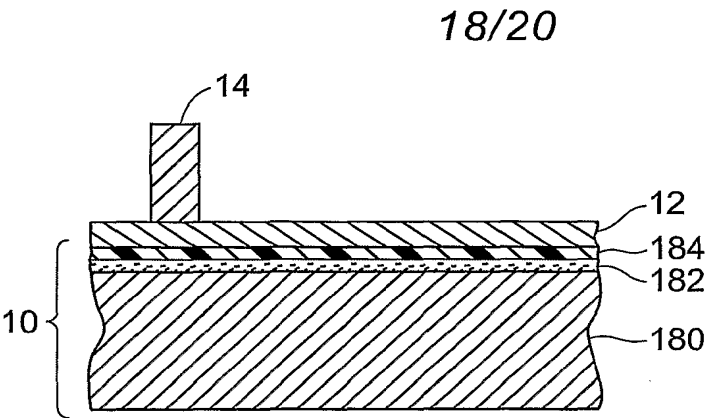


FIG. 17A

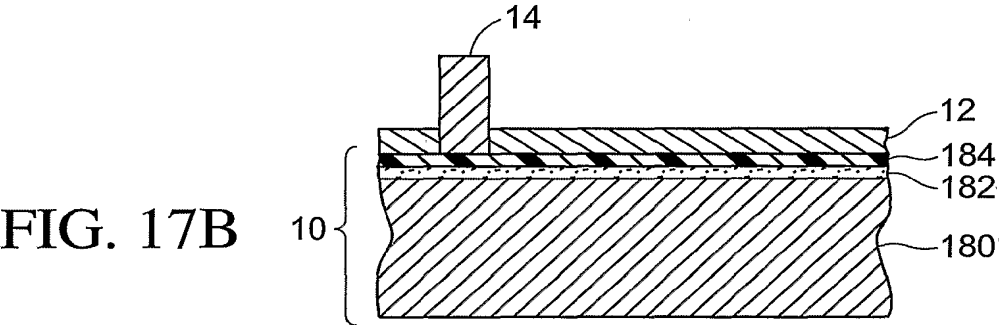


FIG. 17B

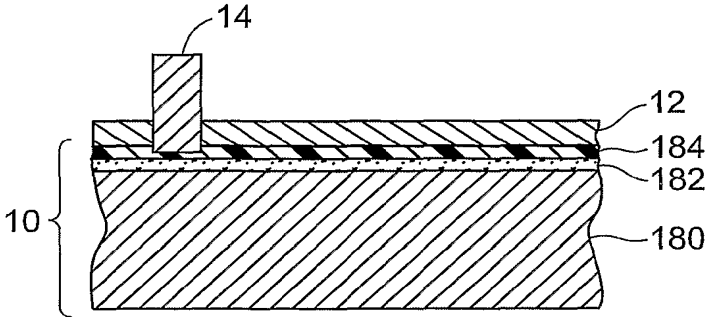


FIG. 17C

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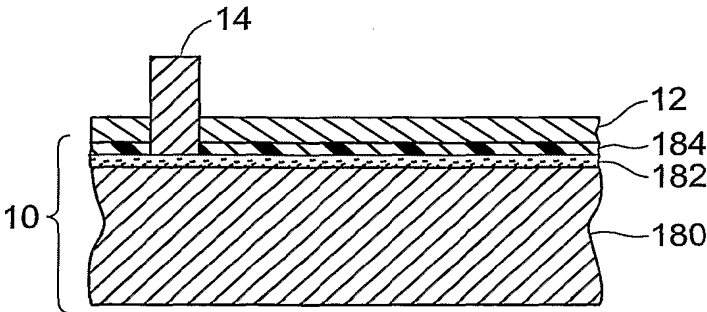


FIG. 17D

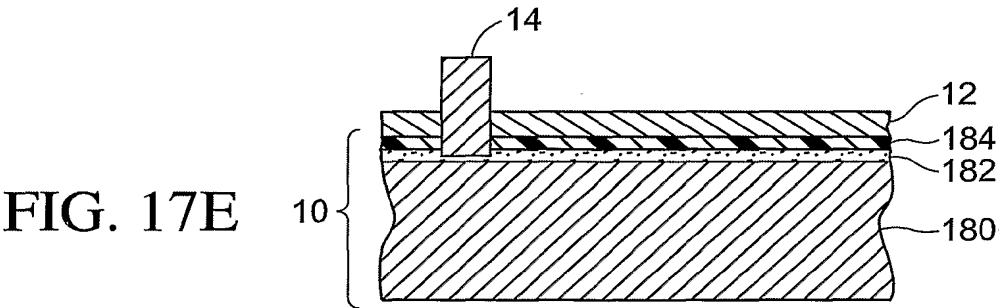


FIG. 17E

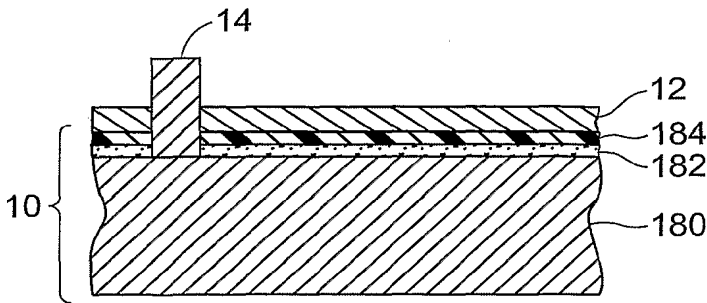
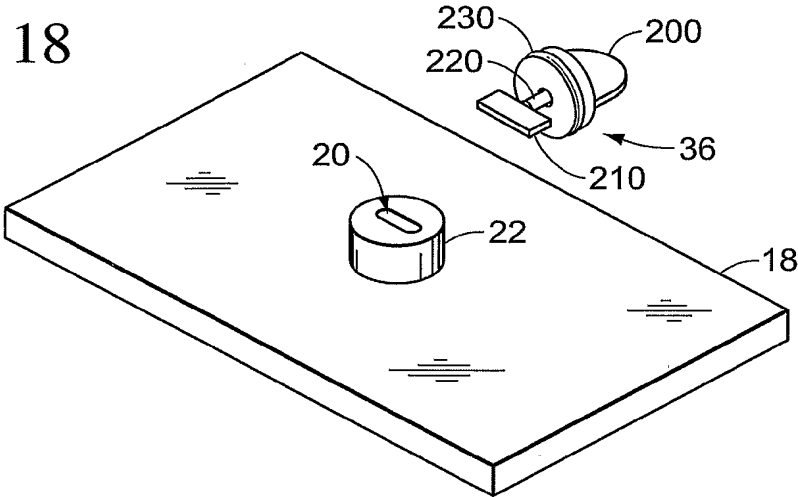


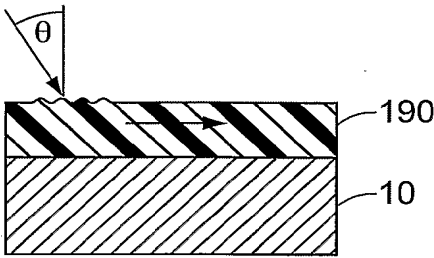
FIG. 17F

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FIG. 18



(a)



(b)

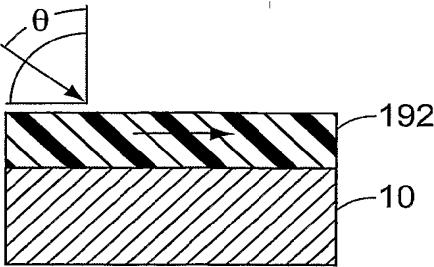


FIG. 19

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US2004/016907

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 B01J19/00 G01N33/543 B01L3/00

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)

IPC 7 B01J G01N B01L

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

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

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 01/31347 A (ALDRIDGE H. ET AL.) 3 May 2001 (2001-05-03) abstract; claims 1-12; figures 2,5,7 -----	1,2, 6-10,12, 13,15, 16,30,32
X	WO 01/54814 A (MCGARRY M.W. ET AL.) 2 August 2001 (2001-08-02) abstract; claims 1,2,10,11; figures 1-12 page 5, line 15 - page 8, line 4 -----	1-3,6, 10,11, 13-16, 24-27, 29-32
X	US 5 188 963 A (STAPLETON M.J.) 23 February 1993 (1993-02-23) abstract; claims 1-12; figures 1,5,6 ----- -/--	1-3,6,16

☒ Further documents are listed in the continuation of box C☒ Patent family members are listed in annex

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Date of the actual completion of the international search

4 November 2004

Date of mailing of the international search report

23/11/2004

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US2004/016907

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 334 980 B1 (HAYES D.J. ET AL.) 1 January 2002 (2002-01-01) abstract; claims 1,14,33,44; figures 1-5,8-10 -----	1,2,6,16
A	US 6 197 595 B1 (ANDERSON R.C. ET AL.) 6 March 2001 (2001-03-06) abstract; figures 2,3,5B,7A -----	1-35
A	FURUKI M. ET AL.: "Surface plasmon resonance sensors utilizing microfabricated channels" SENSORS AND ACTUATORS B, ELSEVIER, vol. 79, no. 1, 25 September 2001 (2001-09-25), pages 63-69, XP004297622 ISSN: 0925-4005 the whole document -----	1-35

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US2004/016907

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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Electronic Acknowledgement Receipt

EFS ID:	40279576
Application Number:	16988284
International Application Number:	
Confirmation Number:	1697
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Tiffany A. Reiter/Lindsey Ericson
Filer Authorized By:	Tiffany A. Reiter
Attorney Docket Number:	47706-0004026
Receipt Date:	13-AUG-2020
Filing Date:	07-AUG-2020
Time Stamp:	17:01:15
Application Type:	Utility under 35 USC 111(a)

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
16/988,284	08/07/2020		1720	47706-0004026	1	1

CONFIRMATION NO. 1697

FILING RECEIPT



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10x GENOMICS
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

Date Mailed: 08/17/2020

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

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

Inventor(s)

Mark S. CHEE, San Diego, CA;

Applicant(s)

Prognosys Biosciences, Inc., San Diego, CA;

Power of Attorney: The patent practitioners associated with Customer Number 159367

Domestic Priority data as claimed by applicant

This application is a CON of 16/414,213 05/16/2019
which is a CON of 16/402,098 05/02/2019 PAT 10472669
which is a CON of 16/276,235 02/14/2019 PAT 10480022
which 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 for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see <http://www.uspto.gov> for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

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

If Required, Foreign Filing License Granted: 08/13/2020

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/988,284**

Projected Publication Date: 11/26/2020

Non-Publication Request: No

Early Publication Request: No

Title

Spatially Encoded Biological Assays

Preliminary Class

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

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at <http://www.uspto.gov/web/offices/pac/doc/general/index.html>.

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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NOT GRANTED

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 16/988,284	
APPLICATION AS FILED - PART I							
(Column 1)		(Column 2)		SMALL ENTITY		OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE(\$)	FEE(\$)		RATE(\$)	FEE(\$)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(j))	1	minus 20 = *			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1	minus 3 = *				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL		TOTAL	1720
APPLICATION AS AMENDED - PART II							
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY	
AMENDMENT A	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)
	Total (37 CFR 1.16(i))	*	Minus **	=	x =	OR	x =
	Independent (37 CFR 1.16(h))	*	Minus ***	=	x =	OR	x =
	Application Size Fee (37 CFR 1.16(s))					OR	
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					OR	
				TOTAL ADD'L FEE		TOTAL ADD'L FEE	
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY	
AMENDMENT B	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)
	Total (37 CFR 1.16(i))	*	Minus **	=	x =	OR	x =
	Independent (37 CFR 1.16(h))	*	Minus ***	=	x =	OR	x =
	Application Size Fee (37 CFR 1.16(s))					OR	
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					OR	
				TOTAL ADD'L FEE		TOTAL ADD'L FEE	
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.							



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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/988,284	08/07/2020	Mark S. CHEE	47706-0004026	1697

159367 7590 08/20/2020

FISH & RICHARDSON P.C.

10x GENOMICS

P.O. BOX 1022

MINNEAPOLIS, MN 55440-1022

EXAMINER

ART UNIT

PAPER NUMBER

1634

NOTIFICATION DATE

DELIVERY MODE

08/20/2020

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

<i>Decision Granting Request for Prioritized Examination (Track I)</i>	Application No. 16/988,284	Applicant(s) CHEE, Mark S.	
	Examiner CHERYL P GIBSON BAYLOR	Art Unit OPET	AIA (FITF) Status No
1. THE REQUEST FILED <u>07 August 2020</u> 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 <u>petition for extension of time</u> to extend the time period for filing a reply; B. filing an <u>amendment to amend the application to contain more than four independent claims, more than thirty total claims</u>, or a multiple dependent claim; C. filing a <u>request for continued examination</u> ; 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 CHERYL GIBSON BAYLOR at (571)272-3213. In his/her absence, calls may be directed to Petition Help Desk at (571) 272-3282.			
/CHERYL GIBSON BAYLOR/ Paralegal Specialist, OPET			

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	16988284	
Filing Date	07-Aug-2020	
First Named Inventor	Mark CHEE	
Attorney Docket Number	47706-0004026	
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)		
16414213 filed on 05/16/2019 16435295 filed on 06/07/2019 16435176 filed on 06/07/2019 16430015 filed on 06/03/2019 16430109 filed on 06/03/2019 16734237 filed on 01/03/2020 16669246 filed on 10/30/2019 16443771 filed on 06/17/2019 16437726 filed on 06/11/2019 16816192 filed on 03/11/2020 16913672 filed on 06/26/2020 16816177 filed on 03/11/2020 16894369 filed on 06/05/2020 16837924 filed on 04/01/2020		

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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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)

10662467

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10308982

9371598

10612079

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10494667

10472669

10619196

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 <u>76370</u> ☐ 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	/Kimberly M. Maize, Reg. No. 76,370/
Name	Kimberly M. Maize

*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:	16988284			
Filing Date:	07-Aug-2020			
Title of Invention:	Spatially Encoded Biological Assays			
First Named Inventor/Applicant Name:	Mark S. CHEE			
Filer:	Kimberly M. Maize/Shannon Stadnick			
Attorney Docket Number:	47706-0004026			
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.: 16988284

Filing Date: 07-Aug-2020

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on August 20, 2020

☒ 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:	40311575
Application Number:	16988284
International Application Number:	
Confirmation Number:	1697
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Kimberly M. Maize/Shannon Stadnick
Filer Authorized By:	Kimberly M. Maize
Attorney Docket Number:	47706-0004026
Receipt Date:	20-AUG-2020
Filing Date:	07-AUG-2020
Time Stamp:	12:06:56
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	E20208JC06541750
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	50604	no	3
			a52cfa4dc3696eeff0e928569cb16316d41ad692		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30507	no	2
			2a0b19ec9f4975294279a292b0f84b826b71d70e		
Warnings:					
Information:					
Total Files Size (in bytes):			81111		
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Mark S. Chee	Art Unit :	Unknown
Application No. :	16/988,284	Examiner :	Unknown
Filed :	August 7, 2020	Conf. No. :	1697
Title :	SPATIALLY ENCODED BIOLOGICAL ASSAYS		

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.

Electronic Acknowledgement Receipt

EFS ID:	40406996
Application Number:	16988284
International Application Number:	
Confirmation Number:	1697
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Peter Poulin/Lisa Gray
Filer Authorized By:	Peter Poulin
Attorney Docket Number:	47706-0004026
Receipt Date:	27-AUG-2020
Filing Date:	07-AUG-2020
Time Stamp:	21:49:57
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		47706-0004026prelimamendment.pdf	79136 c23aadd23fc2b95c9e9526f7c160d6b5a49b25d0	yes	7

Multipart Description/PDF files in .zip description			
Document Description	Start	End	
Applicant Arguments/Remarks Made in an Amendment	7	7	
Claims	2	6	
Preliminary Amendment	1	1	

Warnings:

Information:

Total Files Size (in bytes):	79136
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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.

First Named Inventor : Mark S. Chee
Application No. : 16/988,284
Filed : August 7, 2020
Page : 7 of 7

Attorney Docket: 47706-0004026 / 700-000124US

REMARKS

Applicant has canceled claim 1 and added new claims 2-29, and requests that the application be examined in view of the amendments presented herein.

No fees are believed to be due. Please apply any necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: August 27, 2020

/Peter R. Poulin Reg. No. 74,643/

Peter Poulin
Reg. No. 74,643

Customer Number 159367
Fish & Richardson P.C.
Telephone: (617) 956-5901
Facsimile: (877) 769-7945

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Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1. (Canceled)
2. (New) A method of detecting a target protein in a tissue sample, comprising:
contacting a tissue sample with a plurality of probes, wherein a probe of the plurality of probes comprises an antibody that specifically binds a target protein of the tissue sample and is coupled to an oligonucleotide comprising a sequence;
generating an encoded probe comprising the oligonucleotide and an encoding agent, wherein the encoding agent comprises an oligonucleotide sequence that is associated with a location of the target protein in the tissue sample; and
determining all or a portion of a sequence of the encoded probe or a complement thereof to detect the target protein and determine its associated location in the tissue sample.
3. (New) The method of claim 2, further comprising separating unbound probes of the plurality of probes that do not interact with the target protein from the tissue sample.
4. (New) The method of claim 2, further comprising separating the oligonucleotide of the probe comprising the antibody that specifically binds to the target protein from oligonucleotides of probes of the plurality of probes that do not interact with the target protein.
5. (New) The method of claim 4, further comprising, after separating the oligonucleotide of the probe comprising the antibody that specifically binds to the target protein and before determining all or a portion of a sequence of the encoded probe or a complement thereof, amplifying the separated oligonucleotide.

6. (New) The method of claim 2, further comprising obtaining an image of the tissue sample and determining a region of interest for analysis of the tissue sample based on the image.
7. (New) The method of claim 6, wherein the location of the target protein in the tissue sample corresponds to a location of the region of interest, and wherein the oligonucleotide sequence of the encoding agent is uniquely associated with the region of interest of the tissue sample.
8. (New) The method of claim 2, wherein the probe is a first probe, the antibody is a first antibody, the target protein is a first target protein, the oligonucleotide of the probe is a first oligonucleotide, and the encoding agent is a first encoding agent, and wherein a second probe of the plurality of probes comprises a second antibody that specifically binds a second target protein of the tissue sample and is coupled to a second oligonucleotide comprising a sequence.
9. (New) The method of claim 8, wherein the first and second antibodies are the same and the first and second target proteins are the same.
10. (New) The method of claim 9, wherein the first target protein is located in a first region of interest of the tissue sample, the second target protein is located in a second region of interest of the tissue sample different from the first region, and the encoded probe is a first encoded probe, the method further comprising:
 - generating a second encoded probe that comprises the second oligonucleotide and a second encoding agent, wherein the second encoding agent comprises an oligonucleotide sequence that is associated with a location of the second target protein in the tissue sample; and
 - determining all or a portion of a sequence of the second encoded probe or a complement thereof to detect the second target protein and determine its associated location in the tissue sample,
 - wherein the oligonucleotide sequence of the second encoding agent is different from the oligonucleotide sequence of the first encoding agent.

11. (New) The method of claim 10, wherein the location of the first target protein corresponds to a location of the first region of interest in the tissue sample, and the location of the second target protein corresponds to a location of the second region of interest in the tissue sample.

12. (New) The method of claim 8, wherein the first and second antibodies are different and the first and second target proteins are different.

13. (New) The method of claim 12, wherein the first and second target proteins are located in a common region of interest in the tissue sample, and the encoded probe is a first encoded probe, the method further comprising:

generating a second encoded probe that comprises the second oligonucleotide and a second encoding agent, wherein the second encoding agent comprises an oligonucleotide sequence that is associated with a location of the second target protein in the tissue sample; and
determining all or a portion of a sequence of the second encoded probe or a complement thereof to detect the second target protein and determine its associated location in the tissue sample,

wherein the oligonucleotide sequence of the second encoding agent is the same as the oligonucleotide sequence of the first encoding agent.

14. (New) The method of claim 13, wherein the locations of the first and second target proteins each correspond to a location of the common region of interest in the tissue sample.

15. (New) The method of claim 2, wherein the oligonucleotide sequence of the encoding agent comprises a functional sequence for use in sequencing the encoded probe.

16. (New) The method of claim 15, wherein the functional sequence comprises an adapter sequence.

17. (New) The method of claim 15, wherein the functional sequence is located at an end of the encoded probe.

18. (New) The method of claim 2, wherein the sequence of the encoded probe comprises two functional sequences for use in sequencing the encoded probe.

19. (New) The method of claim 18, wherein the functional sequences are located at opposite ends of the encoded probe.

20. (New) The method of claim 18, wherein the functional sequences comprise adapter sequences.

21. (New) The method of claim 2, further comprising:
 contacting multiple different regions of interest in the tissue sample with the plurality of probes; and
 detecting expression of the target protein in two or more of the multiple different regions of interest in the tissue sample.

22. (New) The method of claim 21, further comprising determining the multiple different regions of interest in the tissue sample based on histological features of the tissue sample.

23. (New) The method of claim 21, further comprising determining the multiple different regions of interest in the tissue sample by identifying locations of the target protein in the tissue sample from an image of the tissue sample.

24. (New) The method of claim 21, further comprising detecting expression of the target protein in multiple different regions of interest in two or more serial sections of the tissue sample.

25. (New) The method of claim 24, further comprising generating a three-dimensional map of expression of the target protein in the tissue sample.
26. (New) The method of claim 21, comprising detecting expression of the target protein in at least 25 different regions of interest in the tissue sample.
27. (New) The method of claim 21, comprising detecting expression of the target protein in at least 100 different regions of interest in the tissue sample.
28. (New) The method of claim 2, wherein the tissue sample is a tissue section.
29. (New) The method of claim 28, wherein the tissue section comprises a fresh-frozen or formalin-fixed paraffin embedded (FFPE) tissue sample.

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/988,284	Filing Date 08/07/2020	☐ To be Mailed
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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)	RATE (\$)	ADDITIONAL FEE (\$)
AMENDMENT	08/27/2020		CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	
	Total (37 CFR 1.16(i))	* 28	Minus	** 20	= 8	x \$100 = 800
	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						800
AMENDMENT			CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	
	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.						LDRC
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".						/EVA V GILLIS/
*** 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.						

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