



June 3, 2019

Attorney Docket No.: 47706-0004008

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

Inventor(s): MARK 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>
Certification and Request for Prioritized Examination (Track I)	2
Application Data Sheet	9
Specification	43
Claims	1
Abstract	1
Drawing(s)	5
Declaration	1
Power of Attorney	2

The applicant claims small entity status under 37 CFR 1.27.

Basic Filing Fee	\$75
Search Fee	\$330
Examination Fee	\$380
Publication fee	\$0
Track I processing fee	\$70
Track I prioritized examination fee	\$2000
Total Claims 1 over 20 0 x \$50	\$0
Independent Claims 1 over 3 0 x \$230	\$0
Fee for Multiple Dependent claims	\$0
Application size fee for each 50 pages over 100	
Total Sheets: 50 x .75 - 100/50 = 0 x	\$0



Commissioner for Patents
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Total Filing fee

\$2855

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
61938140.doc

Electronic Patent Application Fee Transmittal

Application Number:				
Filing Date:				
Title of Invention:	Spatially Encoded Biological Assays			
First Named Inventor/Applicant Name:	Mark Chee			
Filer:	Peter Poulin/Shannon Stadnick			
Attorney Docket Number:	47706-0004008			
Filed as Small 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 FILING FEE (ELECTRONIC FILING)	4011	1	75	75
UTILITY SEARCH FEE	2111	1	330	330
UTILITY EXAMINATION FEE	2311	1	380	380
REQUEST FOR PRIORITIZED EXAMINATION	2817	1	2000	2000
Pages:				
Claims:				
Miscellaneous-Filing:				
PROCESSING FEE, EXCEPT PROV. APPLS.	2830	1	70	70

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 (\$)				2855

Electronic Acknowledgement Receipt

EFS ID:	36186373
Application Number:	16430015
International Application Number:	
Confirmation Number:	8975
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark Chee
Customer Number:	159367
Filer:	Peter Poulin/Alysha Claflin
Filer Authorized By:	Peter Poulin
Attorney Docket Number:	47706-0004008
Receipt Date:	03-JUN-2019
Filing Date:	
Time Stamp:	17:23:49
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$2855
RAM confirmation Number	060419INTEFSW00004392061050
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	TrackOne Request	Track_1.pdf	146632	no	2
			7aa2e71025cadfcdbf9962afb932d77b2ee1f899		
Warnings:					
Information:					
2	Application Data Sheet	ADS.pdf	1256401	no	9
			2b4545d2380aff71bcbfc6404e84acbcc99b9395		
Warnings:					
Information:					
3		Application.pdf	336090	yes	45
			5ea78fd0050190f72b2c8ba0b6b5f1e8b4687066		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Abstract		45	45	
	Claims		44	44	
	Specification		1	43	
Warnings:					
Information:					
4	Drawings-only black and white line drawings	Figures.pdf	802881	no	5
			eef852aec2b44492b526ae07990fe4a4ba746da0		
Warnings:					
Information:					
5	Power of Attorney	POA_.pdf	209544	no	2
			e9d50ba63af995b18c3bc23dc42e68d8ef783fa6		

Warnings:					
Information:					
6	Oath or Declaration filed	Declaration.pdf	48408	no	1
			3388bb26440da2ee37d5c40faa34f853b27bfa29		
Warnings:					
Information:					
7	Transmittal of New Application	Transmittal_.pdf	110699	no	2
			85e3fb93dc7c1cf9f446824e3b05f41ec312517c		
Warnings:					
Information:					
8	Fee Worksheet (SB06)	fee-info.pdf	38632	no	2
			83c4285f973ef5bf437f4f343fef7ed3e892a4d5		
Warnings:					
Information:					
Total Files Size (in bytes):			2949287		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

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

First Named Inventor:	Mark 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 06/03/2019
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	47706-0004008
		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	47706-0004008	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	47706-0004008
		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		47706-0004008	
		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	Pending	Remove			
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)		
16276235	Continuation of	15187661	2016-06-20		
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	47706-0004008
		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	47706-0004008
		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	47706-0004008
		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	47706-0004008
		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:[Remove](#)

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

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

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

Signature	/Peter R. Poulin Reg. No. 74,643/		Date (YYYY-MM-DD)	2019-06-03
First Name	Peter	Last Name	Poulin	Registration Number
				74643
Additional Signature may be generated within this form by selecting the Add button.				Add

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	47706-0004008
		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.**

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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.

I claim:

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

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. Patent Application No. 16/414,213, filed on 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, which is a continuation of 13/080,616 filed April 5, 2011, 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 methylphosphoramidite linkages; or peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids, positive backbones, non-ionic backbones and non-ribose backbones. Modifications of the ribose-phosphate backbone may be done to increase the stability of the molecules; for example, PNA:DNA hybrids can exhibit higher stability in some environments.

[0030] "Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Primers usually are extended by a DNA polymerase.

[0031] The term "SNP" or "single nucleotide polymorphism" refers to a genetic variation between individuals; *e.g.*, a single nitrogenous base position in the DNA of organisms that is variable. SNPs are found across the genome; much of the genetic variation between individuals is due to variation at SNP loci, and often this genetic variation results in phenotypic variation between individuals. SNPs for use in the present invention and their respective alleles may be derived from any number of sources, such as public databases (U.C. Santa Cruz Human Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>) or the NCBI dbSNP website (<http://www.ncbi.nlm.nih.gov/SNP/>), or may be experimentally determined as described in U.S. Pat No. 6,969,589; and US Pub. No. 2006/0188875 entitled "Human Genomic Polymorphisms." Although the use of SNPs is described in some of the embodiments presented herein, it will be understood that other biallelic or multi-allelic genetic markers may also be used. A biallelic genetic marker is one that has two polymorphic forms, or alleles. As mentioned above, for a biallelic genetic marker that is associated with a trait, the allele that is more abundant in the genetic composition of a case group as compared to a control group is termed the "associated allele," and the other allele may be referred to as the "unassociated allele." Thus, for each biallelic polymorphism that is associated with a given trait (*e.g.*, a disease or drug response), there is a corresponding associated allele. Other biallelic polymorphisms that may be used with the methods presented herein include, but are not limited to multinucleotide changes, insertions, deletions, and translocations. It will be further appreciated that references to DNA herein may include genomic DNA, mitochondrial DNA, episomal DNA, and/or derivatives of DNA such as amplicons, RNA transcripts, cDNA, DNA analogs, etc. The polymorphic loci that are screened in an association study may be in a diploid or a haploid state and, ideally, would

be from sites across the genome.

[0032] The term "selectively binds", "selective binding" and the like as used herein, when referring to a binding partner (*e.g.* protein, nucleic acid, antibody or other affinity capture agent, etc.), refers to a binding reaction of two or more binding partners with high affinity and/or complementarity to ensure selective hybridization under designated assay conditions. Typically, specific binding will be at least three times the standard deviation of the background signal. Thus, under designated conditions the binding partner binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

[0033] "Sequencing", "sequence determination" and the like means determination of information relating to the nucleotide base sequence of a nucleic acid. Such information may include the identification or determination of partial as well as full sequence information of the nucleic acid. Sequence information may be determined "with varying degrees of statistical reliability or confidence. In one aspect, the term includes the determination of the identity and ordering of a plurality of contiguous nucleotides in a nucleic acid, "High throughput digital sequencing" or "next generation sequencing" means sequence determination using methods that determine many (typically thousands to billions) of nucleic acid sequences in an intrinsically parallel manner, *i.e.* where DNA templates are prepared for sequencing not one at a time, but in a bulk process, and where many sequences are read out preferably in parallel, or alternatively using an ultra-high throughput serial process that itself may be parallelized. Such methods include but are not limited to pyrosequencing (for example, as commercialized by 454 Life Sciences, Inc., Branford, CT); sequencing by ligation (for example, as commercialized in the SOLiD™ technology, Life Technology, Inc., Carlsbad, CA); sequencing by synthesis using modified nucleotides (such as commercialized in TruSeq™ and HiSeq™ technology by Illumina, Inc., San Diego, CA, HeliScope™ by Helicos Biosciences Corporation, Cambridge, MA, and PacBio RS by Pacific Biosciences of California, Inc., Menlo Park, CA), sequencing by ion detection technologies (Ion Torrent, Inc., South San Francisco, CA); sequencing of DNA nanoballs (Complete Genomics, Inc., Mountain View, CA); nanopore-based sequencing technologies (for example, as developed by Oxford Nanopore Technologies, LTD, Oxford, UK), and like highly parallelized sequencing methods.

[0034] The term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation, $T_m = 81.5 + 0.41 (\% G+C)$, when a nucleic acid is in aqueous solution at 1M NaCl (see *e.g.*, Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985)). Other

references (*e.g.*, Allawi and SantaLucia, Jr., *Biochemistry*, 36:10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m .

DETAILED DESCRIPTION OF THE INVENTION

[0035] The practice of the techniques described herein may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and sequencing technology, which are within the skill of those who practice in the art. Such conventional techniques include polymer array synthesis, hybridization and ligation of polynucleotides, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the examples herein. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Green, et al., Eds., *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (1999); Weiner, Gabriel, Stephens, Eds., *Genetic Variation: A Laboratory Manual* (2007); Dieffenbach, Dveksler, Eds., *PCR Primer: A Laboratory Manual* (2003); Bowtell and Sambrook, *DNA Microarrays: A Molecular Cloning Manual* (2003); Mount, *Bioinformatics: Sequence and Genome Analysis* (2004); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); Stryer, *Biochemistry* (4th Ed.) (1995) W.H. Freeman, New York N.Y.; Gait, *"Oligonucleotide Synthesis: A Practical Approach"* (2002) IRL Press, London; Nelson and Cox, *Lehninger, Principles of Biochemistry* (2000) 3rd Ed., W. H. Freeman Pub., New York, N.Y.; and Berg, et al., *Biochemistry* (2002) 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

[0036] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" refers to one or more nucleic acids, and reference to "the assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0037] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing devices, formulations and methodologies that may be used in connection with the presently described invention.

[0038] Where a range of values is provided, it is understood that each intervening value, between the

upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention,

[0039] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention.

The Invention in General

[0040] The assay systems of the invention provide spatially-encoded, multiplexed assays comprising 1) an assay capable of high levels of multiplexing with an efficient spatial encoding scheme; 2) instrumentation capable of delivering reagents according to a spatial pattern; and 3) decoding determined by a readout that is digital in nature. The assay systems of the invention detect the presence or absence and relative amount of a biological target or biological activity indicative of a biological target, as well as the location of the biological target or activity in a biological sample, *e.g.*, a tissue section or other biological structure disposed upon a support such as a microscope slide or culture dish.

[0041] The assay system further provides instrumentation with an ability to deliver reagents in a spatially-defined pattern. This instrumentation, together "with software, reagents and protocols, provides a key component of the highly innovative assay system of the invention, allowing for measurement of numerous biological targets or activities in a meaningful spatial environment, including gene expression and peptide localization, An encoding scheme used in these assay systems allows one to determine the location of biological targets or activity (or lack thereof) in the biological samples after the products of the multiplexed assay are removed from the biological sample and pooled for analysis. Decoding of the encoding scheme can be performed by, *e.g.*, next-generation sequencing, which easily provides millions to trillions of data points at low cost. The assay results such as the amount or activity of biological targets can then be mapped back to specific location in the biological sample. The assay systems open a new analytical window into the complex spatial patterns of cellular function and regulation in biological samples.

[0042] A simplified overview of the assay system 100 of the present invention is provided at Figure 1. At step 110, a biological sample affixed to a support is provided. The biological sample contains biological targets of interest. Biological targets can include any molecule of interest, such as nucleic acids (including, *e.g.* RNA transcripts, genomic DNA sequences, cDNAs, amplicons, or other nucleic acid sequences) and proteins, enzymes and the like. At step 120, encoded probes are delivered to the biological sample according to a known spatial pattern. Encoded probes comprise probes, which can interact "with biological targets of interest, and coding tags, which identify the positions in the sample of the biological targets being assayed, and thus can be used to link assay results back to locations in the sample. Coding tags in most embodiments are oligonucleotides. However, coding tags may also be mass tags, fluorescent labels, or other moieties.

[0043] In some embodiments, the probe and coding tag portions of the encoded probe are pre-coupled before being delivered to the biological sample. For example, in the case where the encoded probes are oligonucleotides, both the probe and coding tag sequence can be synthesized as a single oligonucleotide. Alternatively, the probe and coding tag portions of the encoding probes can be synthesized or obtained separately and combined before delivery to the biological sample (*e.g.*, two separate oligonucleotides can be synthesized and coupled by, *e.g.*, ligation; or an antibody and an oligonucleotide can be prepared separately and conjugated before delivery to the biological sample). Also, as is described in Figures 2-5, the probes and the coding tags (in encoding oligonucleotides) are synthesized separately, and are delivered to the biological sample at different steps (*e.g.*, probes first and coding tags thereafter, or vice versa) in the assay.

[0044] At step 130, the encoded probes are allowed to react or interact with the biological targets, *i.e.*, conditions are provided to allow *e.g.*, oligonucleotides to hybridize to nucleic acid targets, enzymes to catalyze reactions with protein targets, antibodies to bind epitopes, etc. In the case where the biological targets are nucleic acids, the encoded probes are typically oligonucleotides and hybridize to the target nucleic acids. In the case that the biological targets are proteins, the encoded probes typically are aptamers, small molecules, or oligonucleotide-conjugated proteins that interact with target proteins by binding to them or by reacting with them (that is, one of the proteins is a substrate for the other). Encoding oligonucleotides may be coupled to the probes (proteins) by conjugation, chemical or photo-crosslinking via suitable groups and the like.

[0045] Once encoded probes interact with the biological targets, the encoded probes that interacted with the biological targets must be separated from the encoded probes that did not interact with the biological targets at step 140. In the case where the biological targets are nucleic acids and the encoded probes are oligonucleotides, the separation can be accomplished by, *e.g.*, washing the unhybridized encoded probes from the sample. Similarly, for other assays that are based on affinity

binding, including those using aptamer, small molecule, and protein probes, washing steps can be used to remove low affinity binders. In the case where the probe is transformed via interaction with the target, *e.g.*, in the case of a peptide, *e.g.*, via cleavage by a protease or phosphorylation by a kinase, it is convenient to collect, all encoded probes--both encoded probes that interacted with the biological targets and were transformed and encoded probes that were not transformed. After collection or pooling, an antibody or other affinity capture agent can be used to capture probes that were transformed by addition of a moiety (*e.g.*, a phosphate group). In cases where probes have been transformed via cleavage, the transformed probes can be separated, *e.g.*, by capturing the non-transformed probes via a tag that is removed from the transformed probes during the transformation (*e.g.*, by cleavage), or by adding a new tag at the site of cleavage.

[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.

[0050] Thus, the present invention provides an ability to look at many different biological targets in many locations, providing the resolution of in situ hybridization with the highly-parallel data analysis of sequencing. In some embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 20, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 50, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 100, greater than 500, 1,000, 10,000, 25,000, 100,000, 500,000, 1,000,000. It will be appreciated that, due to the spatial encoding dimension of the invention, even much larger numbers can be contemplated. For example, assaying 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, *a*1 through *a*10 (coding tag 406 on encoded probe 420) and *b*1 through *b*10 (coding tag 408 encoded probe 422). Coding tag *a*1, 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 *a*2 is deposited on the biological sample in ten spots on the second horizontal line in 412. Coding tag *a*3 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 *b*1 is deposited on the biological sample in ten discrete spots in the first vertical row of 414, coding tag *b*2 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 *a*1 and *b*4, *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 *a*1, *b*4.

[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 *a*1, *a*2, *a*3, *a*4 and *b*1, *b*3, *b*3 and *b*4. 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 *a*1 through *a*4 are deposited in spots in a pattern (here, in a vertical pattern), and encoding oligonucleotides *b*1 through *b*4 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, alb3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at alb1 (position shown with vertical lines). A sequence readout was obtained for both target-specific oligonucleotides 1 and 2 at positions a2b1, a3b1, alb2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at alb4, a2b4 or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample, and neither target nucleic acid was detected in the upper right portion of the biological sample. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample, as shown in 550.

[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, pions of a tissue sample can be analyzed in parallel and the data combined to build a 3D map.

[0072] Integral to the assay system of the invention is instrumentation that allows for spatial patterning of reagents onto the biological sample. Technologies for formulating and delivering both biological molecules (*e.g.* oligonucleotides or antibodies) and chemical reagents (*e.g.*, small molecules or dNTPs) are known in the art, and uses of these instrument systems are known to one skilled in the art and easily adaptable to the assay systems of the invention. One example of a suitable reagent delivery system is the LabcyteTM Echo acoustic liquid handier, 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, electroendoosmosis, electrocapillarity, dielectrophoresis (including traveling wave forms thereof), and/or the like.

[0078] Valves generally include any mechanism for regulating the passage of fluid through a channel. Valves can include, for example, deformable members that can be selectively deformed to partially or completely close a channel, a movable projection that can be selectively extended into a channel to partially or completely block a channel, an electrocapillary structure, and/or the like.

[0079] An open gasket can be attached to the top of the biological sample and the sample and reagents can be injected into the gasket. Suitable gasket materials include, but are not limited to, neoprene, nitrile, and silicone rubber. Alternatively, a watertight reaction chamber may be formed by a gasket sandwiched between the biological sample on the substrate and a chemically inert, water resistant material such as, but not limited to, black-anodized aluminum, thermoplastics (*e.g.*, polystyrene, polycarbonate, etc), glass, etc.

[0080] In an optional embodiment, the assay system comprises imaging means to determine features and organization of the biological sample of interest. The images obtained, *e.g.*, may be used to design the deposition pattern of the reagents. Imaging means are optional, as an individual can instead view the biological sample using, *e.g.*, a microscope, analyze the organization of the biological sample, and specify a spatial pattern for delivery assay reagents. If included, the delivery system can comprise a microcircuit arrangement including an imager, such as a CCD or IGFET-based (*e.g.*, CMOS-based) imager and an ultrasonic sprayer for reagent delivery such as described in US Pub. No. 20090197326, which is incorporated herein by reference. Also, it should be noted that although Figures 4 and 5 illustrate using a x,y grid configuration, other configurations can be used, such as, *e.g.*, following the topology of a tissue sample; targeting certain groups of cells, cell layers and/or cell types in a tissue, and the like.

[0081] In yet another alternative, the reagent delivery system controls the delivery of reagents to specific patterns on a biological sample surface using semiconductor techniques such as masking and spraying. Specific areas of a biological sample can be protected from exposure to reagents through use of a mask to protect specific areas from exposure. The reagents may be introduced to the biological sample using conventional techniques such as spraying or fluid flow. The use of masked delivery results in a patterned delivery scheme on the substrate surface.

[0082] In a preferred aspect of the invention, the reagent delivery instrumentation is based on inkjet printing technology. There are a variety of different ink-jetting mechanisms (*e.g.*, thermal, piezoelectric) and compatibility has been shown with aqueous and organic ink formulations. Sets of independently actuated nozzles can be used to deliver multiple reagents at the same time, and very high resolutions are 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. 1 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system 101, two reagents 120, 122 that specifically bind to a biological molecule of interest are associated with coding identifiers 106, 108 that encode for a spatial location in the sample. These coding identifiers 106, 108 are optionally associated with sites that assist in their identification in the assay format, *e.g.*, universal priming sites 104, 110 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section 116 is encoded using the combination of the patterns 112, 114 created using the separate coding identifiers 106, 108 which provide a two dimensional code 118 that shows the location of any positive detection of the biological molecule 102 as well as quantifying the biological molecule 102 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 2, two target-specific assay oligonucleotides are ligated together 202 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides containing tag sequence sets X and Y are ligated 204 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 206, *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 $\sim 1,000$ reads may be sampled, for a total of $\sim 10^9$ 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, electroendoosmosis, 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 $\sim 500 \mu\text{l}$ and can contain $\sim 3 \times 10^{11}$ molecules at a $1 \mu\text{M}$ concentration. To assay 1,000 target sequences in 10,000 cells, $\sim 2,000$ 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.

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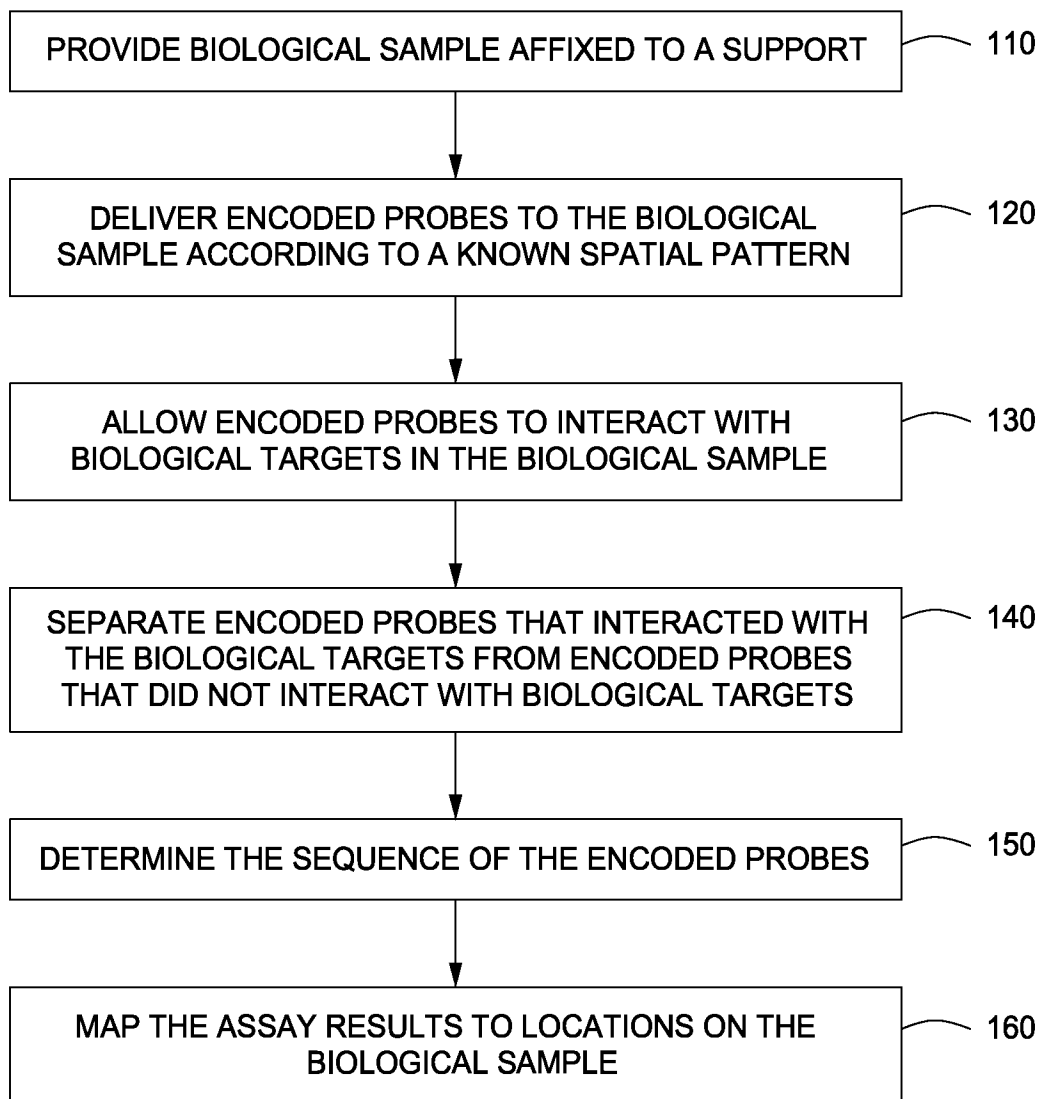


FIG. 1

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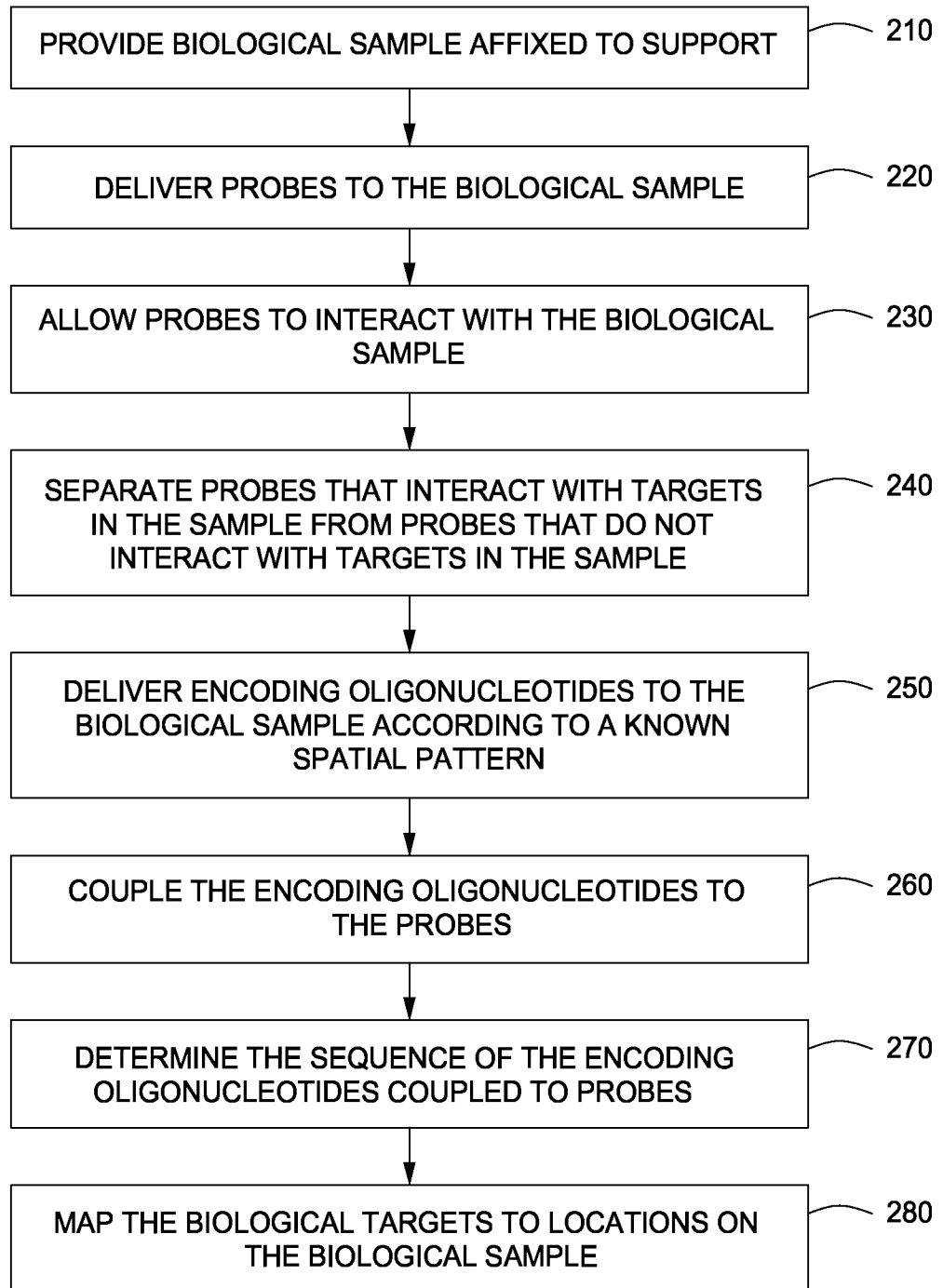


FIG. 2

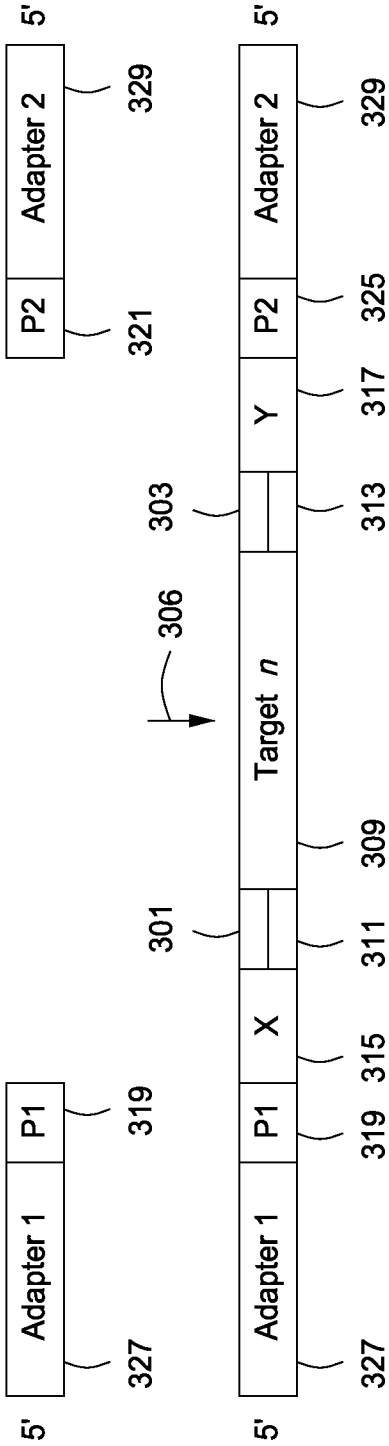


FIG. 3

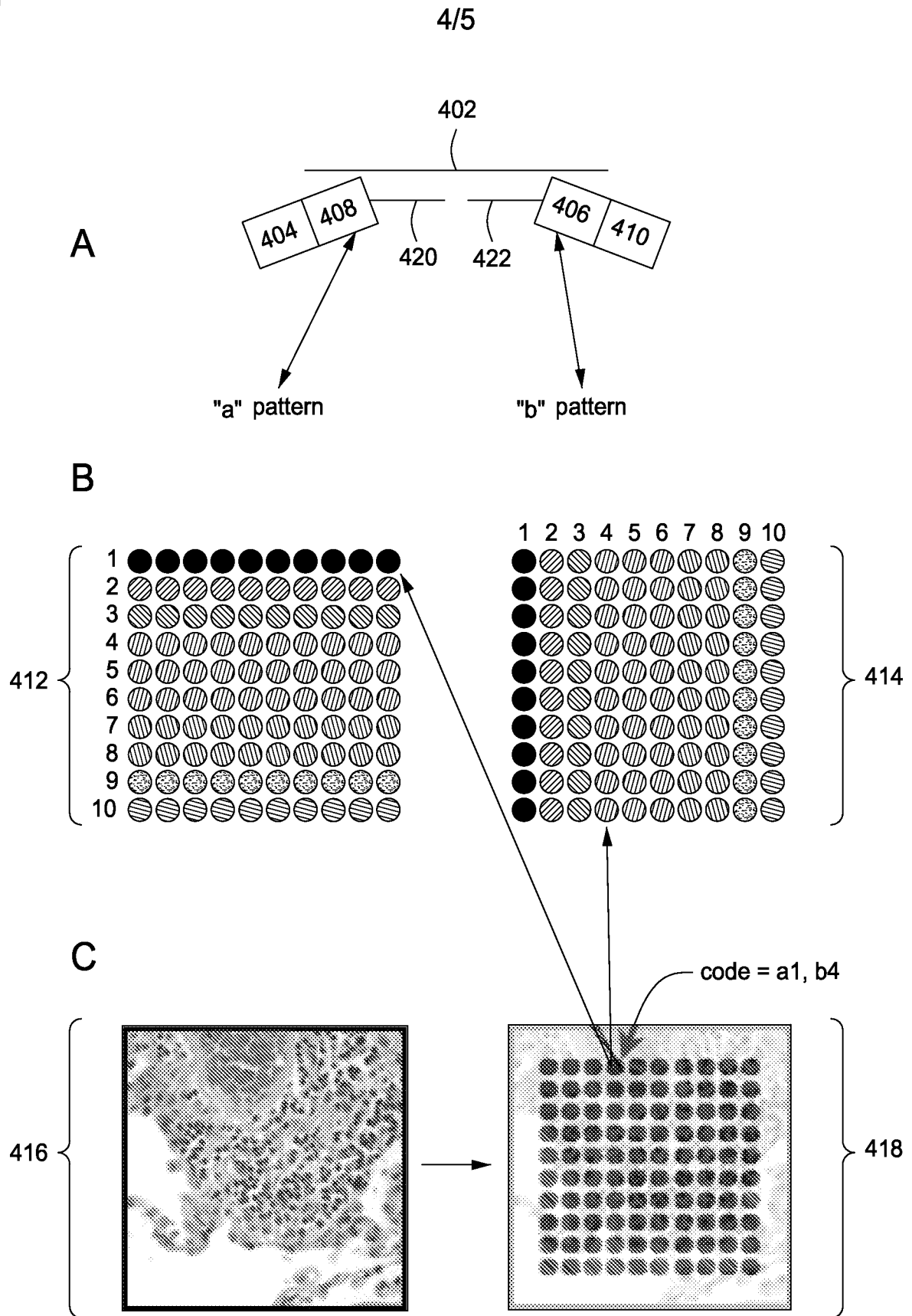


FIG. 4

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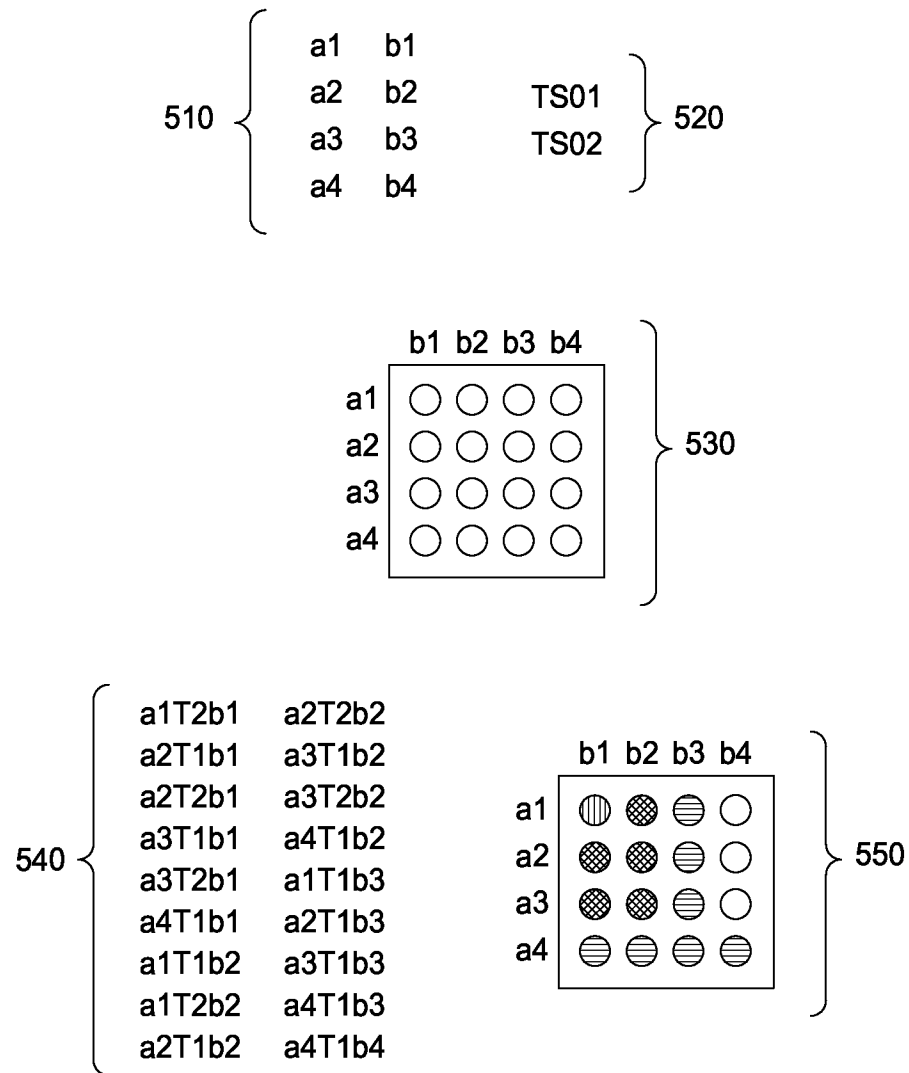


FIG. 5

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

Application Number	
Filing Date	
First Named Inventor	Mark Chee
Title	SPATIALLY ENCODED BIOLOGICAL ASSAYS
Art Unit	
Examiner Name	
Attorney Docket Number	47706-0004008

SIGNATURE of Applicant or Patent Practitioner

Signature	/Peter R. Poulin Reg. No. 74,643/	Date (Optional)	06/03/2019
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			

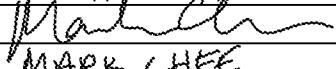
I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

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

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Mark S. Chee	Art Unit :	1636
Application No. :	16/430,015	Examiner :	Unknown
Filed :	June 3, 2019	Conf. No. :	8975
Title :	SPATIALLY ENCODED BIOLOGICAL ASSAYS		

MAIL STOP AMENDMENT

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

INFORMATION DISCLOSURE STATEMENT

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

Under 35 U.S.C. § 120, this application relies on the earlier filing date of application number 16/414,213, filed on 5/16/2019. All 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.

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

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

Respectfully submitted,

Date: June 10, 2019

/Cameron Luitjens/

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

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61947299.doc

First Named Inventor : Mark S. Chee
Application No. : 16/430,015
Filed : June 3, 2019
Page : 2 of 2

Attorney Docket: 47706-0004008 / 700-000108US

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. 47706-0004008	Application No. 16/430,015
	Applicant Prognosys Biosciences, Inc.		
	Filing Date June 3, 2019	Group Art Unit 1636	

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

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		Filing Date June 3, 2019	Group Art Unit 1636
(37 CFR §1.98(b))			

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Prognosis Biosciences, Inc.	
		Filing Date June 3, 2019	Group Art Unit 1636
(37 CFR §1.98(b))			

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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	Applicant Prognosys Biosciences, Inc.		
	Filing Date June 3, 2019	Group Art Unit 1636	

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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	Applicant Prognosis Biosciences, Inc.	
	Filing Date June 3, 2019	Group Art Unit 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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Examiner Signature	Date Considered
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	Applicant Prognosis Biosciences, Inc.	
	Filing Date June 3, 2019	Group Art Unit 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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	532.	WONG et al., "Direct Site-Selective Covalent Protein Immobilization Catalyzed by a Phosphopantetheinyl Transferase," J. Am. Chem Soc., 2008, 130:12456-64
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	534.	WORTHINGTON et al., "Cloning of random oligonucleotides to create single-insert plasmid libraries," Analyt. Biochem, 2001, 294:169-175
	535.	XIAO et al., "Direct determination of haplotypes from single DNA molecules," Nature Methods, 2009, 6(3):199-01

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.

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 47706-0004008	Application No. 16/430,015
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))		Applicant Prognosys Biosciences, Inc.	
		Filing Date June 3, 2019	Group Art Unit 1636

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	536.	YIN et al., "Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase," PNAS, 2005, 102(44):15815-20
	537.	YONEZAWA et al., "DNA display for in vitro selection of diverse peptide libraries," Nucleic Acids Research , 2003, 31 (19):e118
	538.	ZHANG et al., "Binding-induced DNA assembly and its application to yoctomole detection of proteins," Anal Chem (2012) 84(2):877-884
	539.	ZHENG et al., Origins of human mitochondrial point mutations as DNA polymerase mediated errors. Mutat. Res. 599(1-2): 11-20, 2006
	540.	ZHOU et al., "Genetically Encoded Short Peptide Tags for Orthogonal Protein Labeling by Sfp and Ac S Phos ho entethein I Transferases," ACS Chemical Biolo 2007 2 5 : 337-346
	541.	ZILBERMAN et al., "Genome-wide analysis of DNA methylation patterns," Development (2007) 134: 3959-3965

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

Electronic Acknowledgement Receipt

EFS ID:	36269269
Application Number:	16430015
International Application Number:	
Confirmation Number:	8975
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Kirsten Neuman
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004008
Receipt Date:	11-JUN-2019
Filing Date:	
Time Stamp:	17:06:48
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	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	73275	no	2
			5e245f02a3d4211a70dfd0d7a48dcaff3f5d22e7		

Warnings:

Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	PTO_1449.pdf	281207	no	24
			367f6b438c656772bb9cc7191cab36ac474 b0698		
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
			Total Files Size (in bytes):	354482	
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Mark S. Chee	Art Unit :	1636
Application No. :	16/430,015	Examiner :	Unknown
Filed :	June 3, 2019	Conf. No. :	8975
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.

Amendments to the Specification:

Please replace paragraph [00123] with the following amended paragraph:

[00123] ~~Fig. 1~~ Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system ~~101~~ 401, two reagents ~~120~~ 420, ~~122~~ 422 that specifically bind to a biological molecule of interest are associated with coding identifiers ~~106~~ 406, ~~108~~ 408 that encode for a spatial location in the sample. These coding identifiers ~~106~~ 406, ~~108~~ 408 are optionally associated with sites that assist in their identification in the assay format, e.g., universal priming sites ~~104~~ 404, ~~110~~ 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section ~~116~~ 416 is encoded using the combination of the patterns ~~112~~ 412, ~~114~~ 414 created using the separate coding identifiers ~~106~~ 406, ~~108~~ 408 which provide a two dimensional code ~~118~~ 418 that shows the location of any positive detection of the biological molecule ~~102~~ 402 as well as quantifying the biological molecule ~~102~~ 402 at each location assayed in the tissue.

Please replace paragraph [00140] with the following amended paragraph:

[00140] In ~~Figure 2~~ Figure 3, two target-specific assay oligonucleotides are ligated together ~~202~~ 302 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides containing tag sequence sets X and Y are ligated ~~204~~ 304 to the target specific oligonucleotides. Oligonucleotides containing X ligate specifically to one side of the targeting construct and oligonucleotides containing Y ligate to the other. The oligonucleotides contain

First Named Inventor : Mark S. Chee
Application No. : 16/430,015
Filed : June 3, 2019
Page : 3 of 8

Attorney Docket: 47706-0004008 / 700-000108US

universal primer sites P1 and P2. Following ligation, the constructs are eluted and, optionally, sequencing adapters can be attached ~~206~~ 306, e.g., via PCR.

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 substrate comprising (i) a capture agent that binds specifically to a target biological molecule and (ii) a coding identifier, wherein each of the capture agent and the coding identifier are independently attached to a location on a surface of the substrate.
3. **(New)** The substrate of claim 2, wherein the capture agent is attached to the surface of the substrate by a chemical-coupling mechanism.
4. **(New)** The substrate of claim 2, wherein the capture agent is attached to the surface of the substrate through ligation.
5. **(New)** The substrate of claim 2, wherein the capture agent is attached to the surface of the substrate through photolithographic immobilization methods.
6. **(New)** The substrate of claim 2, wherein the coding identifier is attached to the surface of the substrate through a chemical-coupling mechanism.
7. **(New)** The substrate of claim 2, wherein the coding identifier is attached to the surface of the substrate through ligation.
8. **(New)** The substrate of claim 2, wherein the coding identifier is attached to the surface of the substrate through photolithographic immobilization methods.

9. **(New)** The substrate of claim 2, the capture agent is a nucleic acid.
10. **(New)** The substrate of claim 2, wherein the capture agent is a protein.
11. **(New)** The substrate of claim 10, wherein the protein is an antibody.
12. **(New)** The substrate of claim 2, wherein the capture agent is a small molecule.
13. **(New)** The substrate of claim 2, wherein the capture agent is a substrate for an enzyme.
14. **(New)** The substrate of claim 2, wherein the target biological molecule comprises a nucleic acid.
15. **(New)** The substrate of claim 14, wherein the nucleic acid comprises a single nucleotide polymorphism.
16. **(New)** The substrate of claim 14, wherein the nucleic acid comprises a mutation.
17. **(New)** The substrate of claim 14, wherein the nucleic acid is genomic DNA.
18. **(New)** The substrate of claim 14, wherein the nucleic acid is mRNA.
19. **(New)** The substrate of claim 9, wherein the nucleic acid comprises a universal sequence.
20. **(New)** The substrate of claim 9, wherein the nucleic acid is a gene-specific sequence.
21. **(New)** The substrate of claim 2, wherein the substrate comprises a slide.

22. **(New)** The substrate of claim 2, wherein the substrate comprises a flow-cell.
23. **(New)** The substrate of claim 2, wherein the substrate comprises a bead.
24. **(New)** The substrate of claim 2, wherein the target biological molecule comprises a protein.
25. **(New)** The substrate of claim 2, wherein the capture agent comprises a protein conjugated to an oligonucleotide comprising a nucleic acid sequence and wherein the protein specifically binds to the target biological molecule.
26. **(New)** The substrate of claim 2, wherein the capture agent binds specifically to a target biological molecule of a tissue sample.
27. **(New)** The substrate of claim 2, wherein the capture agent further comprises a first primer binding region.
28. **(New)** The substrate of claim 2, wherein the coding identifier further comprises a second primer binding region.
29. **(New)** The substrate of claim 2, wherein the substrate further comprises a cleavable site.

First Named Inventor : Mark S. Chee
Application No. : 16/430,015
Filed : June 3, 2019
Page : 7 of 8

Attorney Docket: 47706-0004008 / 700-000108US

REMARKS

Claim 1 has been canceled without prejudice. Claims 2-29 are newly added. Applicant's specification fully supports newly added claims 2-29. See, e.g.:

Claim	Exemplary Support (specification as filed)
2	[0008], [0052]
3-4, 6-7	[00115]
5, 8	[00116]
9-12	[0009]
13, 25	[0059]
14-16	[0054]
17-18	[0042]
19	[0062]
20	[0066]
21	[0040]
22	[0075]
23	[00120]
24	[0053]
26	[0052]
27-28	[0056]
29	[0045]

The specification has been amended to comport with the figure numbers and reference characters in the drawings.

No new matter has been added. Applicant asks that all claims be examined in view of the amendment to the claims.

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

First Named Inventor : Mark S. Chee
Application No. : 16/430,015
Filed : June 3, 2019
Page : 8 of 8

Attorney Docket: 47706-0004008 / 700-000108US

Respectfully submitted,

Date: June 18, 2019

/Cameron Luitjens/

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

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

61949891.doc

Electronic Patent Application Fee Transmittal

Application Number:	16430015			
Filing Date:	03-Jun-2019			
Title of Invention:	Spatially Encoded Biological Assays			
First Named Inventor/Applicant Name:	Mark S. CHEE			
Filer:	Cameron Mark Luitjens/Judith Witzel			
Attorney Docket Number:	47706-0004008			
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1202	8	100	800
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 (\$)				800

Electronic Acknowledgement Receipt

EFS ID:	36334607
Application Number:	16430015
International Application Number:	
Confirmation Number:	8975
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Judith Witzel
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004008
Receipt Date:	18-JUN-2019
Filing Date:	03-JUN-2019
Time Stamp:	15:48:04
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$800
RAM confirmation Number	061919INTEFSW00003052061050
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		PrelimAmend.pdf	132889	yes	8
			4b36ab2749eed25cd33a31489ab29a53afa1a79c		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Preliminary Amendment		1	1	
	Specification		2	3	
	Claims		4	6	
	Applicant Arguments/Remarks Made in an Amendment		7	8	
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30546	no	2
			109457605214a6a07d76f9459d1401d7a9afadc8		
Warnings:					
Information:					
Total Files Size (in bytes):			163435		

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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.

Document code: WFEE

United States Patent and Trademark Office
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UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/430,015	06/03/2019	Mark S. CHEE	47706-0004008

CONFIRMATION NO. 8975

FORMALITIES LETTER



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

Date Mailed: 06/19/2019

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given **TWO MONTHS** from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
 - The application contains drawings and the specification contains a brief description of the drawings. However, the specification does not contain a brief description of the several views of the drawings as required by 37 CFR 1.74 and 37 CFR 1.77(b)(7) and/or a drawing(s) has not been labeled in accordance with 37 CFR 1.84(u)(1). If each figure is not labeled "Fig." with a consecutive Arabic numeral (1, 2, etc.) or an Arabic numeral and capital letter in the English alphabet (A, B, etc.), then the drawing(s) must be relabeled in accordance with 37 CFR 1.84(u)(1). In addition, if the brief description of the several views of the drawings does not refer to the figure(s) as properly labeled, then the specification must be amended to correspond to the figure(s) as properly labeled and a substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125, is required.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

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Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice".
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Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/tnguyen/

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 16/430,015	
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	75	N/A		
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	330	N/A		
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	380	N/A		
TOTAL CLAIMS (37 CFR 1.16(j))	1	minus 20 = *	x 50 =	0.00			
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1	minus 3 = *	x 230 =	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			
			TOTAL	785	TOTAL		
* If the difference in column 1 is less than zero, enter "0" in column 2.							
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(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus **	=	x =	x =	=
	Independent (37 CFR 1.16(h))	*	Minus ***	=	x =	x =	=
	Application Size Fee (37 CFR 1.16(s))						
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
				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(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus **	=	x =	x =	=
	Independent (37 CFR 1.16(h))	*	Minus ***	=	x =	x =	=
	Application Size Fee (37 CFR 1.16(s))						
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
				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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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
16/430,015	06/03/2019	1636	785	47706-0004008	1	1

CONFIRMATION NO. 8975

FILING RECEIPT



0000000109009160

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

Date Mailed: 06/19/2019

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
which is a CON of 16/276,235 02/14/2019
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: 06/18/2019

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/430,015**

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No

**** SMALL ENTITY ****

Title

Spatially Encoded Biological Assays

Preliminary Class

435

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>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

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

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/430,015	06/03/2019	Mark S. CHEE	47706-0004008

CONFIRMATION NO. 8975

FORMALITIES LETTER



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

Date Mailed: 06/20/2019

NOTICE OF INCOMPLETE REPLY (NONPROVISIONAL)

Filing Date Granted

The U.S. Patent and Trademark Office has received your reply on 06/18/2019 to the NOTICE TO FILE CORRECTED APPLICATION PAPERS (Notice) mailed 06/19/2019 and it has been entered into the application. The reply, however, is not a complete reply for the reason(s) listed below. A complete reply must be timely filed to prevent ABANDONMENT of the above-identified application. Replies should be mailed to: Mail Stop Missing Parts, Commissioner for Patents, P.O.Box 1450, Alexandria VA 22313-1450.

The period for reply continues to run from the date of the Notice mailed 06/19/2019. Applicant must submit all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the appropriate extension fee under the provisions of 37 CFR 1.136(a).

Applicant's reply to the Notice mailed on 06/19/2019 was received in the U.S. Patent and Trademark Office on 06/18/2019, after the expiration of the two month period for reply set forth in the Notice. The application will become abandoned unless applicant obtains an extension of the period for reply, as above. The petition must be accompanied by the appropriate fee as set forth in 37 CFR 1.17 (see the current fees below). The date on which the reply, the petition, and the fee have been filed is the date of the reply and also the date for purposes of determining the period of extension and the corresponding amount of the fee due. The expiration of the time period is determined by the amount of the fee paid. Applicant is advised that extensions may not be granted under 37 CFR 1.136(a) for more than FIVE MONTHS beyond the time period set in the Notice.

Items Required to Avoid Abandonment:

The required items noted below SHOULD be filed along with any items required above.

The application is informal since it does not comply with the regulations for the reason(s) indicated below.

The required item(s) identified below must be timely submitted to avoid abandonment:

- Replacement drawings in compliance with 37 CFR 1.84 and 37 CFR 1.121(d) are required. The drawings submitted are not acceptable because:
 - The application contains drawings and the specification contains a brief description of the drawings. However, the specification does not contain a brief description of the several views of the drawings as required by 37 CFR 1.74 and 37 CFR 1.77(b)(7) and/or a drawing(s) has not been labeled in accordance with 37 CFR 1.84(u)(1). If each figure is not labeled "Fig." with a consecutive Arabic numeral (1, 2, etc.) or an Arabic numeral and capital letter in the English alphabet (A, B, etc.), then the drawing(s) must be relabeled in accordance with 37 CFR 1.84(u)(1). In addition, if the brief description of the several views of

the drawings does not refer to the figure(s) as properly labeled, then the specification must be amended to correspond to the figure(s) as properly labeled and a substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125, is required.

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Mail date of Notice: 06/19/2019

Last date that extension may be obtained: (Note: The petition and fee must be received 01/19/2020 by this date, or include a proper certificate of mailing under 37 CFR 1.8 with a date on or before this date, and extend the time to include this date.)

Length of Extension of Time (response due on or before)	Fee under 37 CFR 1.17(a) effective March 19, 2013		
	Undiscounted	Small Entity	Micro Entity
One Month (09/19/2019)	\$200	\$100	\$50
Two Months (10/19/2019)	\$600	\$300	\$150
Three Months (11/19/2019)	\$1400	\$700	\$350
Four Months (12/19/2019)	\$2200	\$1100	\$550
Five Months (01/19/2020)	\$3000	\$1500	\$750

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice".
<https://portal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html>

For more information about EFS-Web please call the USPTO Electronic Business Center at 1-866-217-9197 or visit our website at <http://www.uspto.gov/ebc>.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

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

/sareebuddin/

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 16/430,015	
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	75		N/A	
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	330		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	380		N/A	
TOTAL CLAIMS (37 CFR 1.16(j))	28	minus 20 = * 8	x 50 =	400	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1	minus 3 = *	x 230 =	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			
			TOTAL	1185		TOTAL	
* If the difference in column 1 is less than zero, enter "0" in column 2.							
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		OR	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		OR	TOTAL ADD'L FEE
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.							

Doc Code: 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	16430015	
Filing Date	03-Jun-2019	
First Named Inventor	Mark CHEE	
Attorney Docket Number	47706-0004008	
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)		
16276235 filed on 02/14/2019 16430109 filed on 06/03/2019 16414213 filed on 05/16/2019 16402098 filed on 05/02/2019 16276260 filed on 02/14/2019		

as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

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9371598

as the term of said prior patent is presently shortened by any terminal disclaimer. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:

- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.

☐ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.

Applicants claims the following fee status:

☐ Small Entity

☐ Micro Entity

☒ Regular Undiscounted

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES

I certify, in accordance with 37 CFR 1.4(d)(4) that I am:

- ☒ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application
- Registration Number 58674
- ☐ A sole inventor
- ☐ A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application
- ☐ A joint inventor; all of whom are signing this request

Signature	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	16430015			
Filing Date:	03-Jun-2019			
Title of Invention:	Spatially Encoded Biological Assays			
First Named Inventor/Applicant Name:	Mark S. CHEE			
Filer:	Cameron Mark Luitjens/Shannon Stadnick			
Attorney Docket Number:	47706-0004008			
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.: 16430015

Filing Date: 03-Jun-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on June 20, 2019

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt

EFS ID:	36362179
Application Number:	16430015
International Application Number:	
Confirmation Number:	8975
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004008
Receipt Date:	20-JUN-2019
Filing Date:	03-JUN-2019
Time Stamp:	15:33:40
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	062119INTEFSW00002174061050
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	39467	no	3
			2731bbc417f65c90a7ffe4ee40261d083adc4d6b		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30605	no	2
			9540b231a518fab98ab2bd63d8a9e7db2503af9		
Warnings:					
Information:					
Total Files Size (in bytes):			70072		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Mark S. Chee	Art Unit :	1636
Application No. :	16/430,015	Examiner :	Unknown
Filed :	June 3, 2019	Conf. No. :	8975
Title :	SPATIALLY ENCODED BIOLOGICAL ASSAYS		

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

REPLY TO NOTICE TO FILE CORRECTED APPLICATION PAPERS DATED

JUNE 19, 2019

The applicant as a large entity submits:

☒ Substitute drawings (1 sheet) in compliance with 37 CFR §1.84.

The applicant understands that this perfects the application and no additional papers or filing fees are required.

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

Respectfully submitted,

Date: June 21, 2019

/Cameron Luitjens/

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

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

61959580.doc

4/5

FIG. 4A

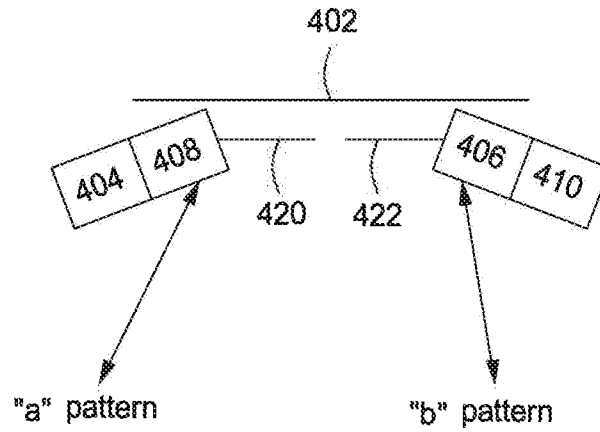


FIG. 4B

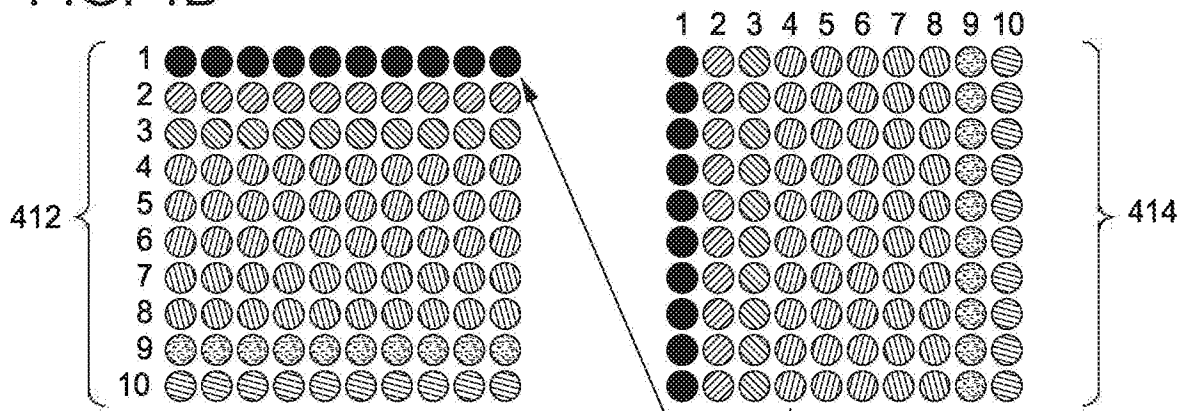
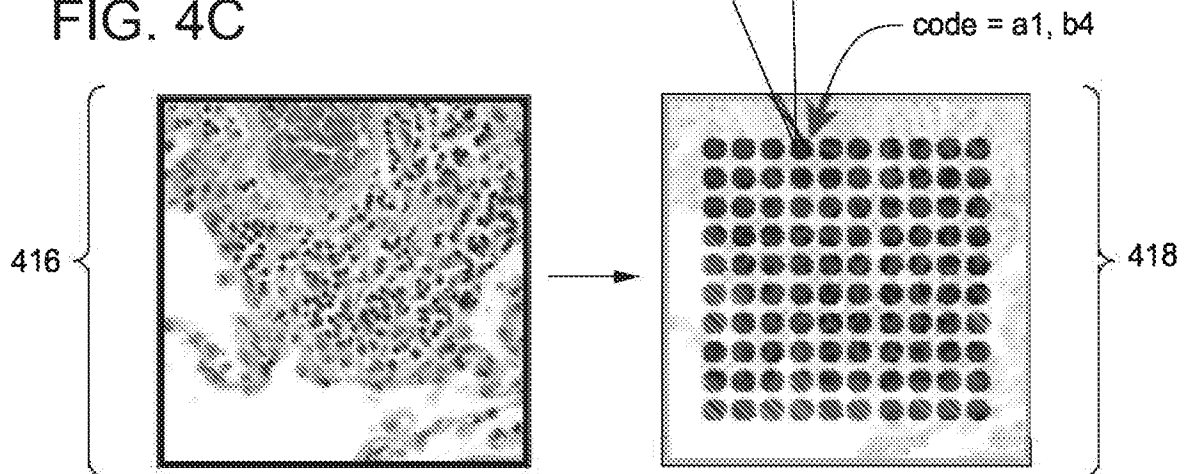


FIG. 4C



Electronic Acknowledgement Receipt

EFS ID:	36378898
Application Number:	16430015
International Application Number:	
Confirmation Number:	8975
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004008
Receipt Date:	21-JUN-2019
Filing Date:	03-JUN-2019
Time Stamp:	17:23:50
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
------------------------	----

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	RESPONSE.pdf	59832	no	1
			4d0fc2a7a8792710969eed1b70cd97e1bd4ba6f4		

Warnings:

Information:					
2	Drawings-only black and white line drawings	DRAWING_REPLACE.pdf	1608433	no	1
			93b20db61eea4ad4343abc28f36f827bdf9f2064		
Warnings:					
Information:					
Total Files Size (in bytes):				1668265	
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

Doc Code: DIST.E.FILE Document Description: Electronic Terminal Disclaimer - Filed		PTO/SB/25 U.S. Patent and Trademark Office Department of Commerce	
Electronic Petition Request	TERMINAL DISCLAIMER TO OBIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION		
Application Number	16430015		
Filing Date	03-Jun-2019		
First Named Inventor	Mark CHEE		
Attorney Docket Number	47706-0004008 / 700-00010		
Title of Invention	Spatially Encoded Biological Assays		
☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action ☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.			
Owner	Percent Interest		
Prognosys Biosciences, Inc.	100%		
The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Number(s)			
16435176 filed on 06/07/2019 as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns. In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.			
☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.			

☐ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.	
Applicant claims the following fee status: ☐ Small Entity ☐ Micro Entity ☒ Regular Undiscounted	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.	
THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES I certify, in accordance with 37 CFR 1.4(d)(4) that I am: ☒ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application Registration Number <u>58674</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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
 Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	16430015			
Filing Date:	03-Jun-2019			
Title of Invention:	Spatially Encoded Biological Assays			
First Named Inventor/Applicant Name:	Mark S. CHEE			
Filer:	Cameron Mark Luitjens/Shannon Stadnick			
Attorney Docket Number:	47706-0004008 / 700-00010			
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.: 16430015

Filing Date: 03-Jun-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on June 25, 2019

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt

EFS ID:	36398085
Application Number:	16430015
International Application Number:	
Confirmation Number:	8975
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004008 / 700-00010
Receipt Date:	25-JUN-2019
Filing Date:	03-JUN-2019
Time Stamp:	12:09:16
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	062519INTEFSW00013049061050
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	33903	no	2
			787efb7f28e6b427b17d9410cb135b3ccb4ac7fd		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30611	no	2
			a2a639fccdcaddb8a6ed35ef71798ef5e142cec9		
Warnings:					
Information:					
Total Files Size (in bytes):			64514		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					



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United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY. DOCKET NO	TOT CLAIMS	IND CLAIMS
16/430,015	06/03/2019	1636	1185	47706-0004008 / 700-00010	28	1

CONFIRMATION NO. 8975
UPDATED FILING RECEIPT

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



Date Mailed: 06/26/2019

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
which is a CON of 16/276,235 02/14/2019
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: 06/18/2019

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/430,015**

Projected Publication Date: 10/03/2019

Non-Publication Request: No

Early Publication Request: No

**** SMALL ENTITY ****

Title

Spatially Encoded Biological Assays

Preliminary Class

435

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>.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, <http://www.stopfakes.gov>. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

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

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

technology, manufacture products, deliver services, and grow your business, visit <http://www.SelectUSA.gov> or call +1-202-482-6800.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875						Application or Docket Number 16/430,015				
APPLICATION AS FILED - PART I										
(Column 1)		(Column 2)		SMALL ENTITY		OR 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	75		N/A				
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	330		N/A				
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	380		N/A				
TOTAL CLAIMS (37 CFR 1.16(j))	28 minus 20 =	*	x 50 =	400	OR					
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*	x 230 =	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						
			TOTAL	1185		TOTAL				
* If the difference in column 1 is less than zero, enter "0" in column 2.										
APPLICATION AS AMENDED - PART II										
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY		
AMENDMENT A		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))							OR		
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	
(Column 1)		(Column 2)		(Column 3)		SMALL ENTITY		OR OTHER THAN SMALL ENTITY		
AMENDMENT B		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE(\$)	ADDITIONAL FEE(\$)		RATE(\$)	ADDITIONAL FEE(\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))							OR		
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3. ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.										

Doc Code: DIST.E.FILE Document Description: Electronic Terminal Disclaimer - Filed		PTO/SB/25 U.S. Patent and Trademark Office Department of Commerce	
Electronic Petition Request	TERMINAL DISCLAIMER TO OBIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION		
Application Number	16430015		
Filing Date	03-Jun-2019		
First Named Inventor	Mark CHEE		
Attorney Docket Number	47706-0004008 / 700-00010		
Title of Invention	Spatially Encoded Biological Assays		
☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action ☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.			
Owner	Percent Interest		
Prognosys Biosciences, Inc.	100%		
The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Number(s)			
16437726 filed on 06/11/2019 as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns. In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.			
☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.			

☐ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.	
Applicant claims the following fee status: ☐ Small Entity ☐ Micro Entity ☒ Regular Undiscounted	
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>58674</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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
 Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	16430015			
Filing Date:	03-Jun-2019			
Title of Invention:	Spatially Encoded Biological Assays			
First Named Inventor/Applicant Name:	Mark S. CHEE			
Filer:	Cameron Mark Luitjens/Shannon Stadnick			
Attorney Docket Number:	47706-0004008 / 700-00010			
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.: 16430015

Filing Date: 03-Jun-2019

Applicant/Patent under Reexamination: CHEE

Electronic Terminal Disclaimer filed on June 27, 2019

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt

EFS ID:	36426213
Application Number:	16430015
International Application Number:	
Confirmation Number:	8975
Title of Invention:	Spatially Encoded Biological Assays
First Named Inventor/Applicant Name:	Mark S. CHEE
Customer Number:	159367
Filer:	Cameron Mark Luitjens/Shannon Stadnick
Filer Authorized By:	Cameron Mark Luitjens
Attorney Docket Number:	47706-0004008 / 700-00010
Receipt Date:	27-JUN-2019
Filing Date:	03-JUN-2019
Time Stamp:	12:37:14
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	062719INTEFSW00013923061050
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	33903	no	2
			caf84c8a8426e37e9ffe2f7fe347af078e7fb6bf		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30612	no	2
			c278f5e5935deed28c79056471709e924b8d59a0		
Warnings:					
Information:					
Total Files Size (in bytes):			64515		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/430,015	06/03/2019	Mark S. CHEE	47706-0004008 / 700-00010	8975
159367	7590	07/01/2019	EXAMINER	
FISH & RICHARDSON P.C. 10x Genomics P.O. BOX 1022 MINNEAPOLIS, MN 55440-1022			ART UNIT	PAPER NUMBER
			1634	
			NOTIFICATION DATE	DELIVERY MODE
			07/01/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com
ipadmin@10xgenomics.com

<i>Decision Granting Request for Prioritized Examination (Track I)</i>	Application No. 16/430,015	Applicant(s) CHEE, Mark S.	
	Examiner CHERYL P GIBSON BAYLOR	Art Unit OPET	AIA (FITF) Status No
1. THE REQUEST FILED <u>03 June 2019</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			

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Mark S. Chee	Art Unit :	1636
Application No. :	16/430,015	Examiner :	Unknown
Filed :	June 3, 2019	Conf. No. :	8975
Title :	SPATIALLY ENCODED BIOLOGICAL ASSAYS		

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

REPLY TO NOTICE OF INCOMPLETE REPLY DATED JUNE 20, 2019

The applicant as a large entity submits:

- ☒ A substitute specification in compliance with 37 CFR §1.52. No new matter has been added.

Replacement drawings in compliance with 37 CFR §1.84 and §1.121(d) were filed on June 21, 2019.

The applicant understands that this perfects the application and no additional papers or filing fees are required.

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

Respectfully submitted,

Date: July 2, 2019

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

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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 on 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, which is a continuation of 13/080,616 filed April 5, 2011, 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 methylphosphoramidite linkages; or peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids, positive backbones, non-ionic backbones and non-ribose backbones. Modifications of the ribose-phosphate backbone may be done to increase the stability of the molecules; for example, PNA:DNA hybrids can exhibit higher stability in some environments.

[0030] "Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Primers usually are extended by a DNA polymerase.

[0031] The term "SNP" or "single nucleotide polymorphism" refers to a genetic variation between individuals; *e.g.*, a single nitrogenous base position in the DNA of organisms that is variable. SNPs are found across the genome; much of the genetic variation between individuals is due to variation at SNP loci, and often this genetic variation results in phenotypic variation between individuals. SNPs for use in the present invention and their respective alleles may be derived from any number of sources, such as public databases (U.C. Santa Cruz Human Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>) or the NCBI dbSNP website (<http://www.ncbi.nlm.nih.gov/SNP/>), or may be experimentally determined as described in U.S. Pat No. 6,969,589; and US Pub. No. 2006/0188875 entitled "Human Genomic Polymorphisms." Although the use of SNPs is described in some of the embodiments presented herein, it will be understood that other biallelic or multi-allelic genetic markers may also be used. A biallelic genetic marker is one that has two polymorphic forms, or alleles. As mentioned above, for a biallelic genetic marker that is associated with a trait, the allele that is more abundant in the genetic composition of a case group as compared to a control group is termed the "associated allele," and the other allele may be referred to as the "unassociated allele." Thus, for each biallelic polymorphism that is associated with a given trait (*e.g.*, a disease or drug response), there is a corresponding associated allele. Other biallelic polymorphisms that may be used with the methods presented herein include, but are not limited to multinucleotide changes, insertions, deletions, and translocations. It will be further appreciated that references to DNA herein may include genomic DNA, mitochondrial DNA, episomal DNA, and/or derivatives of DNA such as amplicons, RNA transcripts, cDNA, DNA analogs, etc. The polymorphic loci that are screened in an association study may be in a diploid or a haploid state and, ideally, would

be from sites across the genome.

[0032] The term "selectively binds", "selective binding" and the like as used herein, when referring to a binding partner (*e.g.* protein, nucleic acid, antibody or other affinity capture agent, etc.), refers to a binding reaction of two or more binding partners with high affinity and/or complementarity to ensure selective hybridization under designated assay conditions. Typically, specific binding will be at least three times the standard deviation of the background signal. Thus, under designated conditions the binding partner binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

[0033] "Sequencing", "sequence determination" and the like means determination of information relating to the nucleotide base sequence of a nucleic acid. Such information may include the identification or determination of partial as well as full sequence information of the nucleic acid. Sequence information may be determined "with varying degrees of statistical reliability or confidence. In one aspect, the term includes the determination of the identity and ordering of a plurality of contiguous nucleotides in a nucleic acid, "High throughput digital sequencing" or "next generation sequencing" means sequence determination using methods that determine many (typically thousands to billions) of nucleic acid sequences in an intrinsically parallel manner, *i.e.* where DNA templates are prepared for sequencing not one at a time, but in a bulk process, and where many sequences are read out preferably in parallel, or alternatively using an ultra-high throughput serial process that itself may be parallelized. Such methods include but are not limited to pyrosequencing (for example, as commercialized by 454 Life Sciences, Inc., Branford, CT); sequencing by ligation (for example, as commercialized in the SOLiD™ technology, Life Technology, Inc., Carlsbad, CA); sequencing by synthesis using modified nucleotides (such as commercialized in TruSeq™ and HiSeq™ technology by Illumina, Inc., San Diego, CA, HeliScope™ by Helicos Biosciences Corporation, Cambridge, MA, and PacBio RS by Pacific Biosciences of California, Inc., Menlo Park, CA), sequencing by ion detection technologies (Ion Torrent, Inc., South San Francisco, CA); sequencing of DNA nanoballs (Complete Genomics, Inc., Mountain View, CA); nanopore-based sequencing technologies (for example, as developed by Oxford Nanopore Technologies, LTD, Oxford, UK), and like highly parallelized sequencing methods.

[0034] The term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation, $T_m = 81.5 + 0.41 (\% G+C)$, when a nucleic acid is in aqueous solution at 1M NaCl (see *e.g.*, Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985)). Other

references (*e.g.*, Allawi and SantaLucia, Jr., *Biochemistry*, 36:10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m .

DETAILED DESCRIPTION OF THE INVENTION

[0035] The practice of the techniques described herein may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and sequencing technology, which are within the skill of those who practice in the art. Such conventional techniques include polymer array synthesis, hybridization and ligation of polynucleotides, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the examples herein. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Green, et al., Eds., *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (1999); Weiner, Gabriel, Stephens, Eds., *Genetic Variation: A Laboratory Manual* (2007); Dieffenbach, Dveksler, Eds., *PCR Primer: A Laboratory Manual* (2003); Bowtell and Sambrook, *DNA Microarrays: A Molecular Cloning Manual* (2003); Mount, *Bioinformatics: Sequence and Genome Analysis* (2004); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); Stryer, *Biochemistry* (4th Ed.) (1995) W.H. Freeman, New York N.Y.; Gait, *"Oligonucleotide Synthesis: A Practical Approach"* (2002) IRL Press, London; Nelson and Cox, *Lehninger, Principles of Biochemistry* (2000) 3rd Ed., W. H. Freeman Pub., New York, N.Y.; and Berg, et al., *Biochemistry* (2002) 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

[0036] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" refers to one or more nucleic acids, and reference to "the assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0037] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing devices, formulations and methodologies that may be used in connection with the presently described invention.

[0038] Where a range of values is provided, it is understood that each intervening value, between the

upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention,

[0039] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention.

The Invention in General

[0040] The assay systems of the invention provide spatially-encoded, multiplexed assays comprising 1) an assay capable of high levels of multiplexing with an efficient spatial encoding scheme; 2) instrumentation capable of delivering reagents according to a spatial pattern; and 3) decoding determined by a readout that is digital in nature. The assay systems of the invention detect the presence or absence and relative amount of a biological target or biological activity indicative of a biological target, as well as the location of the biological target or activity in a biological sample, *e.g.*, a tissue section or other biological structure disposed upon a support such as a microscope slide or culture dish.

[0041] The assay system further provides instrumentation with an ability to deliver reagents in a spatially-defined pattern. This instrumentation, together "with software, reagents and protocols, provides a key component of the highly innovative assay system of the invention, allowing for measurement of numerous biological targets or activities in a meaningful spatial environment, including gene expression and peptide localization. An encoding scheme used in these assay systems allows one to determine the location of biological targets or activity (or lack thereof) in the biological samples after the products of the multiplexed assay are removed from the biological sample and pooled for analysis. Decoding of the encoding scheme can be performed by, *e.g.*, next-generation sequencing, which easily provides millions to trillions of data points at low cost. The assay results such as the amount or activity of biological targets can then be mapped back to specific location in the biological sample. The assay systems open a new analytical window into the complex spatial patterns of cellular function and regulation in biological samples.

[0042] A simplified overview of the assay system 100 of the present invention is provided at Figure 1. At step 110, a biological sample affixed to a support is provided. The biological sample contains biological targets of interest. Biological targets can include any molecule of interest, such as nucleic acids (including, *e.g.* RNA transcripts, genomic DNA sequences, cDNAs, amplicons, or other nucleic acid sequences) and proteins, enzymes and the like. At step 120, encoded probes are delivered to the biological sample according to a known spatial pattern. Encoded probes comprise probes, which can interact "with biological targets of interest, and coding tags, which identify the positions in the sample of the biological targets being assayed, and thus can be used to link assay results back to locations in the sample. Coding tags in most embodiments are oligonucleotides. However, coding tags may also be mass tags, fluorescent labels, or other moieties.

[0043] In some embodiments, the probe and coding tag portions of the encoded probe are pre-coupled before being delivered to the biological sample. For example, in the case where the encoded probes are oligonucleotides, both the probe and coding tag sequence can be synthesized as a single oligonucleotide. Alternatively, the probe and coding tag portions of the encoding probes can be synthesized or obtained separately and combined before delivery to the biological sample (*e.g.*, two separate oligonucleotides can be synthesized and coupled by, *e.g.*, ligation; or an antibody and an oligonucleotide can be prepared separately and conjugated before delivery to the biological sample). Also, as is described in Figures 2-5, the probes and the coding tags (in encoding oligonucleotides) are synthesized separately, and are delivered to the biological sample at different steps (*e.g.*, probes first and coding tags thereafter, or vice versa) in the assay.

[0044] At step 130, the encoded probes are allowed to react or interact with the biological targets, *i.e.*, conditions are provided to allow *e.g.*, oligonucleotides to hybridize to nucleic acid targets, enzymes to catalyze reactions with protein targets, antibodies to bind epitopes, etc. In the case where the biological targets are nucleic acids, the encoded probes are typically oligonucleotides and hybridize to the target nucleic acids. In the case that the biological targets are proteins, the encoded probes typically are aptamers, small molecules, or oligonucleotide-conjugated proteins that interact with target proteins by binding to them or by reacting with them (that is, one of the proteins is a substrate for the other). Encoding oligonucleotides may be coupled to the probes (proteins) by conjugation, chemical or photo-crosslinking via suitable groups and the like.

[0045] Once encoded probes interact with the biological targets, the encoded probes that interacted with the biological targets must be separated from the encoded probes that did not interact with the biological targets at step 140. In the case where the biological targets are nucleic acids and the encoded probes are oligonucleotides, the separation can be accomplished by, *e.g.*, washing the unhybridized encoded probes from the sample. Similarly, for other assays that are based on affinity

binding, including those using aptamer, small molecule, and protein probes, washing steps can be used to remove low affinity binders. In the case where the probe is transformed via interaction with the target, *e.g.*, in the case of a peptide, *e.g.*, via cleavage by a protease or phosphorylation by a kinase, it is convenient to collect, all encoded probes--both encoded probes that interacted with the biological targets and were transformed and encoded probes that were not transformed. After collection or pooling, an antibody or other affinity capture agent can be used to capture probes that were transformed by addition of a moiety (*e.g.*, a phosphate group). In cases where probes have been transformed via cleavage, the transformed probes can be separated, *e.g.*, by capturing the non-transformed probes via a tag that is removed from the transformed probes during the transformation (*e.g.*, by cleavage), or by adding a new tag at the site of cleavage.

[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.

[0050] Thus, the present invention provides an ability to look at many different biological targets in many locations, providing the resolution of in situ hybridization with the highly-parallel data analysis of sequencing. In some embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 20, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 50, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 100, greater than 500, 1,000, 10,000, 25,000, 100,000, 500,000, 1,000,000. It will be appreciated that, due to the spatial encoding dimension of the invention, even much larger numbers can be contemplated. For example, assaying 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, *a*1 through *a*10 (coding tag 406 on encoded probe 420) and *b*1 through *b*10 (coding tag 408 encoded probe 422). Coding tag *a*1, 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 *a*2 is deposited on the biological sample in ten spots on the second horizontal line in 412. Coding tag *a*3 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 *b*1 is deposited on the biological sample in ten discrete spots in the first vertical row of 414, coding tag *b*2 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 *a*1 and *b*4, *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 *a*1, *b*4.

[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 *a*1, *a*2, *a*3, *a*4 and *b*1, *b*3, *b*3 and *b*4. 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 *a*1 through *a*4 are deposited in spots in a pattern (here, in a vertical pattern), and encoding oligonucleotides *b*1 through *b*4 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, alb3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at alb1 (position shown with vertical lines). A sequence readout was obtained for both target-specific oligonucleotides 1 and 2 at positions a2b1, a3b1, alb2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at alb4, a2b4 or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample, and neither target nucleic acid was detected in the upper right portion of the biological sample. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample, as shown in 550.

[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, pions of a tissue sample can be analyzed in parallel and the data combined to build a 3D map.

[0072] Integral to the assay system of the invention is instrumentation that allows for spatial patterning of reagents onto the biological sample. Technologies for formulating and delivering both biological molecules (*e.g.* oligonucleotides or antibodies) and chemical reagents (*e.g.*, small molecules or dNTPs) are known in the art, and uses of these instrument systems are known to one skilled in the art and easily adaptable to the assay systems of the invention. One example of a suitable reagent delivery system is the LabcyteTM 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. 1~~ Fig. 4 illustrates such a target-specific assay system for identification of nucleic acid sequences in a sample. In this system ~~101~~ 401, two reagents ~~120~~ 420, ~~122~~ 422 that specifically bind to a biological molecule of interest are associated with coding identifiers ~~106~~ 406, ~~108~~ 408 that encode for a spatial location in the sample. These coding identifiers ~~106~~ 406, ~~108~~ 408 are optionally associated with sites that assist in their identification in the assay format, e.g., universal priming sites ~~104~~ 404, ~~110~~ 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section ~~116~~ 416 is encoded using the combination of the patterns ~~112~~ 412, ~~114~~ 414 created using the separate coding identifiers ~~106~~ 406, ~~108~~ 408 which provide a two dimensional code ~~118~~ 418 that shows the location of any positive detection of the biological molecule ~~102~~ 402 as well as quantifying the biological molecule ~~102~~ 402 at each location assayed in the tissue.

[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 2~~ Figure 3, two target-specific assay oligonucleotides are ligated together ~~202~~ 302 following *in situ* hybridization to target sequences. At the same time, encoding oligonucleotides containing tag sequence sets X and Y are ligated ~~204~~ 304 to the target specific oligonucleotides. Oligonucleotides containing X ligate specifically to one side of the targeting construct and oligonucleotides containing Y ligate to the other. The oligonucleotides contain universal primer sites P1 and P2. Following ligation, the constructs are eluted and, optionally, sequencing adapters can be attached ~~206~~ 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 $\sim 1,000$ reads may be sampled, for a total of $\sim 10^9$ 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, electroendosmosis, 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 $\sim 500 \mu\text{l}$ and can contain $\sim 3 \times 10^{11}$ molecules at a $1 \mu\text{M}$ concentration. To assay 1,000 target sequences in 10,000 cells, $\sim 2,000$ 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.

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 on 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, which is a continuation of 13/080,616 filed April 5, 2011, 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 methylphosphoramidite linkages; or peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with bicyclic structures including locked nucleic acids, positive backbones, non-ionic backbones and non-ribose backbones. Modifications of the ribose-phosphate backbone may be done to increase the stability of the molecules; for example, PNA:DNA hybrids can exhibit higher stability in some environments.

[0030] "Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Primers usually are extended by a DNA polymerase.

[0031] The term "SNP" or "single nucleotide polymorphism" refers to a genetic variation between individuals; *e.g.*, a single nitrogenous base position in the DNA of organisms that is variable. SNPs are found across the genome; much of the genetic variation between individuals is due to variation at SNP loci, and often this genetic variation results in phenotypic variation between individuals. SNPs for use in the present invention and their respective alleles may be derived from any number of sources, such as public databases (U.C. Santa Cruz Human Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>) or the NCBI dbSNP website (<http://www.ncbi.nlm.nih.gov/SNP/>), or may be experimentally determined as described in U.S. Pat No. 6,969,589; and US Pub. No. 2006/0188875 entitled "Human Genomic Polymorphisms." Although the use of SNPs is described in some of the embodiments presented herein, it will be understood that other biallelic or multi-allelic genetic markers may also be used. A biallelic genetic marker is one that has two polymorphic forms, or alleles. As mentioned above, for a biallelic genetic marker that is associated with a trait, the allele that is more abundant in the genetic composition of a case group as compared to a control group is termed the "associated allele," and the other allele may be referred to as the "unassociated allele." Thus, for each biallelic polymorphism that is associated with a given trait (*e.g.*, a disease or drug response), there is a corresponding associated allele. Other biallelic polymorphisms that may be used with the methods presented herein include, but are not limited to multinucleotide changes, insertions, deletions, and translocations. It will be further appreciated that references to DNA herein may include genomic DNA, mitochondrial DNA, episomal DNA, and/or derivatives of DNA such as amplicons, RNA transcripts, cDNA, DNA analogs, etc. The polymorphic loci that are screened in an association study may be in a diploid or a haploid state and, ideally, would

be from sites across the genome.

[0032] The term "selectively binds", "selective binding" and the like as used herein, when referring to a binding partner (*e.g.* protein, nucleic acid, antibody or other affinity capture agent, etc.), refers to a binding reaction of two or more binding partners with high affinity and/or complementarity to ensure selective hybridization under designated assay conditions. Typically, specific binding will be at least three times the standard deviation of the background signal. Thus, under designated conditions the binding partner binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

[0033] "Sequencing", "sequence determination" and the like means determination of information relating to the nucleotide base sequence of a nucleic acid. Such information may include the identification or determination of partial as well as full sequence information of the nucleic acid. Sequence information may be determined "with varying degrees of statistical reliability or confidence. In one aspect, the term includes the determination of the identity and ordering of a plurality of contiguous nucleotides in a nucleic acid, "High throughput digital sequencing" or "next generation sequencing" means sequence determination using methods that determine many (typically thousands to billions) of nucleic acid sequences in an intrinsically parallel manner, *i.e.* where DNA templates are prepared for sequencing not one at a time, but in a bulk process, and where many sequences are read out preferably in parallel, or alternatively using an ultra-high throughput serial process that itself may be parallelized. Such methods include but are not limited to pyrosequencing (for example, as commercialized by 454 Life Sciences, Inc., Branford, CT); sequencing by ligation (for example, as commercialized in the SOLiD™ technology, Life Technology, Inc., Carlsbad, CA); sequencing by synthesis using modified nucleotides (such as commercialized in TruSeq™ and HiSeq™ technology by Illumina, Inc., San Diego, CA, HeliScope™ by Helicos Biosciences Corporation, Cambridge, MA, and PacBio RS by Pacific Biosciences of California, Inc., Menlo Park, CA), sequencing by ion detection technologies (Ion Torrent, Inc., South San Francisco, CA); sequencing of DNA nanoballs (Complete Genomics, Inc., Mountain View, CA); nanopore-based sequencing technologies (for example, as developed by Oxford Nanopore Technologies, LTD, Oxford, UK), and like highly parallelized sequencing methods.

[0034] The term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation, $T_m = 81.5 + 0.41 (\% G+C)$, when a nucleic acid is in aqueous solution at 1M NaCl (see *e.g.*, Anderson and Young, Quantitative Filter Hybridization, in *Nucleic Acid Hybridization* (1985)). Other

references (*e.g.*, Allawi and SantaLucia, Jr., *Biochemistry*, 36:10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m .

DETAILED DESCRIPTION OF THE INVENTION

[0035] The practice of the techniques described herein may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and sequencing technology, which are within the skill of those who practice in the art. Such conventional techniques include polymer array synthesis, hybridization and ligation of polynucleotides, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the examples herein. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as Green, et al., Eds., *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) (1999); Weiner, Gabriel, Stephens, Eds., *Genetic Variation: A Laboratory Manual* (2007); Dieffenbach, Dveksler, Eds., *PCR Primer: A Laboratory Manual* (2003); Bowtell and Sambrook, *DNA Microarrays: A Molecular Cloning Manual* (2003); Mount, *Bioinformatics: Sequence and Genome Analysis* (2004); Sambrook and Russell, *Condensed Protocols from Molecular Cloning: A Laboratory Manual* (2006); and Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* (2002) (all from Cold Spring Harbor Laboratory Press); Stryer, *Biochemistry* (4th Ed.) (1995) W.H. Freeman, New York N.Y.; Gait, *"Oligonucleotide Synthesis: A Practical Approach"* (2002) IRL Press, London; Nelson and Cox, *Lehninger, Principles of Biochemistry* (2000) 3rd Ed., W. H. Freeman Pub., New York, N.Y.; and Berg, et al., *Biochemistry* (2002) 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

[0036] Note that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" refers to one or more nucleic acids, and reference to "the assay" includes reference to equivalent steps and methods known to those skilled in the art, and so forth.

[0037] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing devices, formulations and methodologies that may be used in connection with the presently described invention.

[0038] Where a range of values is provided, it is understood that each intervening value, between the

upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention,

[0039] In the following description, numerous specific details are set forth to provide a more thorough understanding of the present invention. However, it will be apparent to one of skill in the art that the present invention may be practiced without one or more of these specific details. In other instances, well-known features and procedures well known to those skilled in the art have not been described in order to avoid obscuring the invention.

The Invention in General

[0040] The assay systems of the invention provide spatially-encoded, multiplexed assays comprising 1) an assay capable of high levels of multiplexing with an efficient spatial encoding scheme; 2) instrumentation capable of delivering reagents according to a spatial pattern; and 3) decoding determined by a readout that is digital in nature. The assay systems of the invention detect the presence or absence and relative amount of a biological target or biological activity indicative of a biological target, as well as the location of the biological target or activity in a biological sample, *e.g.*, a tissue section or other biological structure disposed upon a support such as a microscope slide or culture dish.

[0041] The assay system further provides instrumentation with an ability to deliver reagents in a spatially-defined pattern. This instrumentation, together "with software, reagents and protocols, provides a key component of the highly innovative assay system of the invention, allowing for measurement of numerous biological targets or activities in a meaningful spatial environment, including gene expression and peptide localization, An encoding scheme used in these assay systems allows one to determine the location of biological targets or activity (or lack thereof) in the biological samples after the products of the multiplexed assay are removed from the biological sample and pooled for analysis. Decoding of the encoding scheme can be performed by, *e.g.*, next-generation sequencing, which easily provides millions to trillions of data points at low cost. The assay results such as the amount or activity of biological targets can then be mapped back to specific location in the biological sample. The assay systems open a new analytical window into the complex spatial patterns of cellular function and regulation in biological samples.

[0042] A simplified overview of the assay system 100 of the present invention is provided at Figure 1. At step 110, a biological sample affixed to a support is provided. The biological sample contains biological targets of interest. Biological targets can include any molecule of interest, such as nucleic acids (including, *e.g.* RNA transcripts, genomic DNA sequences, cDNAs, amplicons, or other nucleic acid sequences) and proteins, enzymes and the like. At step 120, encoded probes are delivered to the biological sample according to a known spatial pattern. Encoded probes comprise probes, which can interact "with biological targets of interest, and coding tags, which identify the positions in the sample of the biological targets being assayed, and thus can be used to link assay results back to locations in the sample. Coding tags in most embodiments are oligonucleotides. However, coding tags may also be mass tags, fluorescent labels, or other moieties.

[0043] In some embodiments, the probe and coding tag portions of the encoded probe are pre-coupled before being delivered to the biological sample. For example, in the case where the encoded probes are oligonucleotides, both the probe and coding tag sequence can be synthesized as a single oligonucleotide. Alternatively, the probe and coding tag portions of the encoding probes can be synthesized or obtained separately and combined before delivery to the biological sample (*e.g.*, two separate oligonucleotides can be synthesized and coupled by, *e.g.*, ligation; or an antibody and an oligonucleotide can be prepared separately and conjugated before delivery to the biological sample). Also, as is described in Figures 2-5, the probes and the coding tags (in encoding oligonucleotides) are synthesized separately, and are delivered to the biological sample at different steps (*e.g.*, probes first and coding tags thereafter, or vice versa) in the assay.

[0044] At step 130, the encoded probes are allowed to react or interact with the biological targets, *i.e.*, conditions are provided to allow *e.g.*, oligonucleotides to hybridize to nucleic acid targets, enzymes to catalyze reactions with protein targets, antibodies to bind epitopes, etc. In the case where the biological targets are nucleic acids, the encoded probes are typically oligonucleotides and hybridize to the target nucleic acids. In the case that the biological targets are proteins, the encoded probes typically are aptamers, small molecules, or oligonucleotide-conjugated proteins that interact with target proteins by binding to them or by reacting with them (that is, one of the proteins is a substrate for the other). Encoding oligonucleotides may be coupled to the probes (proteins) by conjugation, chemical or photo-crosslinking via suitable groups and the like.

[0045] Once encoded probes interact with the biological targets, the encoded probes that interacted with the biological targets must be separated from the encoded probes that did not interact with the biological targets at step 140. In the case where the biological targets are nucleic acids and the encoded probes are oligonucleotides, the separation can be accomplished by, *e.g.*, washing the unhybridized encoded probes from the sample. Similarly, for other assays that are based on affinity

binding, including those using aptamer, small molecule, and protein probes, washing steps can be used to remove low affinity binders. In the case where the probe is transformed via interaction with the target, *e.g.*, in the case of a peptide, *e.g.*, via cleavage by a protease or phosphorylation by a kinase, it is convenient to collect, all encoded probes--both encoded probes that interacted with the biological targets and were transformed and encoded probes that were not transformed. After collection or pooling, an antibody or other affinity capture agent can be used to capture probes that were transformed by addition of a moiety (*e.g.*, a phosphate group). In cases where probes have been transformed via cleavage, the transformed probes can be separated, *e.g.*, by capturing the non-transformed probes via a tag that is removed from the transformed probes during the transformation (*e.g.*, by cleavage), or by adding a new tag at the site of cleavage.

[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.

[0050] Thus, the present invention provides an ability to look at many different biological targets in many locations, providing the resolution of in situ hybridization with the highly-parallel data analysis of sequencing. In some embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 20, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 50, in other embodiments, the sum of the multiple biological targets being assayed and the multiple sites in the biological sample is greater than 100, greater than 500, 1,000, 10,000, 25,000, 100,000, 500,000, 1,000,000. It will be appreciated that, due to the spatial encoding dimension of the invention, even much larger numbers can be contemplated. For example, assaying 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, alb3, a2b3, a3b3, a4b3 and a4b4 (positions shown with horizontal lines). A sequence readout was obtained for target-specific oligonucleotide 2 only at alb1 (position shown with vertical lines). A sequence readout was obtained for both target-specific oligonucleotides 1 and 2 at positions a2b1, a3b1, alb2, a2b2, and a3b2 (positions shown with cross-hatching). No sequence readout was obtained for either target-specific oligonucleotides at alb4, a2b4 or a3b4 (positions shown without shading). Thus, in the biological sample on which the assay took place the first target nucleic acid was detected in a large portion of the left side and at the bottom of the biological sample, the second target nucleic acid was detected only in the upper left portion of the biological sample, and neither target nucleic acid was detected in the upper right portion of the biological sample. The differential expression of the two target nucleic acids now can be mapped back to the biological sample and to the biological structures or cell types in these locations in the biological sample, as shown in 550.

[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, pions of a tissue sample can be analyzed in parallel and the data combined to build a 3D map.

[0072] Integral to the assay system of the invention is instrumentation that allows for spatial patterning of reagents onto the biological sample. Technologies for formulating and delivering both biological molecules (*e.g.* oligonucleotides or antibodies) and chemical reagents (*e.g.*, small molecules or dNTPs) are known in the art, and uses of these instrument systems are known to one skilled in the art and easily adaptable to the assay systems of the invention. One example of a suitable reagent delivery system is the LabcyteTM Echo acoustic liquid handier, 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 401, two reagents 420, 422 that specifically bind to a biological molecule of interest are associated with coding identifiers 406, 408 that encode for a spatial location in the sample. These coding identifiers 406, 408 are optionally associated with sites that assist in their identification in the assay format, *e.g.*, universal priming sites 404, 410 for amplification of the assay products or adapters to enable identification of the coding identifiers and the binding agents using sequencing technologies. The sample that is tested, here shown as a tissue section 416 is encoded using the combination of the patterns 412, 414 created using the separate coding identifiers 406, 408 which provide a two dimensional code 418 that shows the location of any positive detection of the biological molecule 402 as well as quantifying the biological molecule 402 at each location assayed in the tissue.

[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 $\sim 1,000$ reads may be sampled, for a total of $\sim 10^9$ 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, electroendoosmosis, 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 $\sim 500 \mu\text{l}$ and can contain $\sim 3 \times 10^{11}$ molecules at a $1 \mu\text{M}$ concentration. To assay 1,000 target sequences in 10,000 cells, $\sim 2,000$ 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.

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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			79366d3fd1e96279bb90346f715c1dd28c2d240c		

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Information:					
3	Specification	CLEAN_SPECIFICATION.pdf	382928	no	43
			a0f3914f8f298ebb126fb15d244e5c1b645011cd		
Warnings:					
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Total Files Size (in bytes):			988391		
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Doc Code: DIST.E.FILE Document Description: Electronic Terminal Disclaimer - Filed		PTO/SB/25 U.S. Patent and Trademark Office Department of Commerce	
Electronic Petition Request	TERMINAL DISCLAIMER TO OBIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION		
Application Number	16430015		
Filing Date	03-Jun-2019		
First Named Inventor	Mark CHEE		
Attorney Docket Number	47706-0004008 / 700-00010		
Title of Invention	Spatially Encoded Biological Assays		
☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action ☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.			
Owner	Percent Interest		
Prognosys Biosciences, Inc.	100%		
The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Number(s)			
16435295 filed on 06/07/2019 as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns. In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.			
☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.			

☐ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.	
Applicant claims the following fee status: ☐ Small Entity ☐ Micro Entity ☒ Regular Undiscounted	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.	
THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES I certify, in accordance with 37 CFR 1.4(d)(4) that I am: ☒ An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application Registration Number <u>58674</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	/Cameron Mark Luitjens/
Name	Cameron Mark Luitjens

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
 Form PTO/SB/96 may be used for making this certification. See MPEP § 324.