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Nordman et al.

(54) **METHOD AND SYSTEM FOR MULTIPLEX GENETIC ANALYSIS**

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(60) Provisional application No. 60/689,692, filed on Jun. 10, 2005.

Publication Classification

(51) **Int. Cl.**
C12Q 1/68 (2006.01)

(52) **U.S. Cl.** **435/6**

(57) **ABSTRACT**

A method for identifying nucleotides in a nucleic acid sequence is disclosed. A plurality of polymerization complexes are provided within a plurality of confined reaction environments. Each complex comprises a polymerase enzyme and a template nucleic acid. The plurality of complexes are contacted with a plurality of types of nucleotide analogs labeled with distinguishable fluorescent labels under conditions suitable for polymerization. Fluorescent signals associated with incorporation of a nucleotide analog are transmitted to a detector, wherein the location of the fluorescent signal on the detector is indicative of the individual confined reaction environment and the type of nucleotide incorporated. The nucleotide in a nucleic acid sequence is identified based upon the type of nucleotide incorporated and the confined reaction environment.

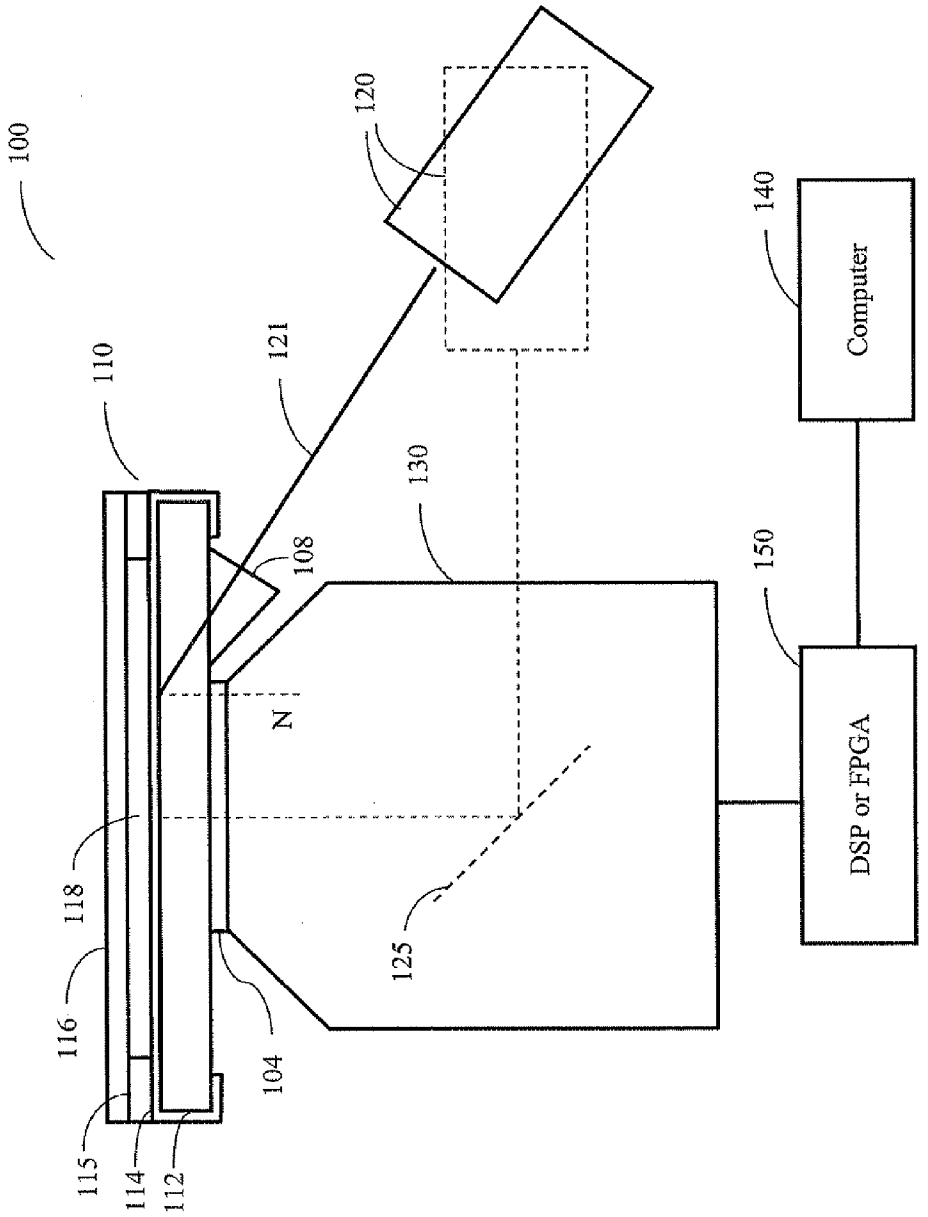
100
120
140
Computer
110
121
130
108
150
N
DSP or FPGA
118
125
116
104
115
114
112

FIG. 1

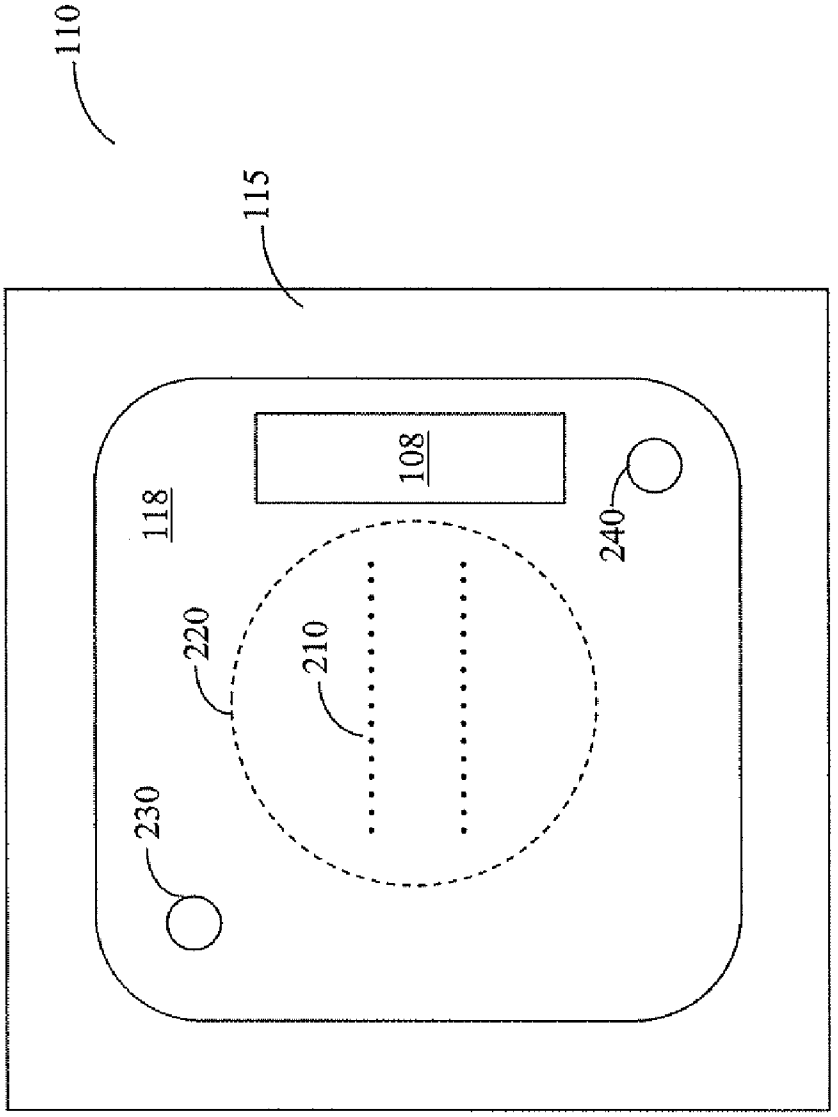

FIG. 2

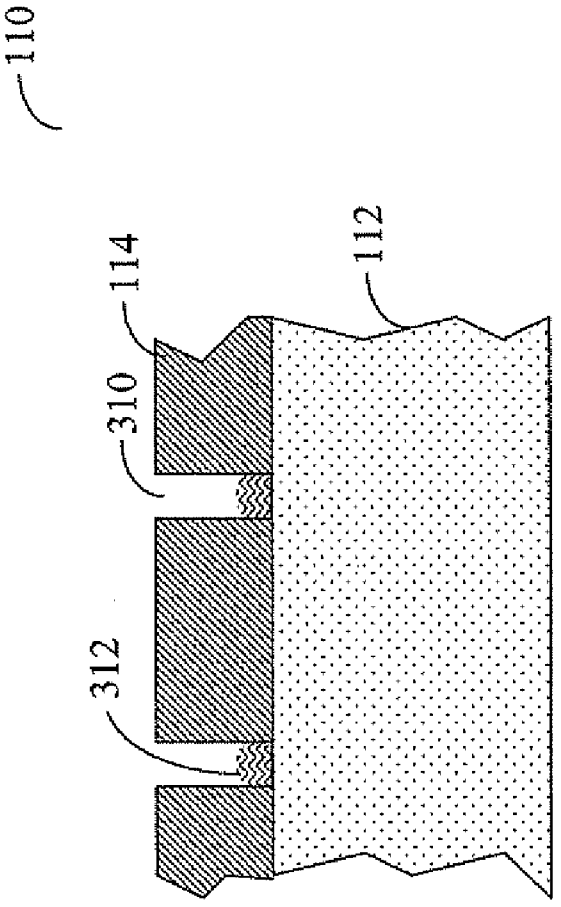

FIG. 3

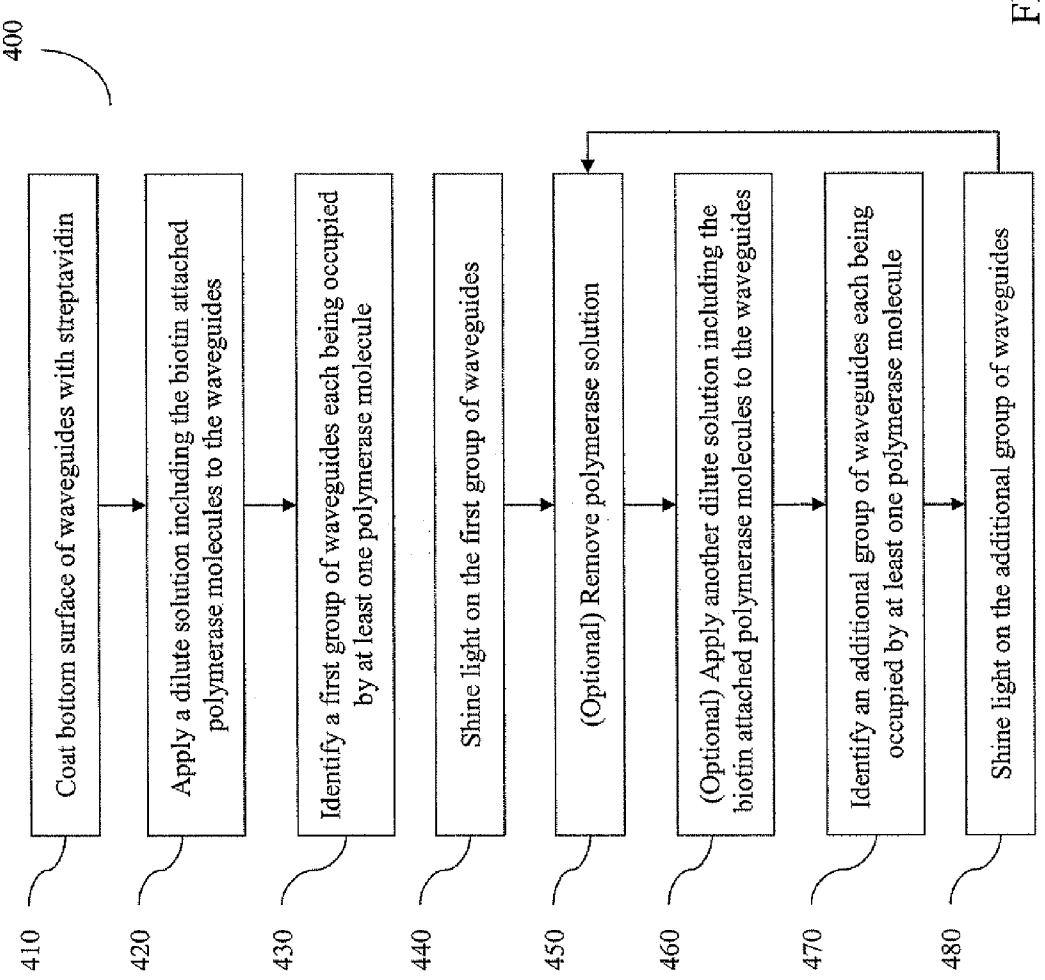
400
Coat bottom surface of waveguides with streptavidin
Apply a dilute solution including the biotin attached polymerase molecules to the waveguides
Identify a first group of waveguides each being occupied by at least one polymerase molecule
Shine light on the first group of waveguides
(Optional) Remove polymerase solution
(Optional) Apply another dilute solution including the biotin attached polymerase molecules to the waveguides
Identify an additional group of waveguides each being occupied by at least one polymerase molecule
Shine light on the additional group of waveguides
410
420
430
440
450
460
470
480

FIG. 4

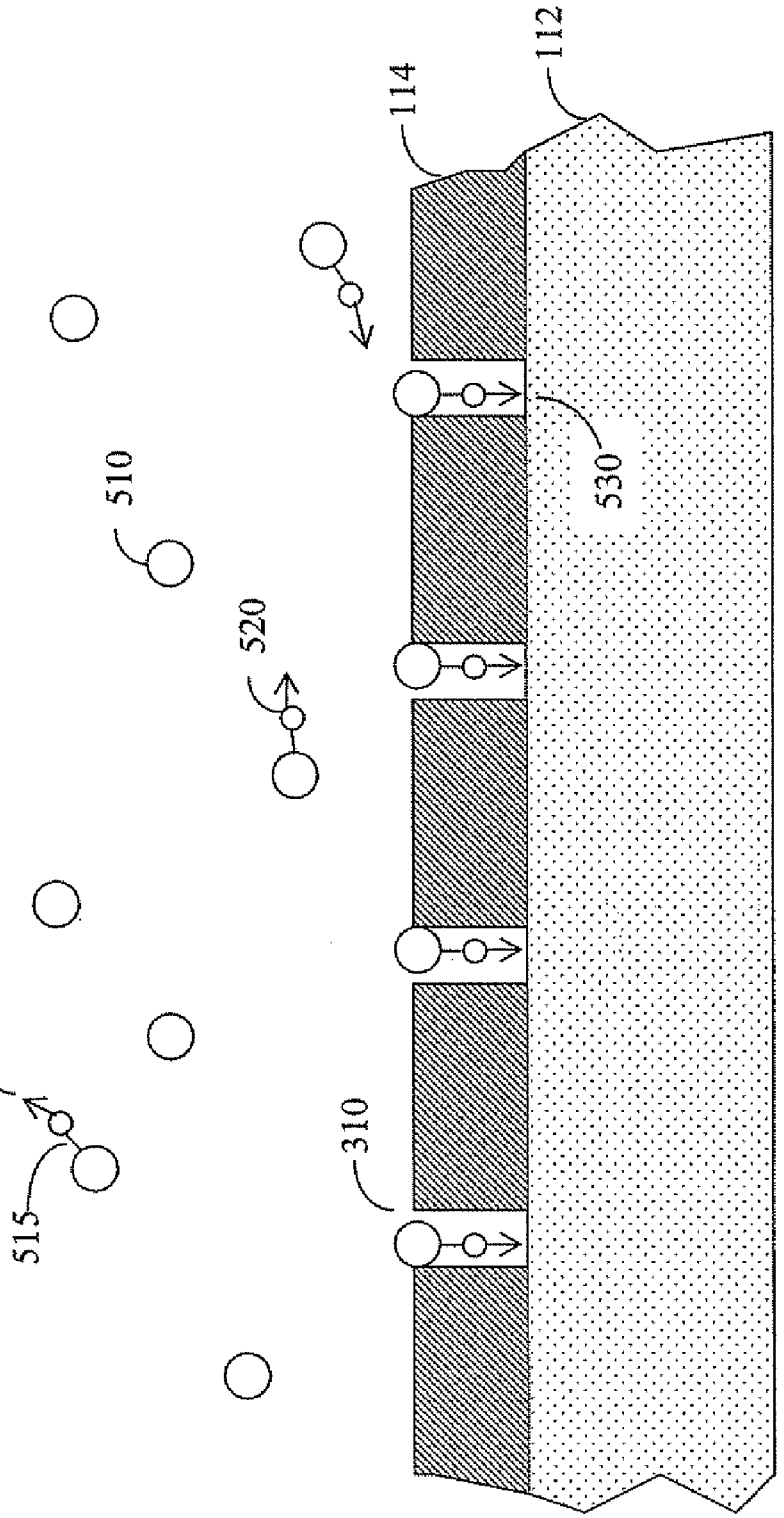

FIG. 5

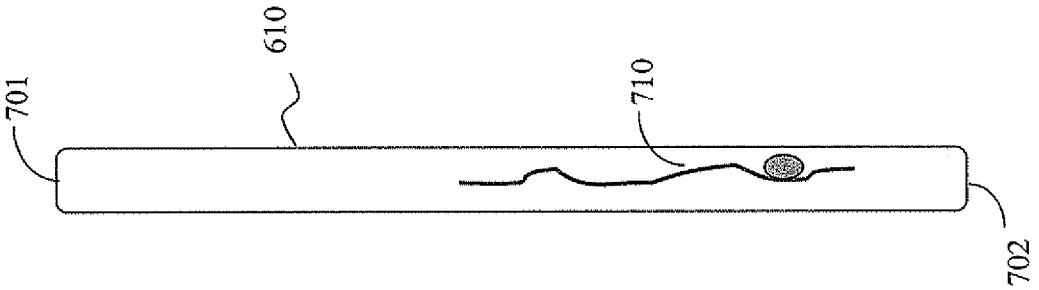

FIG. 7

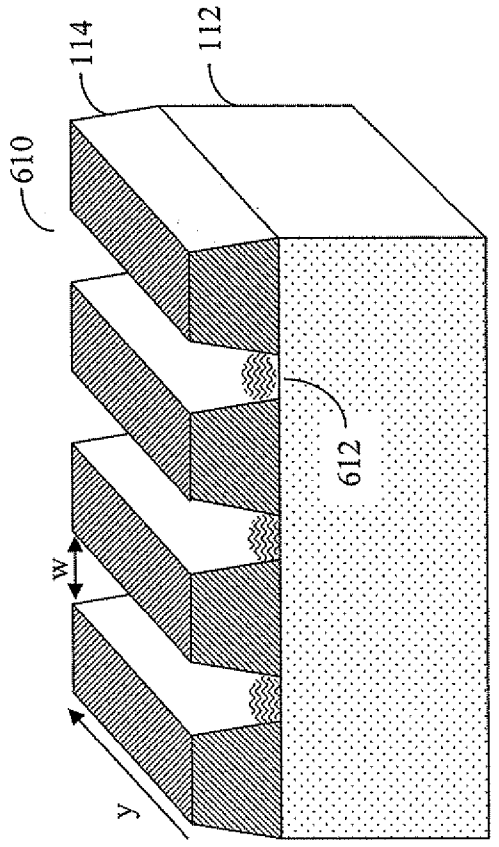

FIG. 6

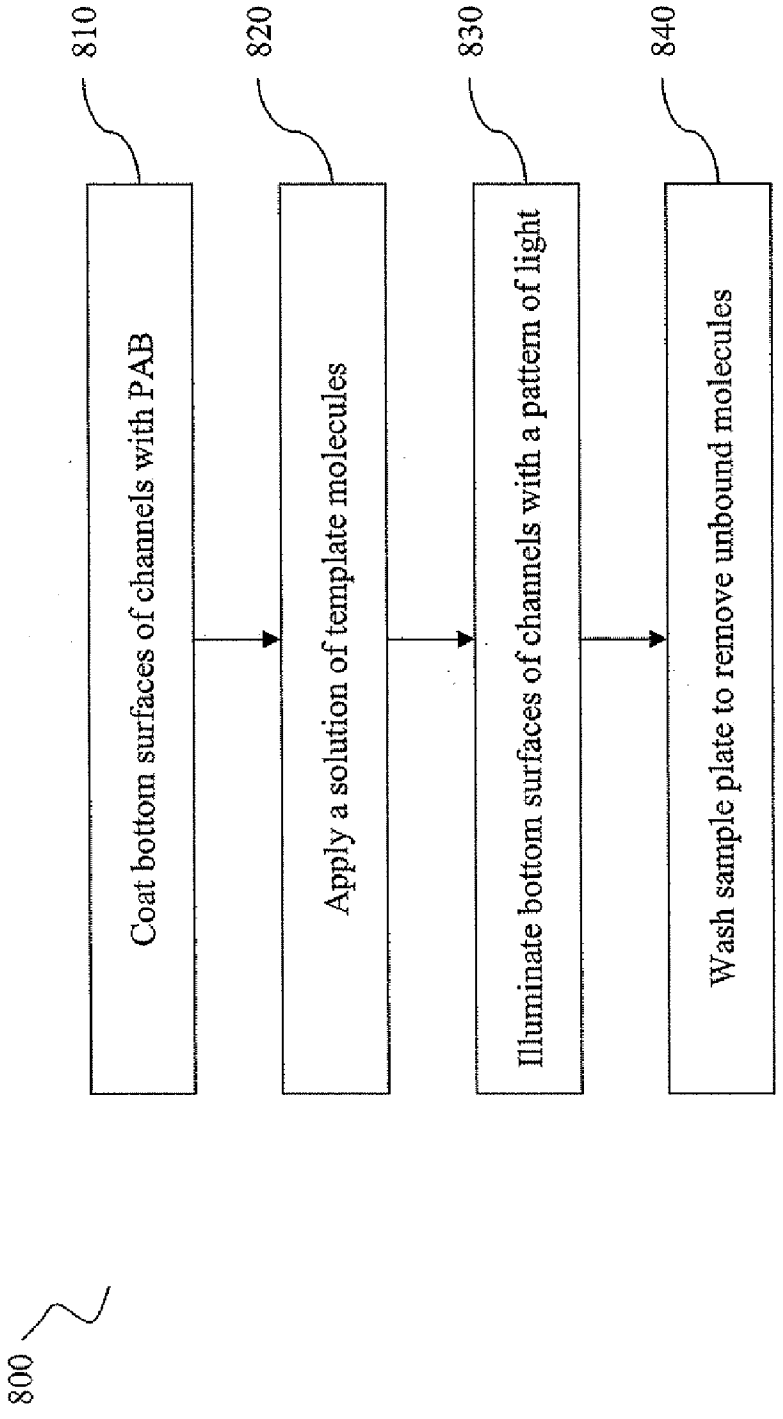
800
810
Coat bottom surfaces of channels with PAB
820
Apply a solution of template molecules
830
Illuminate bottom surfaces of channels with a pattern of light
840
Wash sample plate to remove unbound molecules

FIG. 8A

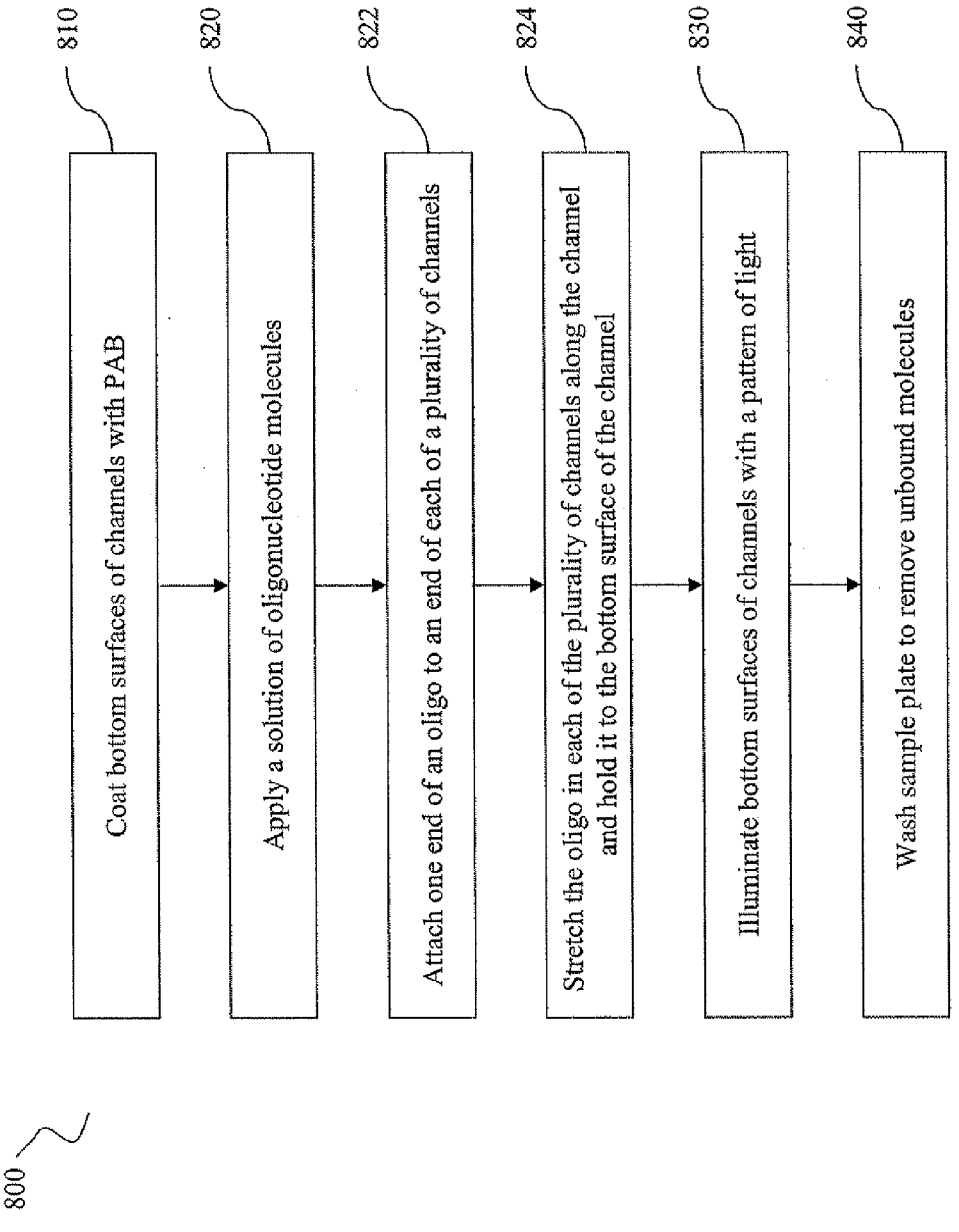
800
810
Coat bottom surfaces of channels with PAB
820
Apply a solution of oligonucleotide molecules
822
Attach one end of an oligo to an end of each of a plurality of channels
824
Stretch the oligo in each of the plurality of channels along the channel and hold it to the bottom surface of the channel
830
Illuminate bottom surfaces of channels with a pattern of light
840
Wash sample plate to remove unbound molecules

FIG. 8B

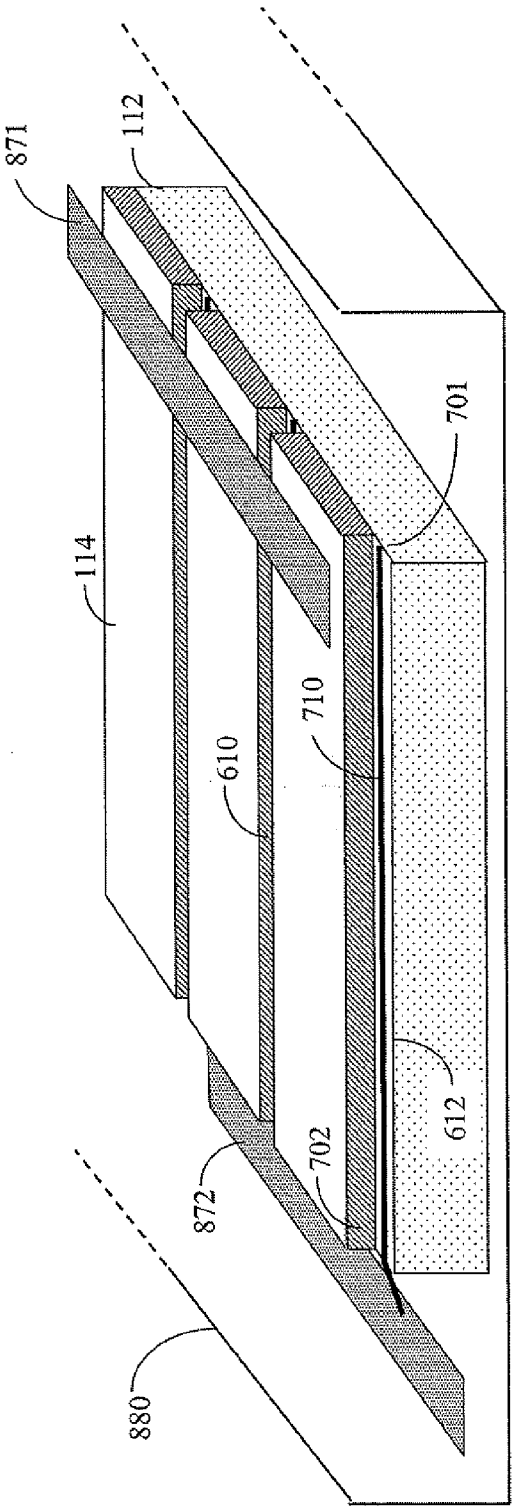

FIG. 8C

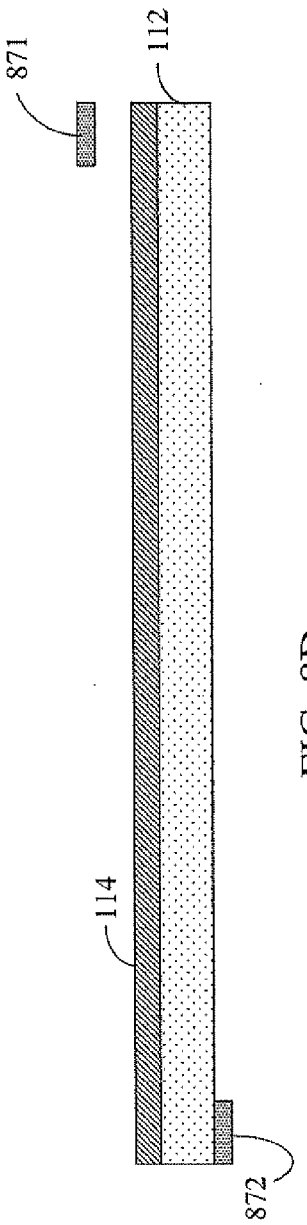

FIG. 8D

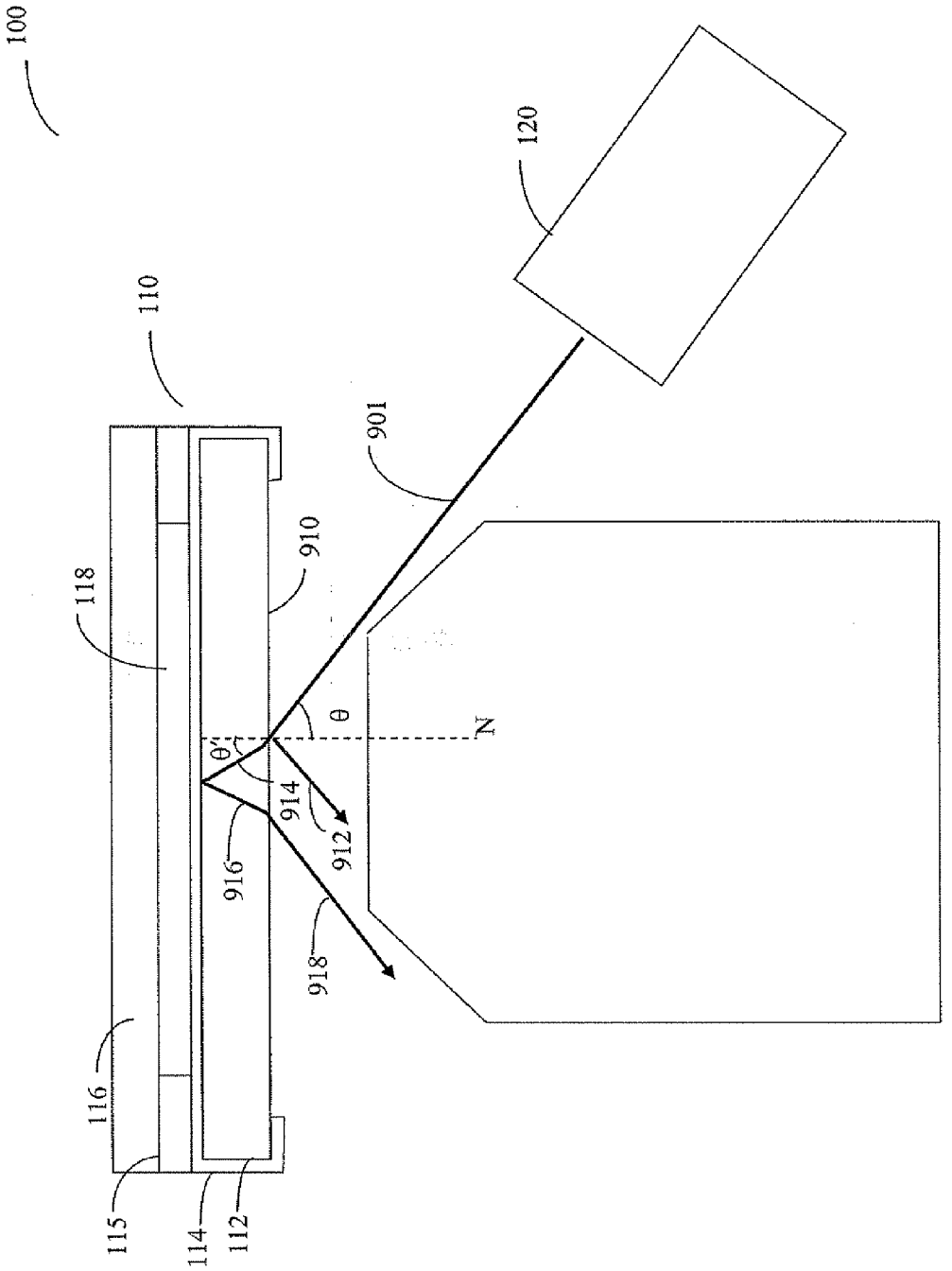

FIG. 9

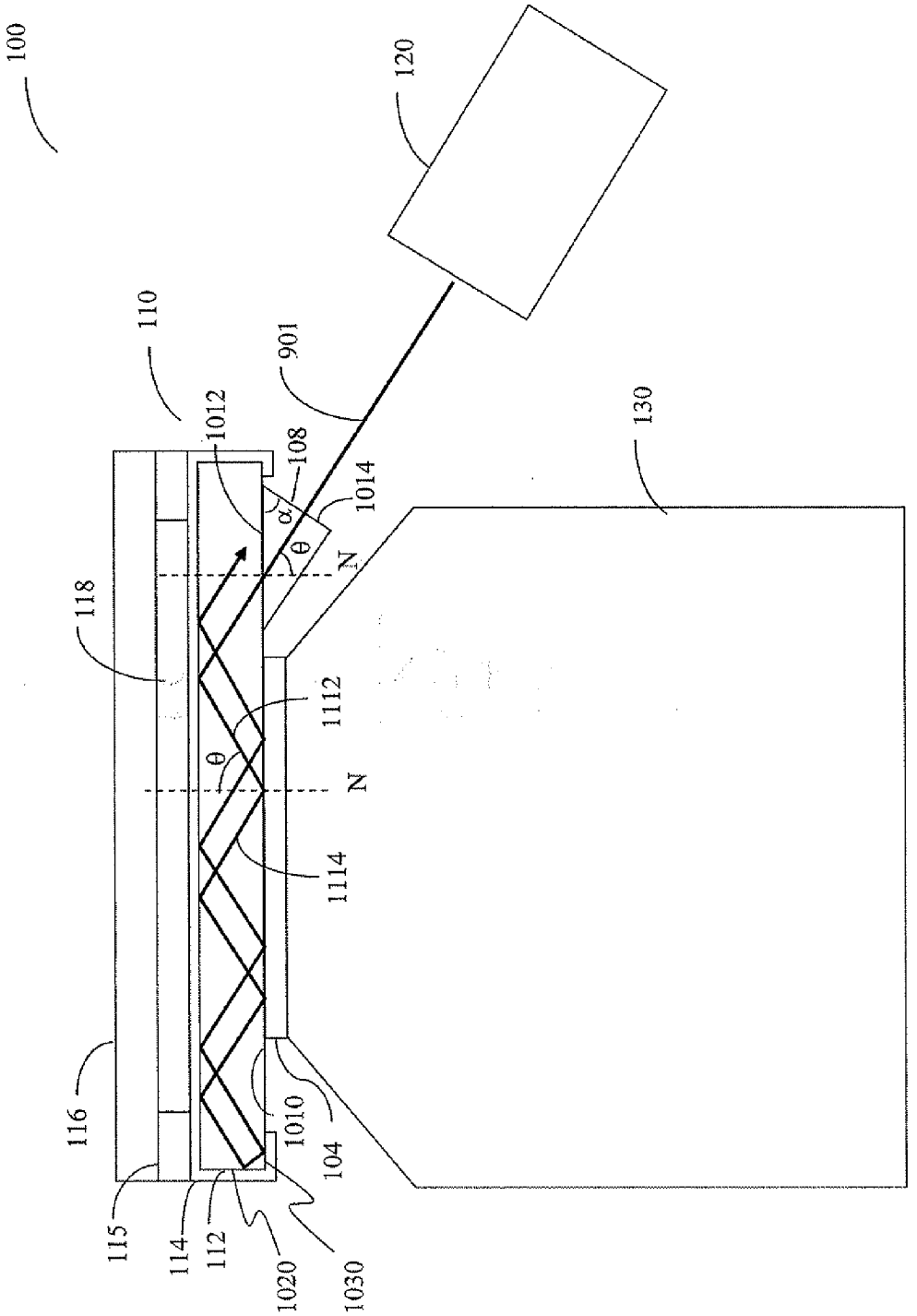
100
120
110
901
1012
108
130
1014
α
θ
N
118
1112
θ
N
1114
116
1010
104
115
114
112
1020
1030

FIG. 10A

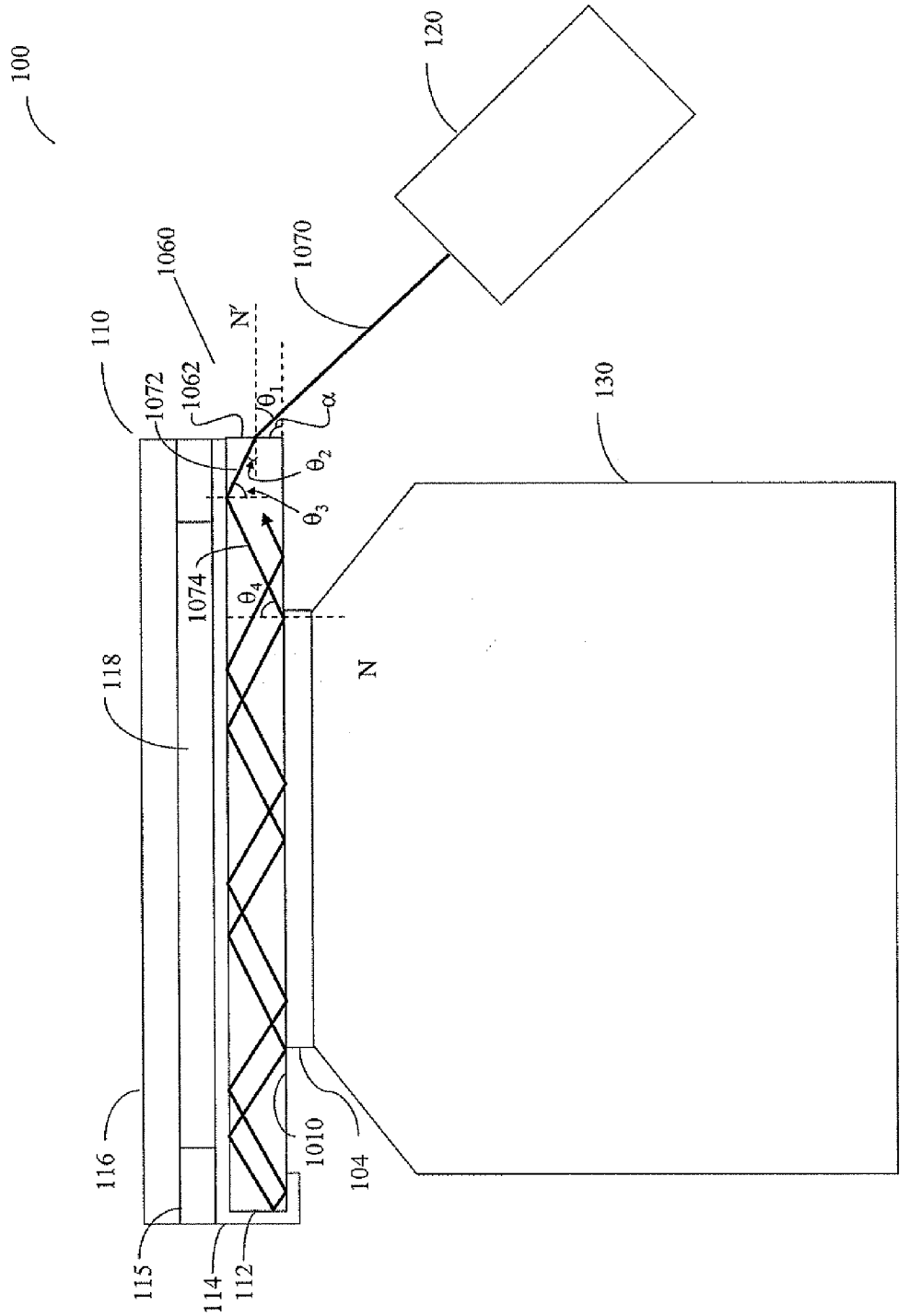
100
120
1060
1070
110
N'
1072
1062
θ_1
α
130
θ_2
θ_3
1074
θ_4
118
N
116
1010
104
115
114
112

FIG. 10B

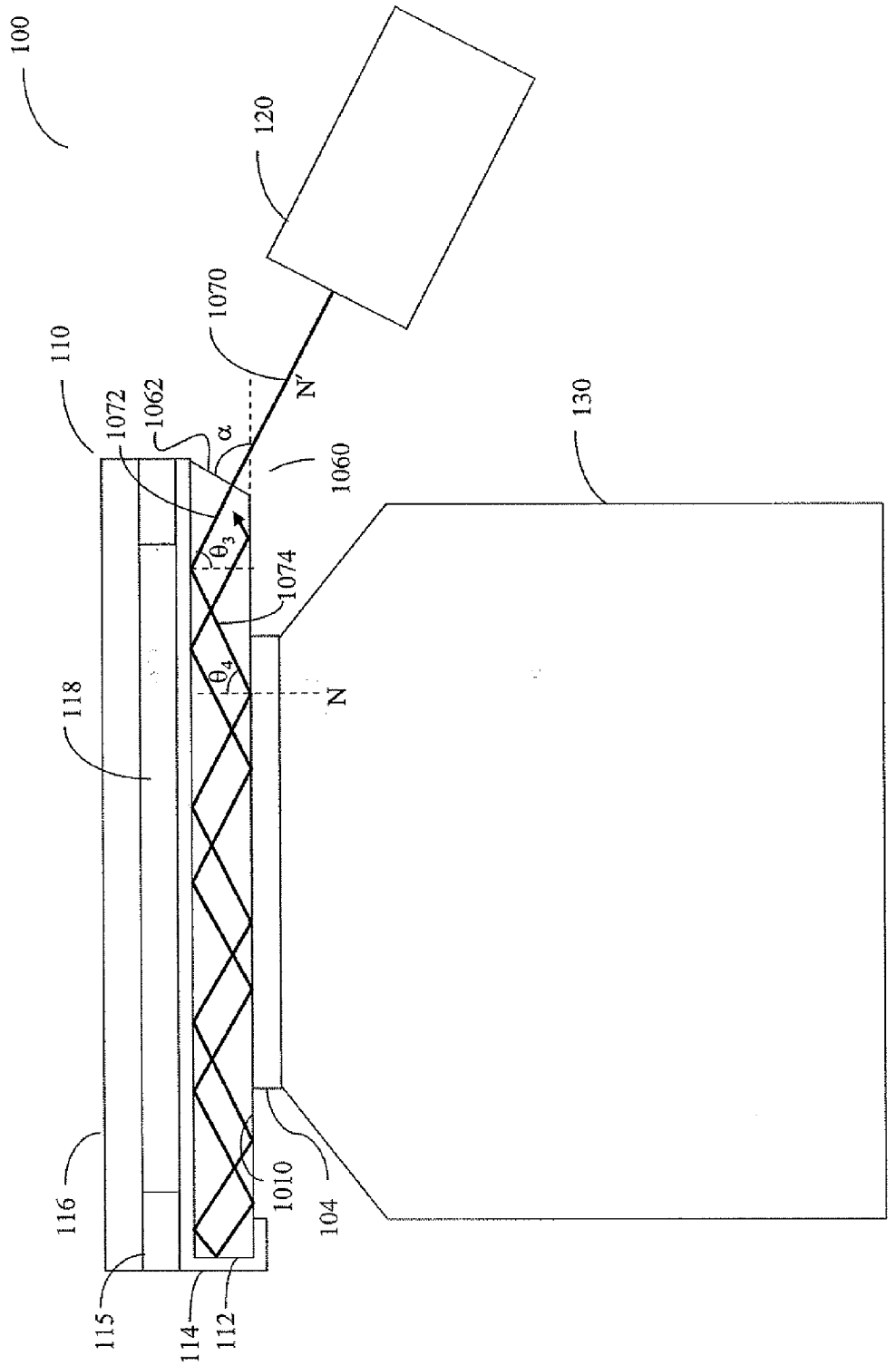
100
120
1070
110
1072
1062
α
N'
130
1060
θ3
1074
θ4
N
118
116
1010
104
115
114
112

FIG. 10C

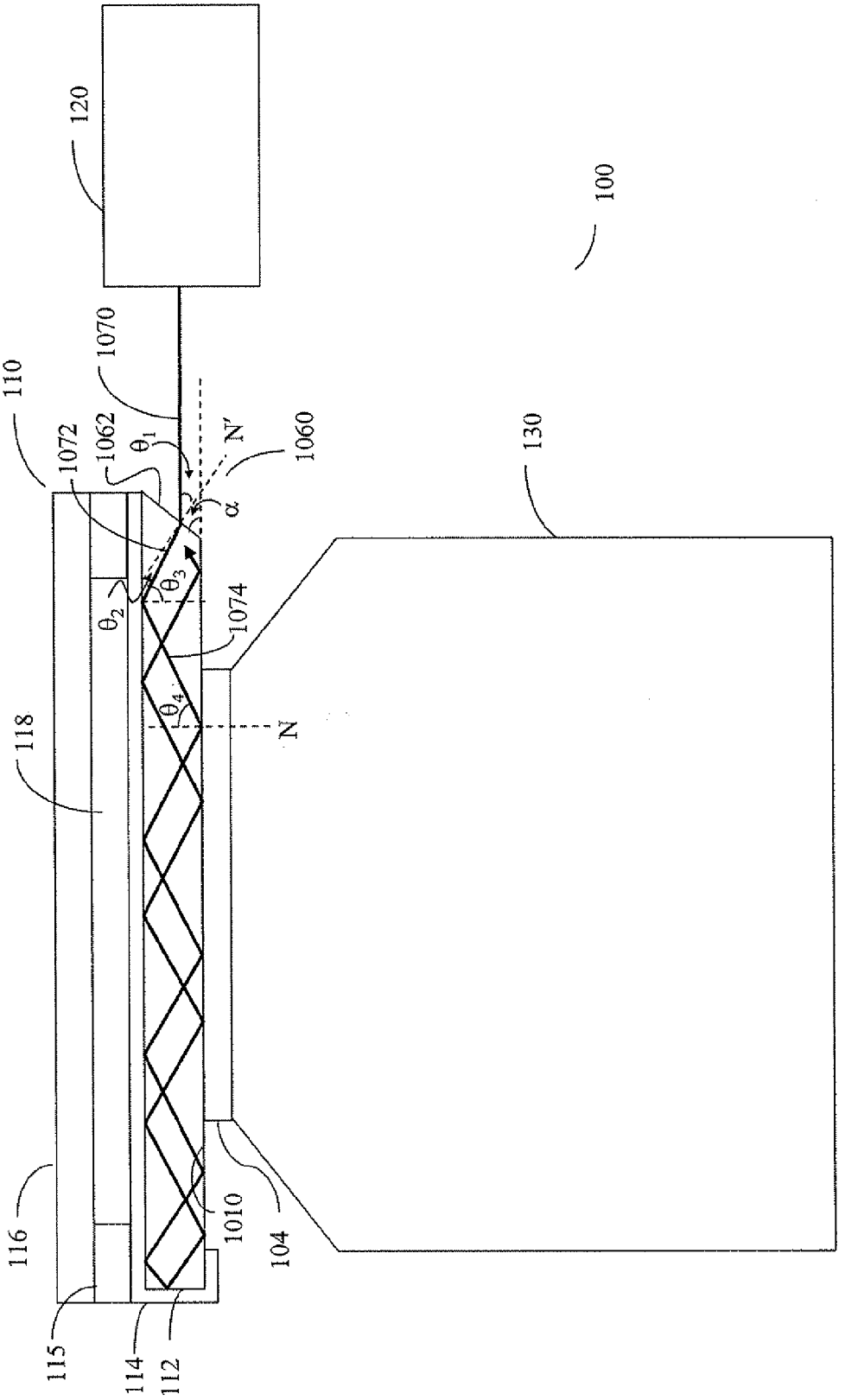
120
100
1070
110
1072
1062
θ1
N'
1060
α
130
θ3
θ2
1074
θ4
N
118
116
1010
104
115
114
112

FIG. 10D

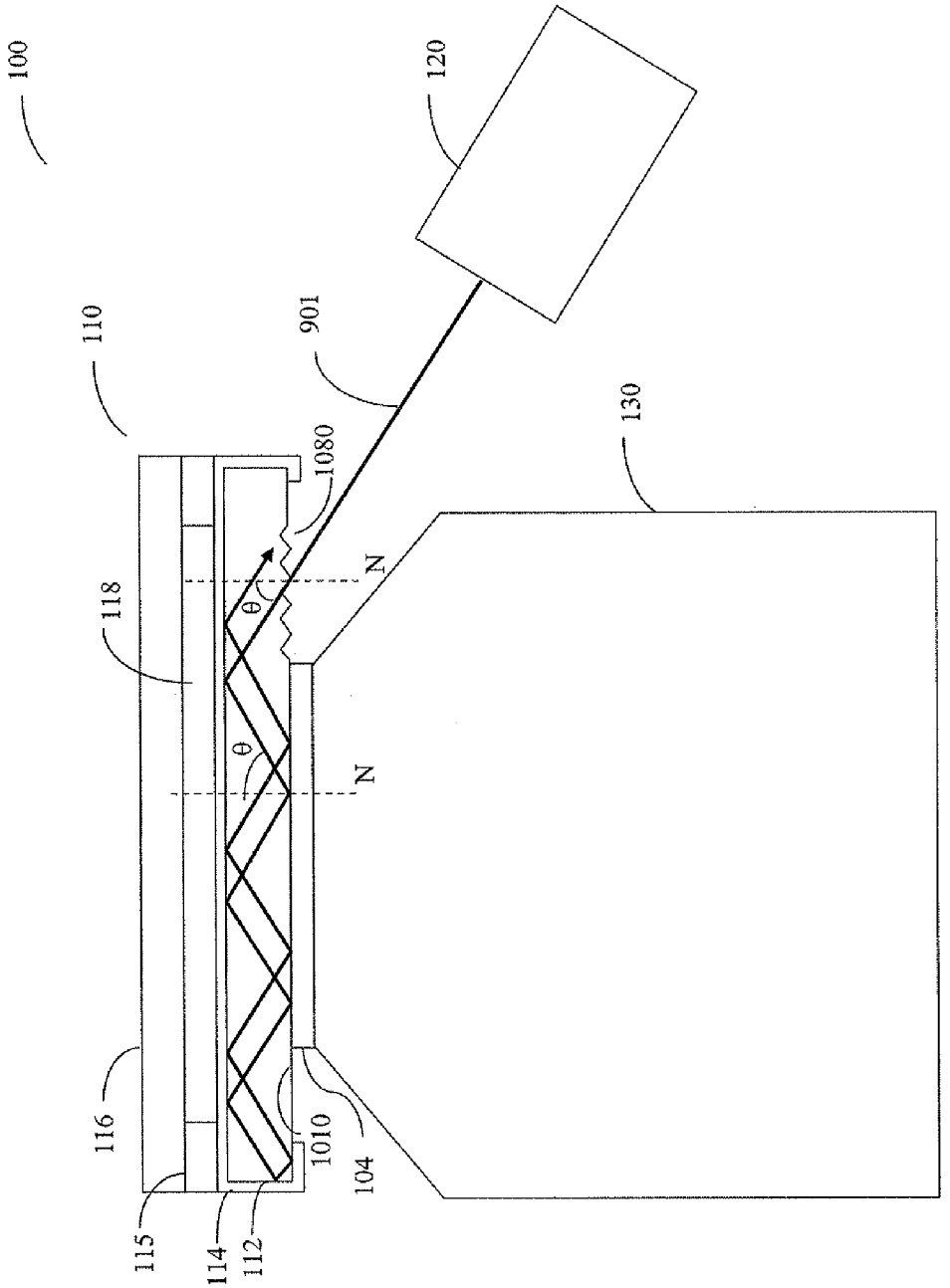

FIG. 10E

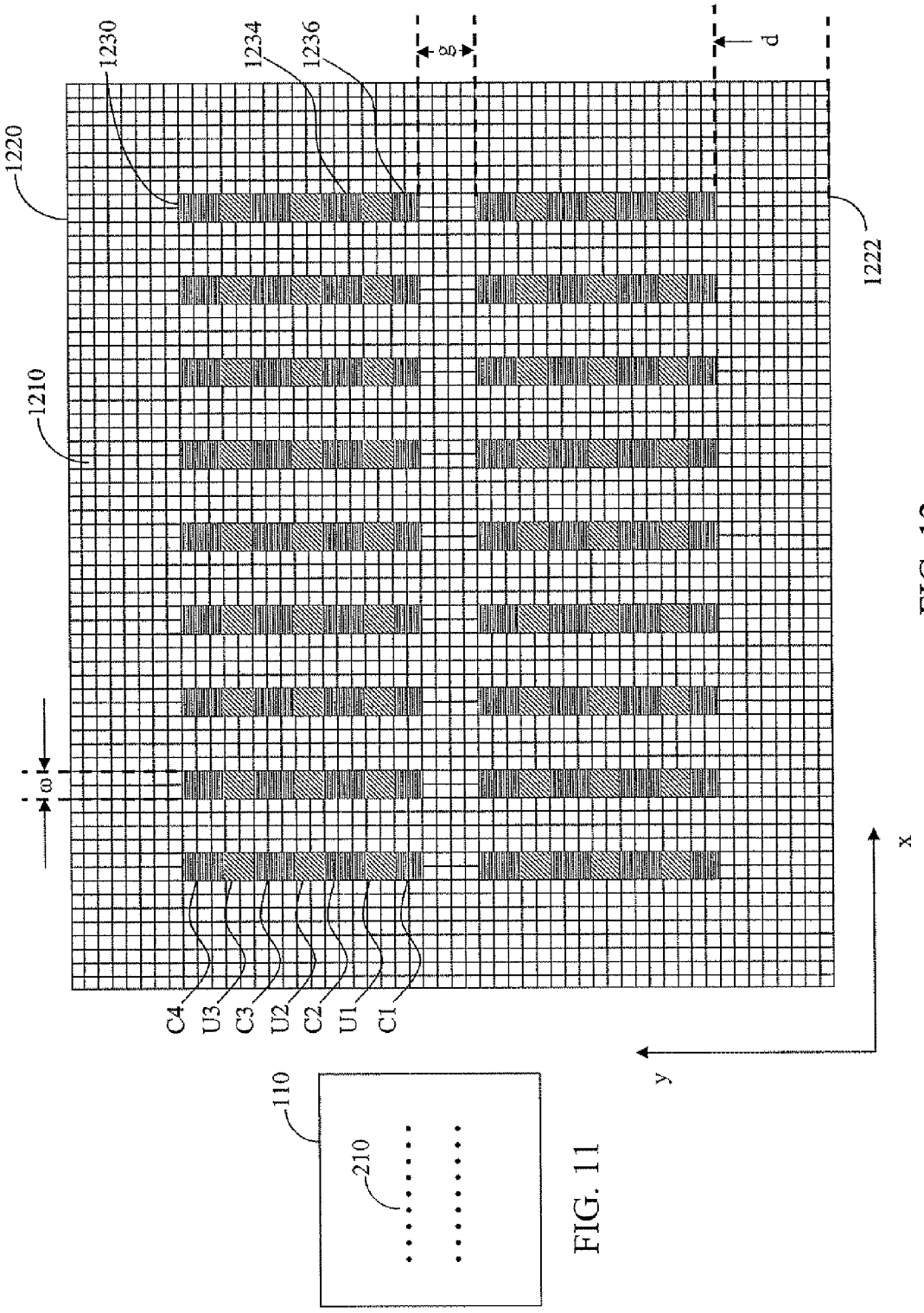

FIG. 12

FIG. 11

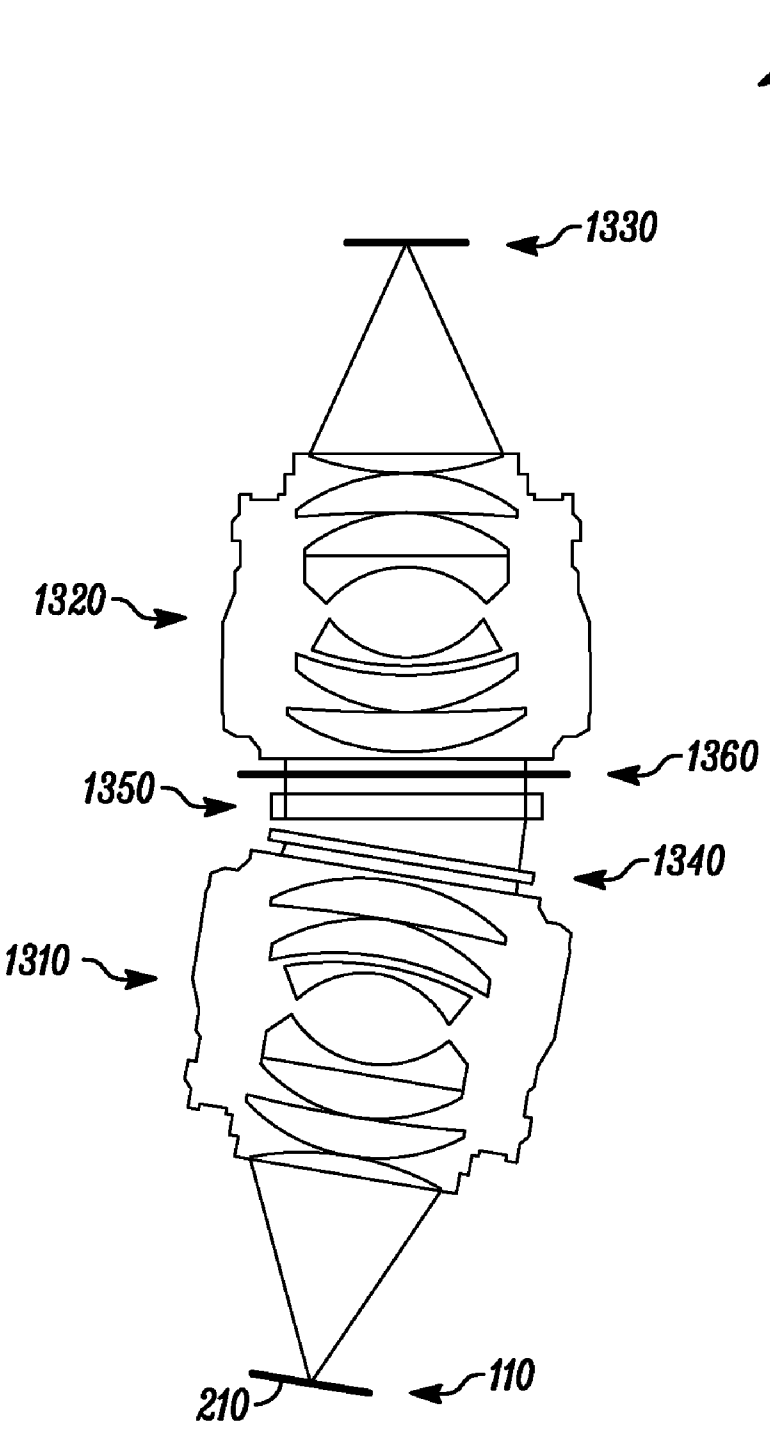

FIG. 13

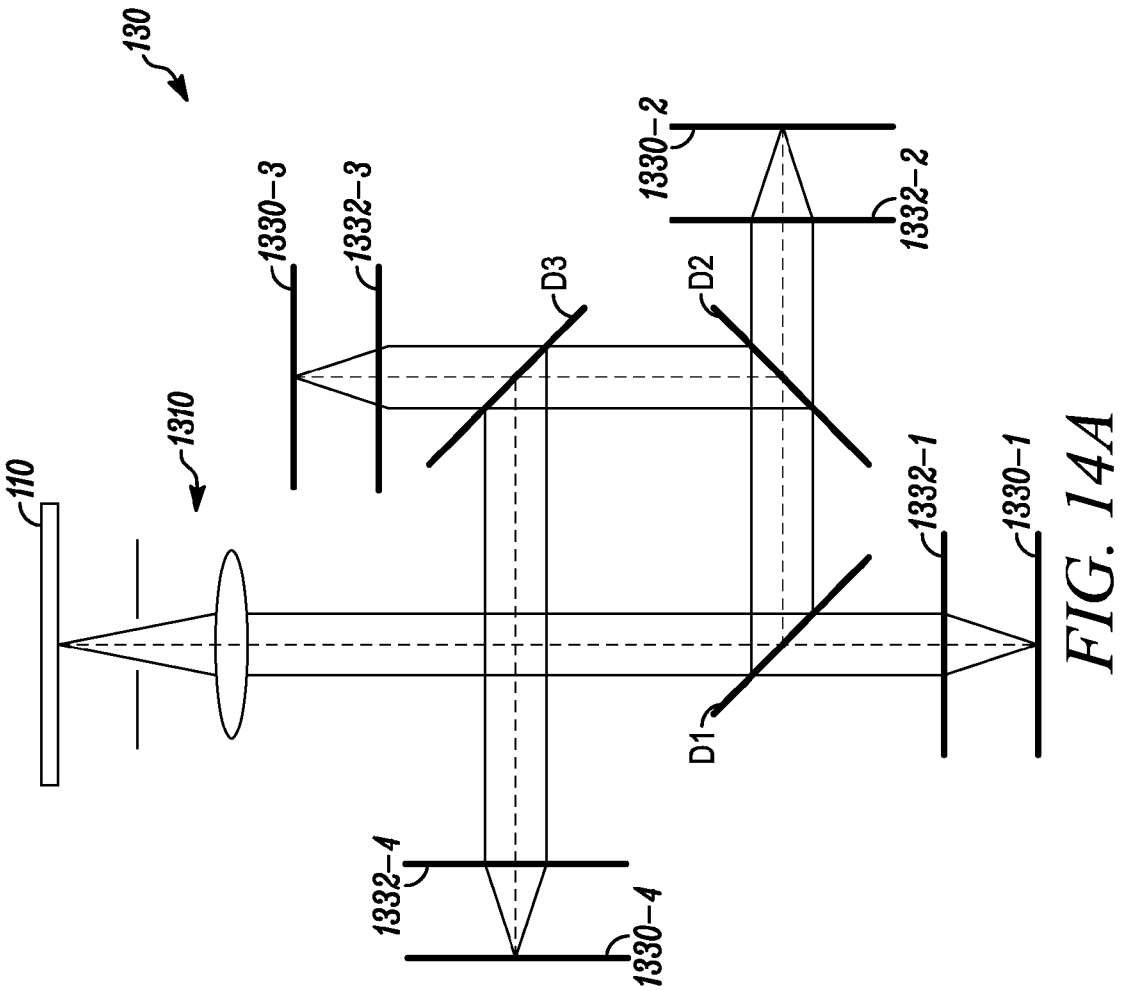
130
1330-3
1332-3
1330-2
1332-2
D3
D2
1310
110
1332-1
1330-1
D1
1332-4
1330-4

FIG. 14A

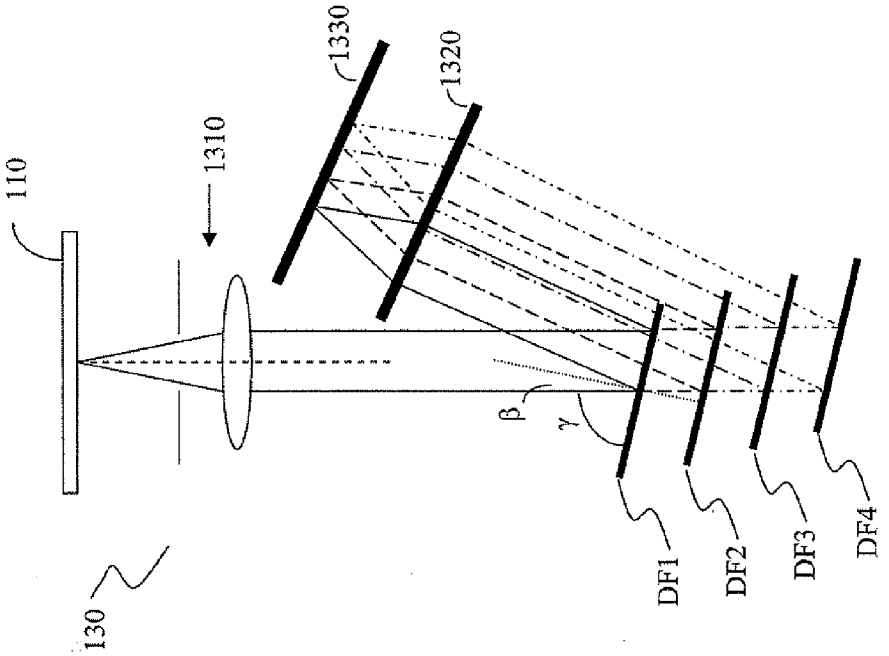

FIG. 14C

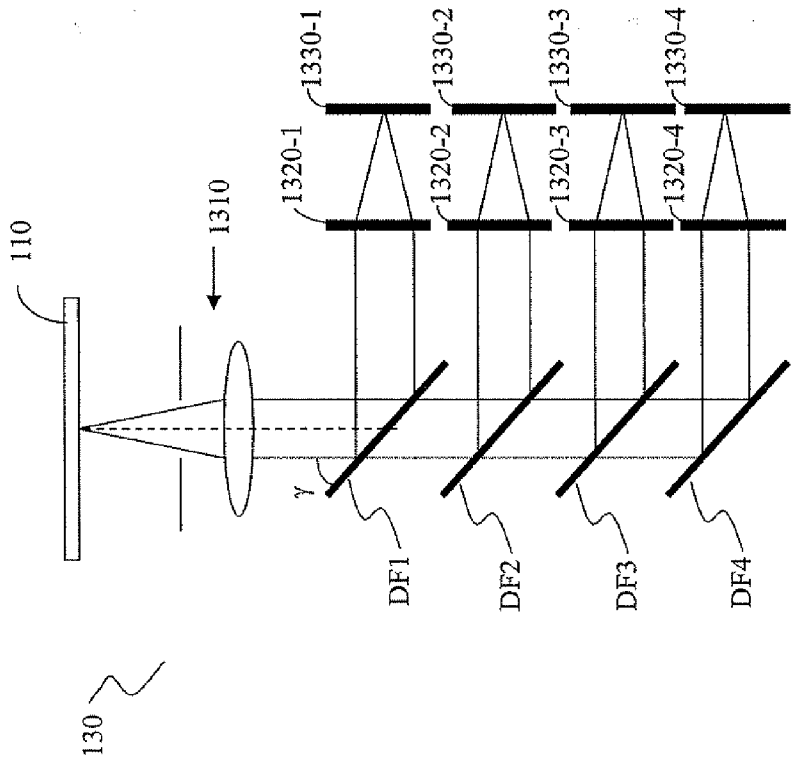

FIG. 14B

FIG. 15A

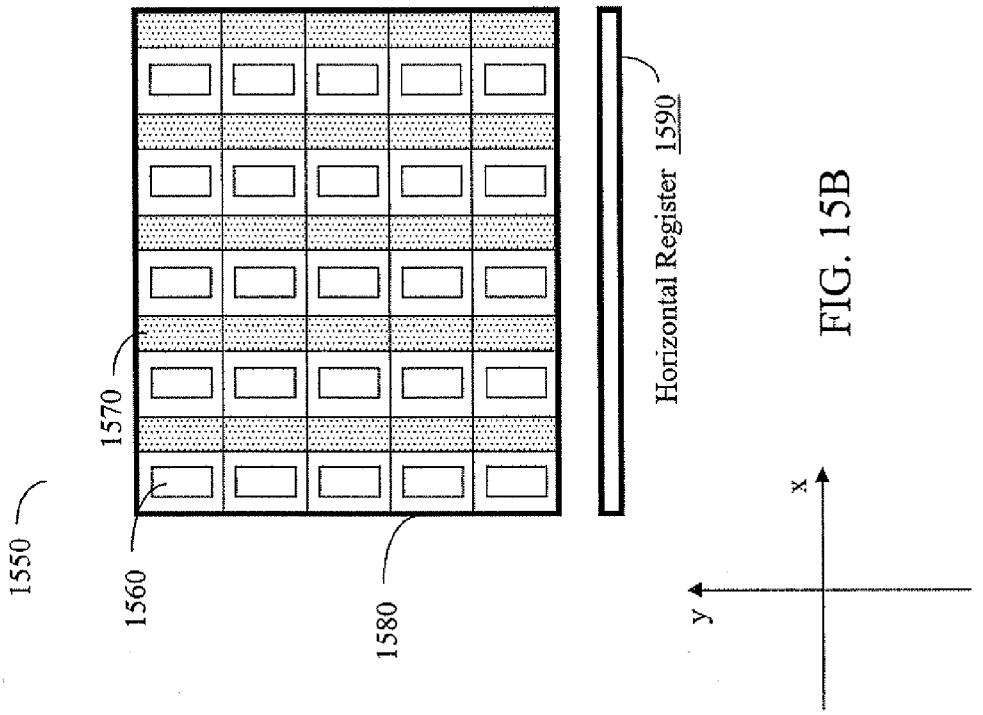

FIG. 15B

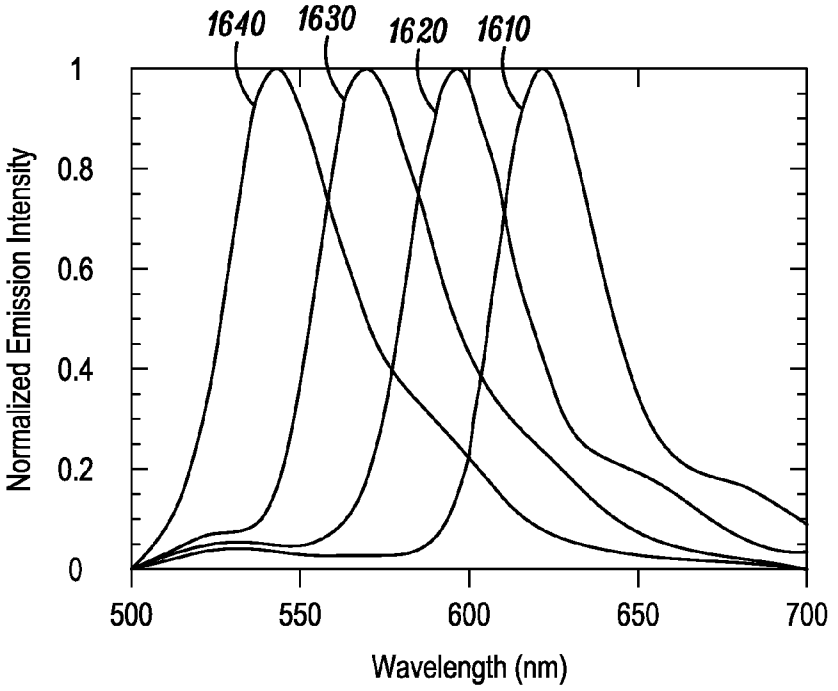

FIG. 16A

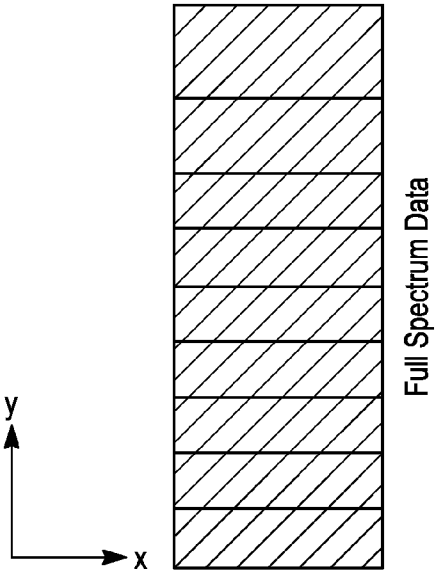

FIG. 16B

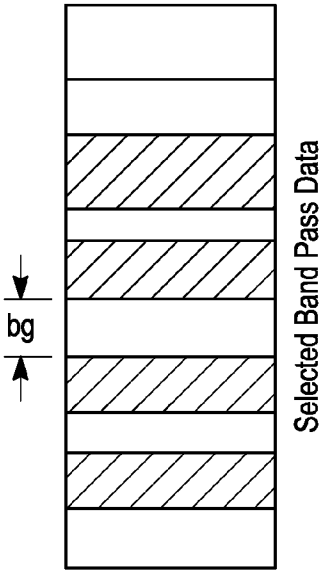

FIG. 16C

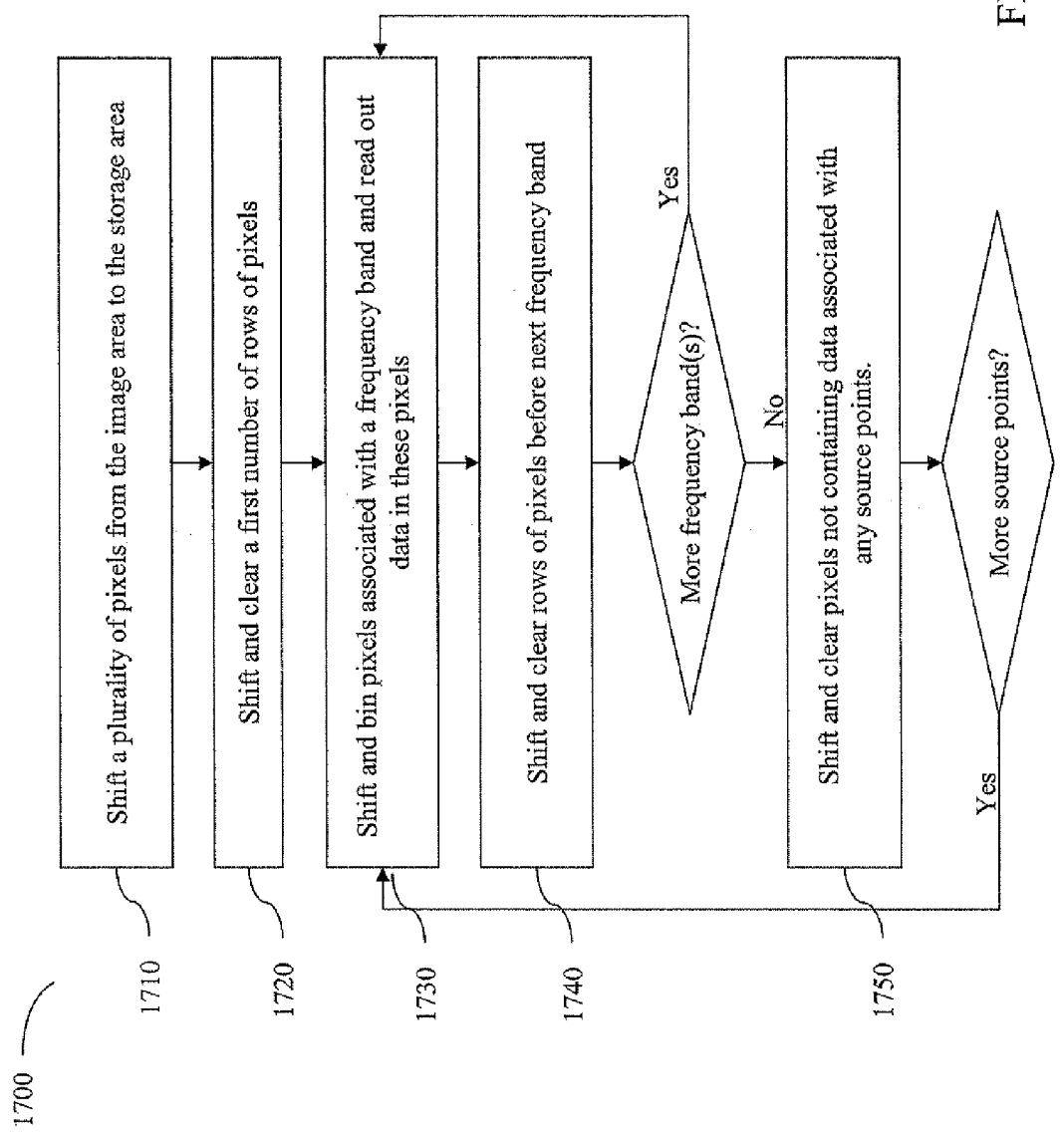
1700
1710
Shift a plurality of pixels from the image area to the storage area
1720
Shift and clear a first number of rows of pixels
1730
Shift and bin pixels associated with a frequency band and read out data in these pixels
1740
Shift and clear rows of pixels before next frequency band
More frequency band(s)?
Yes
No
1750
Shift and clear pixels not containing data associated with any source points.
More source points?
Yes

FIG. 17A

Source Point Thruput (Bold items are adjustable)		
CCD Specifications		
Horizontal Pixels =	**512**	
Vertical Pixels =	**512**	
Readout Time =	**1.00E-07**	sec
Vertical shift Time =	**4.00E-07**	sec
Horiz Reg Clear Time =	**5.00E-06**	sec
Source Point Image		
Pitch between source points (*p*) =	**4**	pixels
Buffer rows (*d*) cleared =	**8**	rows
Bin color =	**3**	
Colors Read =	**4**	
Gap rows cleared =	**2**	rows
Bin gap or band gap (*bg*) =	**2**	rows
Source Point Gap (*g*) =	**4**	rows
Rows of source points =	4	
Readout Time per source point =	0.000241	sec
Readout Time =	0.0009656	
# Source points =	512	
% good =	**25%**	
# good source points =	128	
Polymerase Rate =	**100**	bp/sec
Thruput =	1105.92	Mbp/day
Data rate =	530240	Bytes/sec
Number of rows/read =	128	

FIG. 17B

Source Point Thruput (Bold items are adjustable)		
CCD Specifications		
Horizontal Pixels =	**1004**	
Vertical Pixels =	**1002**	
Readout Time =	**2.86E-08**	sec
Vertical shift Time =	**5.00E-07**	sec
Horiz Reg Clear Time =	**NA**	sec
Source Points Image		
Pitch between source points (*p*) =	**4**	pixels
Buffer rows (*d*) cleared =	**8**	rows
Bin color =	**6**	
Colors Read =	**4**	
Gap rows cleared =	**4**	rows
Bin gap or band gap (*bg*) =	**4**	rows
Source Point Gap (*g*) =	**8**	rows
Rows of source points =	**4**	
Clear =	2.87E-05	sec
Readout Time per source point =	0.000282171	sec
Readout Time =	0.001132686	
# source points =	1004	
% good =	**25%**	
# good source points =	251	
Polymerase Rate =	**100**	bp/sec
Thruput =	2168.64	Mbp/day
Data rate	886389	Bytes/sec
Number of rows/read =	224	

FIG. 17C

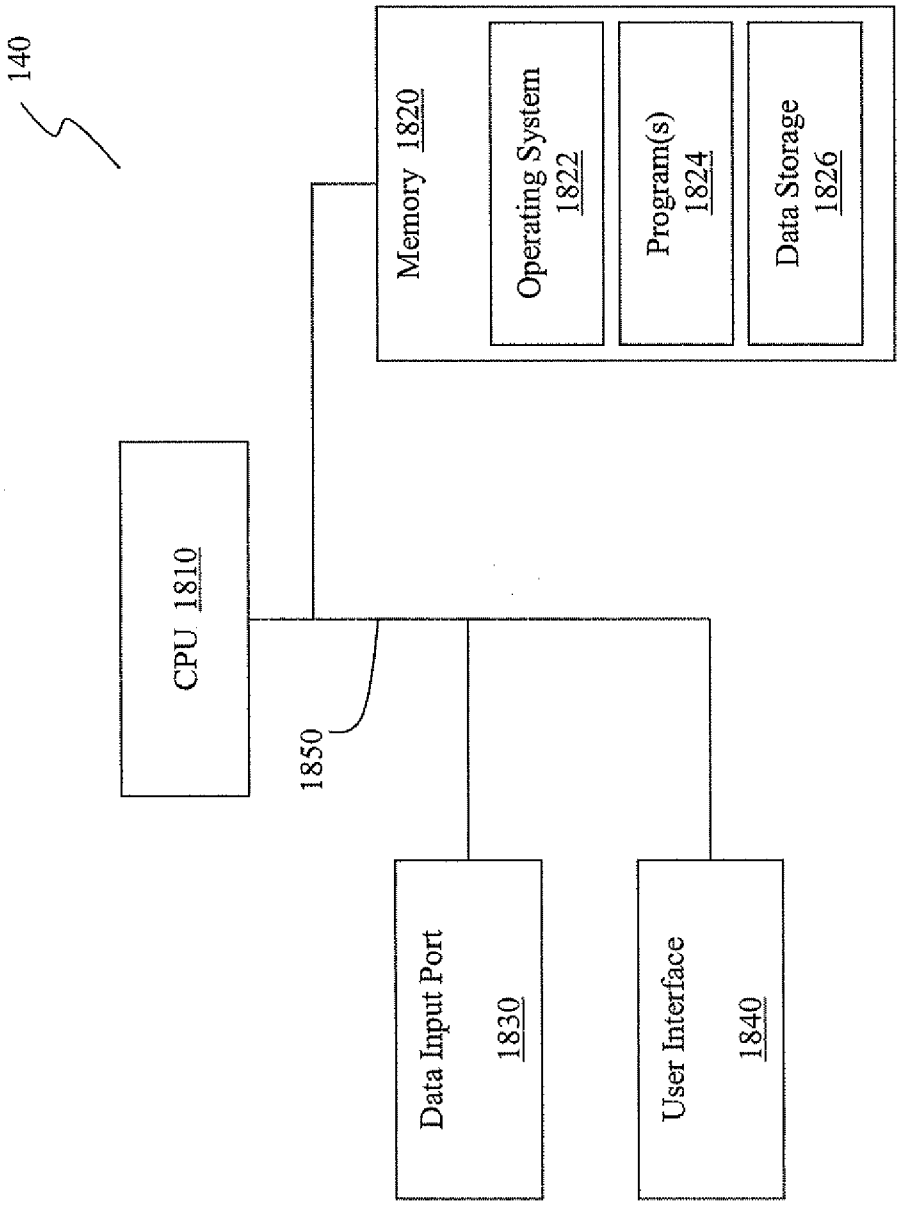
140
Memory 1820
Operating System 1822
Program(s) 1824
Data Storage 1826
CPU 1810
1850
Data Input Port 1830
User Interface 1840

FIG. 18

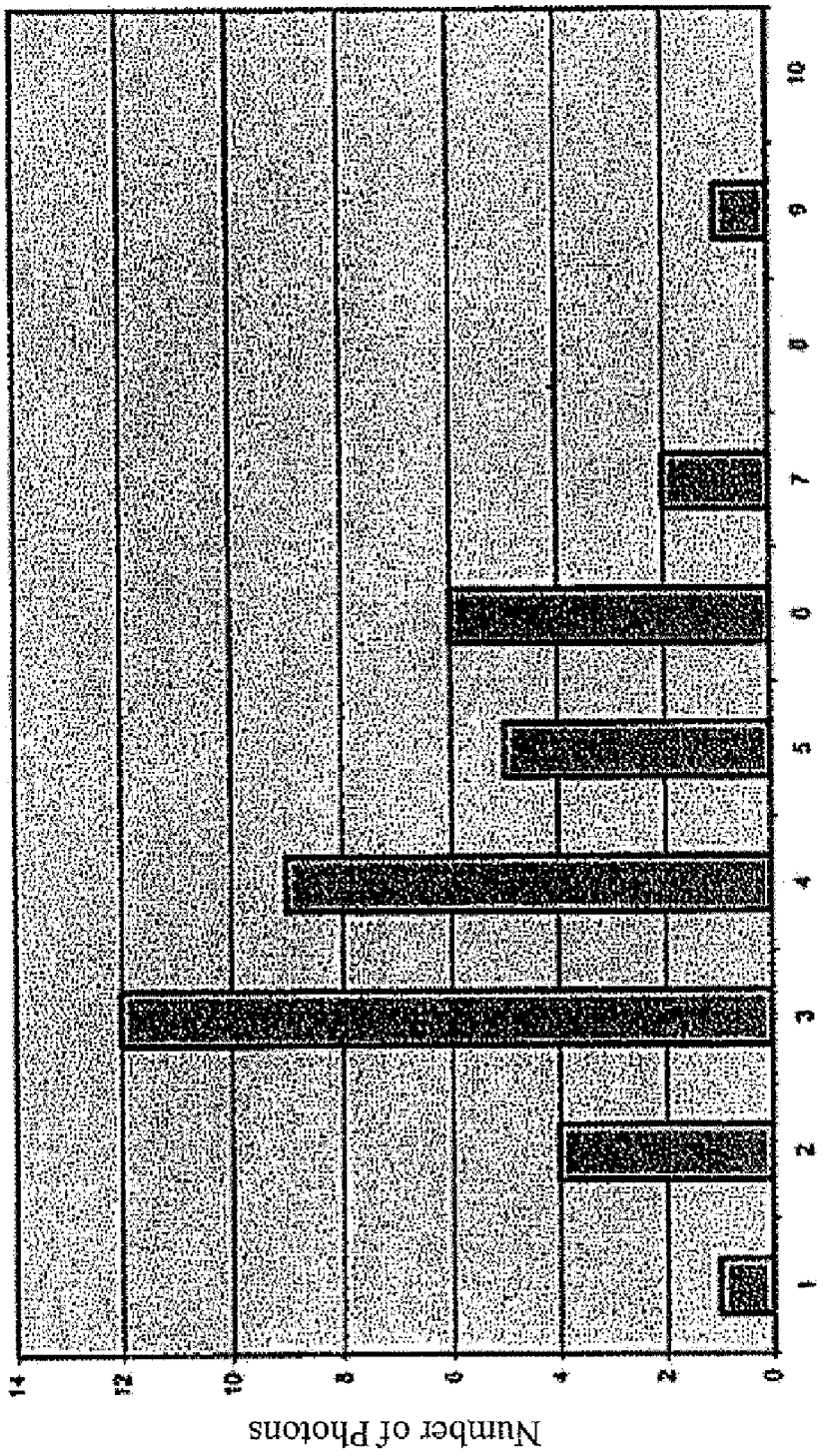
Number of Photons
0
2
4
6
8
10
12
14
Spectral Bin Number
1
2
3
4
5
6
7
8
9
10

FIG. 19

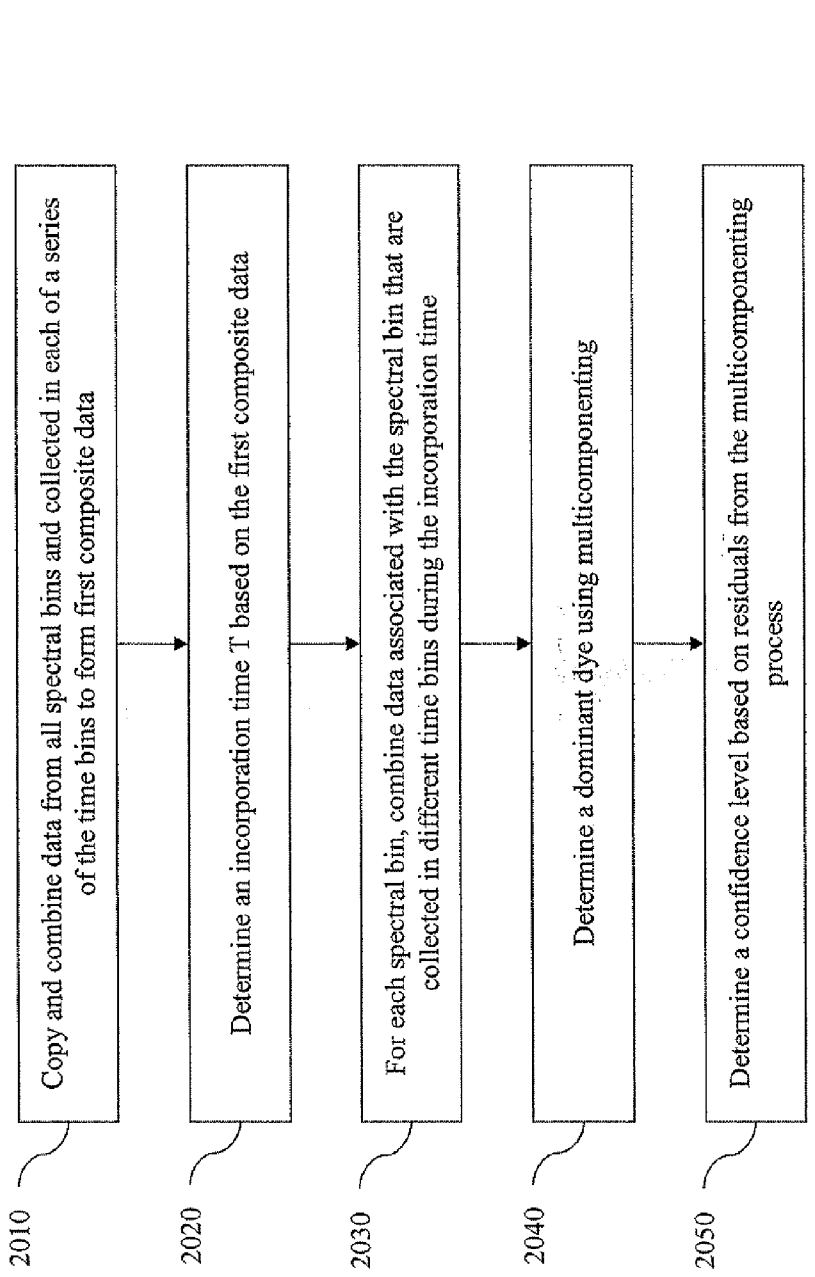
2000
Copy and combine data from all spectral bins and collected in each of a series of the time bins to form first composite data
2010
Determine an incorporation time T based on the first composite data
2020
For each spectral bin, combine data associated with the spectral bin that are collected in different time bins during the incorporation time
2030
Determine a dominant dye using multicomponenting
2040
Determine a confidence level based on residuals from the multicomponenting process
2050

FIG. 20

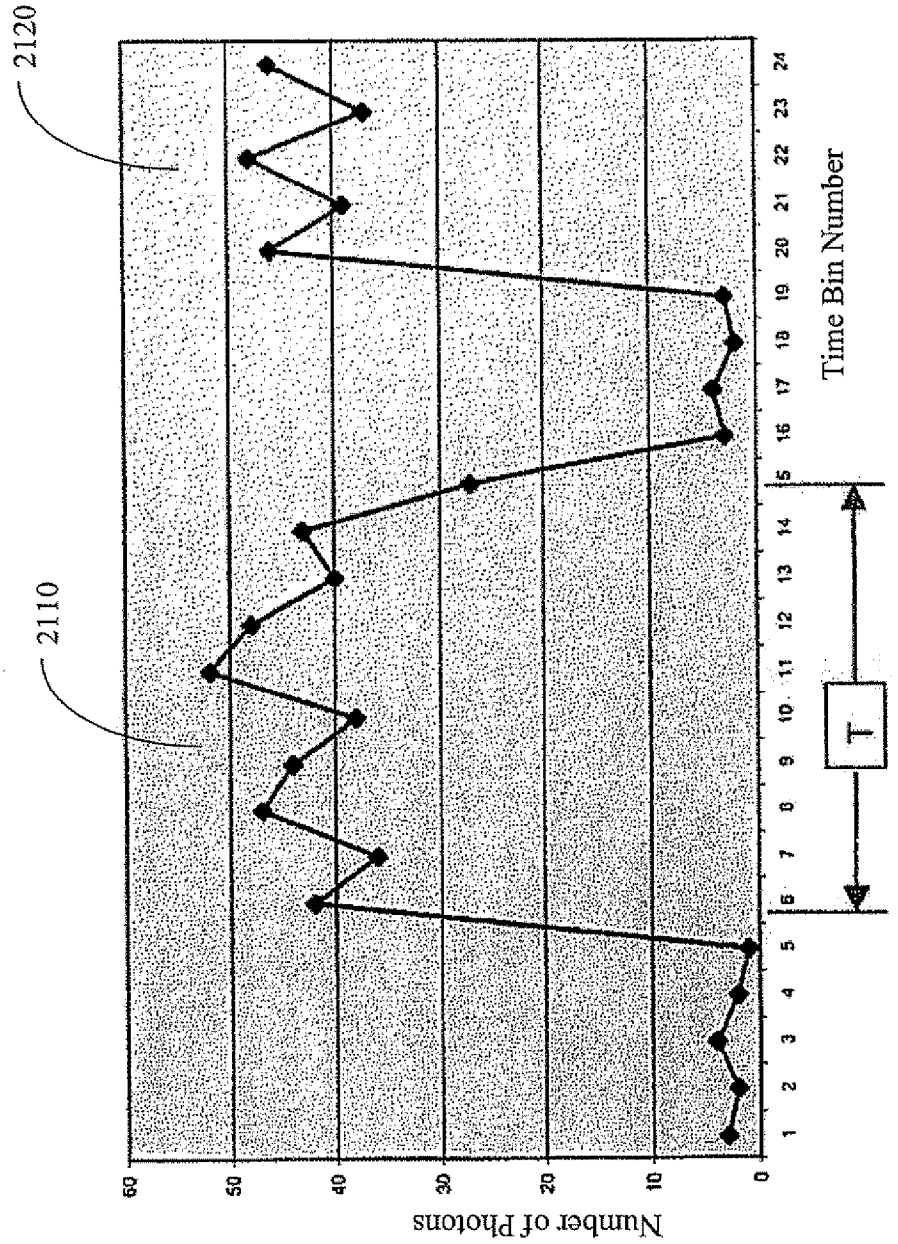

FIG. 21

METHOD AND SYSTEM FOR MULTIPLEX GENETIC ANALYSIS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority as a continuation application under 35 U.S.C. §120 or §365(c) to co-pending U.S. patent application Ser. No. 11/423,403, entitled "Method and System for Multiplex Genetic Analysis," filed Jun. 9, 2006, which in turn claims priority to U.S. Provisional Application No. 60/689,692 filed Jun. 10, 2005. The entireties of the disclosures of the above-identified applications are incorporated herein by reference as though set forth in full.

FIELD

[0002] The present application relates to molecular analysis, and more particularly to methods and systems for multiplex genetic analysis of single molecule nucleic acid synthesis.

INTRODUCTION

[0003] The information stored in a DNA molecule depends on particular sequences of nucleotides, which are bases or building blocks of the DNA molecule. DNA sequencing allows the determination of the nucleotide sequence of a particular DNA segment. A conventional method of DNA sequencing starts with a defined fragment of a DNA molecule as a template. Based on this template, a population of molecules differing in size by one base of a known composition is generated. The population of molecules is then fractioned based on size using, for example, acrylamide or agarose gel electrophoresis of single-stranded DNA molecules. The base at the truncated end of each of the fractionated molecules is thereafter determined to establish the nucleotide sequence.

[0004] A sequencing method called dideoxy sequencing was developed by Fred Sanger. His method is based on DNA synthesis in the presence of dideoxy nucleotides (ddNTP), which differ from normal deoxynucleotides (dNTP) in that they lack a 3'-hydroxyl group so that once a dideoxy nucleotide is incorporated, it will terminate strand synthesis. The procedure for dideoxy sequencing starts with setting up four reactions each in a different tube containing the single strand DNA to be sequenced, labeled (tagged) primer, DNA polymerase, normal dNTPs, and a different ddNTP (i.e. for A,T,C, or G). A dideoxy nucleotide will be incorporated, randomly, at each point the corresponding nucleotide occurs in the template strand. Each time a dideoxy nucleotide is incorporated, it will stop further DNA replication. This will generate a set of fragments of various lengths, each fragment corresponding to the point at which there is a nucleotide complementary to the dideoxy nucleotide. The fragments are then separated based on their length by electrophoresis. With the smaller fragments migrating faster, the sequence can be determined by associating the base composition with each fragment.

[0005] The above technique for DNA sequencing suffer from the disadvantage that sample preparation is relatively complex in order to ensure that the tubes contain the same DNA molecules or fragments of the same DNA molecules to be sequenced. This leads to increased costs and the possibility of error. A simpler method results if molecule-based investigation techniques are used to observe the synthesis of a single DNA molecule. Because only one molecule is being observed, there is no need to ensure that all of the surrounding molecules are the same.

[0006] Specialized tools for imaging and spectroscopy have been developed to characterize nanomaterials and nanomaterials-related phenomenon. Techniques for constructing these tools comprise near-field scanning optical microscopy (NSOM) and single molecule spectroscopy (SMS). These techniques offer unique capabilities for investigating properties at the molecular level owing to their high spatial resolution, chemical sensitivity, and their ability to determine dynamical properties such as molecule binding/unbinding kinetics and the structural dynamics of polymers. For example, a sample-scanning confocal fluorescence microscope using SMS developed by McNeil et al. has demonstrated spatial resolution of ~400 nm, and single molecule sensitivity. It uses a detector system having a single-photon avalanche diode and a sensitive TE-cooled CCD spectrometer, permitting the ability to monitor fluorescence in the range of 400 to 1100 nm at a resolution of 20 nm and the ability to conduct time-lapse fluorescence spectroscopy with single molecule sensitivity.

[0007] The single-molecule techniques described above, however, often employ femtoliter-scale observation volumes and require the use of picomolar to nanomolar sample concentrations to ensure that on average only one molecule will be present in the sample volume. These concentrations are far lower than those that normally occur in nature. Thus, molecule dynamics that are affected by concentration cannot be suitably tested using the techniques. To overcome the deficiencies of NSOM and SMS techniques, other developments have been proposed. For example, Levene et al. describes a device for single molecule analysis employing a sample plate, which has 50 nm-diameter holes in a 100 nm thick aluminum film on a fused silica coverslip. When the holes are illuminated from under the fused silica coverslip, the holes act as zero-mode waveguides prohibiting the light from going through the aluminum film because the diameter of the holes are much smaller than the wavelength of the light, which is about 400-700 nm. The light, however, does generate an evanescent field that extends about 10 nm into the cavity of each illuminated hole producing a zeptoliter-scale effective observation volume near the opening of the hole. See Levene et al., US Patent Application Publication Number 2003/0174992 A1, which is incorporated herein by reference.

[0008] The small observation volume provided by the zero-mode waveguides described by Levene, however, raises other challenges spanning from sample preparation, signal detection, noise or background suppression, data collection and data analysis algorithms. Accordingly, significant further developments are needed.

[0009] The present teaching in one aspect comprises an affordable high-sensitivity and high-throughput system and method for single-molecule analysis that performs at a lower cost relative to conventional systems used in sequencing, resequencing, and SNP detection. These and other features of the present teaching are set forth herein.

SUMMARY

[0010] The present disclosure provides apparatuses, systems and methods for analyzing a plurality of molecules by detecting separately and substantially simultaneously light emissions from a plurality of localized light-emitting analytes each including a single one of the plurality of molecules. The

detected light emissions, after being properly analyzed, can be used to deduce the structure or properties of each of the plurality of molecules. In some embodiments, the apparatus, systems and methods can be used for nucleic acid sequencing, nucleic acid re-sequencing, and/or detection and/or characterization of single nucleotide polymorphism (SNP analysis) including gene expression.

[0011] In one aspect, a method for identifying nucleotides in a nucleic acid sequence is disclosed. A plurality of polymerization complexes are provided within a plurality of confined reaction environments. Each complex comprises a polymerase enzyme and a template nucleic acid. The plurality of complexes are contacted with a plurality of types of nucleotide analogs labeled with distinguishable fluorescent labels under conditions suitable for polymerization. Fluorescent signals associated with incorporation of a nucleotide analog are transmitted to a detector, wherein the location of the fluorescent signal on the detector is indicative of the individual confined reaction environment and the type of nucleotide incorporated. The nucleotide in a nucleic acid sequence is identified based upon the type of nucleotide incorporated and the confined reaction environment.

[0012] In another aspect, a method for identifying nucleotides incorporated in polymerase mediated, template dependent nucleic acid synthesis reactions is disclosed. A plurality of template nucleic acid/polymerase complexes are provided on to a substrate. The plurality of complexes is contacted with a plurality of nucleotide analogs having spectrally distinct fluorescent labels, wherein incorporation of nucleotides produces characteristic incorporation signals. The incorporation signals are directed to a single detector, wherein the location of the signal on the detector is indicative of a complex incorporating the nucleotide and the type of nucleotide incorporated. The nucleotide incorporated into the template mediated nucleic acid synthesis reaction is identified from the location of the signal on the detector.

[0013] In still another aspect, a method for analyzing a reaction is disclosed. A first reactant immobilized on a substrate is provided. The first reactant is contacted with a second reactant and a third reactant, wherein each of the second and third reactants bear a spectrally distinct label. The signals form the spectrally distinct labels that are characteristic of interaction of the first reactant with one of the second and third reactants are directed to a location on a first detector, wherein the location on the detector is indicative of the first reaction location on the substrate and the second or third reactant with which the first reactant interacted. The first reactant and the second or third reactant with which the first reactant interacted are identified.

[0014] These and other features of the present teaching are set forth herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The skilled artisan will understand that the drawings, described below, are for purposes of illustration only, and are not intended to limit the scope of the present teaching in any way.

[0016] FIG. **1** is a block diagram of an exemplary embodiment of a high throughput system for single molecule analysis.

[0017] FIG. **2** is a top view of a sample holder in the system.

[0018] FIG. **3** is a cross-sectional view of a portion of the sample holder.

[0019] FIG. **4** is a flowchart illustrating an exemplary embodiment of a method useful for enriching the sample holder with a plurality of spatially restrained source points.

[0020] FIG. **5** is a diagram illustrating further embodiments of a method useful for enriching the sample holder with a plurality of spatially constrained source points.

[0021] FIG. **6** is a 3-dimensional view of a portion of the sample holder according to further embodiments of the present teaching.

[0022] FIG. **7** is a diagram illustrating a DNA sequencing process along a channel on the sample holder according to exemplary embodiments of the present teaching.

[0023] FIGS. **8**A and **8**B are flowcharts illustrating further embodiments of a method useful for placing a plurality of source points on the sample holder in FIG. **6**.

[0024] FIGS. **8**C and **8**D are diagrams illustrating embodiments of a setup for stretching each of a plurality of oligonucleotide molecules along a bottom surface of a channel on the sample holder.

[0025] FIG. **9** is a block diagram illustrating exemplary embodiments of an optical arrangement useful for illuminating the source points placed on the sample holder.

[0026] FIGS. **10**A-**10**E are block diagrams illustrating further embodiments of an optical arrangement useful for illuminating the source points placed on the sample holder.

[0027] FIG. **11** is top view of a sample holder showing a plurality of source points.

[0028] FIG. **12** is a top view of an image plane in a detector in the system in FIG. **1** according to exemplary embodiments.

[0029] FIG. **13** is a diagram illustrating an optical assembly for detecting light signals from the source points on the sample holder according to exemplary embodiments.

[0030] FIGS. **14**A-**14**C are diagrams illustrating further embodiments of an optical assembly for detecting light signals from the source points on the sample holder.

[0031] FIG. **15**A is a block diagram illustrating a frame transfer CCD array in a detector in the system in FIG. **1** according to exemplary embodiments of the present teaching.

[0032] FIG. **15**B is a block diagram illustrating an interline CCD array in the detector in the system in FIG. **1** according to exemplary embodiments of the present teaching.

[0033] FIG. **16**A are examples of normalized fluorescent spectra corresponding to four different fluorescent dyes.

[0034] FIG. **16**B is a block diagram illustrating full spectrum data.

[0035] FIG. **16**C is a block diagram of the full spectrum data with the most informative wavelengths distinguished from less informative wavelengths.

[0036] FIG. **17**A is a flowchart illustrating a method for reading out CCD data associated with a plurality of source points according to exemplary embodiments.

[0037] FIGS. **17**B-**17**C are each a spreadsheet for estimating the throughput of reading out data from a CCD array using the method in FIG. **17**A.

[0038] FIG. **18** is a block diagram of a computer system used in the system in FIG. **1** according to exemplary embodiments of the present teaching;

[0039] FIG. **19** is a histogram illustrating the number of photons in different spectral bins detected from a single incorporation event;

[0040] FIG. **20** is a flowchart of an exemplary embodiment of a method useful for base determination according to the present teaching; and

[0041] FIG. **21** is a plot of composite data over a plurality of time bins according to exemplary embodiments of the present teaching.

DESCRIPTION OF VARIOUS EMBODIMENTS

[0042] It is to be understood that both the foregoing summary and the following description of various embodiments are exemplary and explanatory only and are not restrictive of the present teachings. In this application, the use of the singular comprises the plural unless specifically stated otherwise. Also, the use of "or" means "and/or" unless stated otherwise. Similarly, "comprise," "comprises," "comprising," "comprise," "comprises," and "including" are not intended to be limiting.

[0043] Additionally, while certain embodiments are described in detail herein, particularly embodiments suitable for analysis of single molecule nucleic acid synthesis, it is to be understood the apparatus, systems and methods of the present disclosure may be employed in other applications for analysis of single molecules, such as but not limited to directed resequencing, SNP detection, and gene expression.

[0044] Furthermore, the figures in this application are for illustration purposes and many of the figures are not to scale with corresponding hardware. Many parts of the features in the figures in this application are drawn out of scale purposefully for ease of illustration.

[0045] Systems according to some embodiments of the present disclosure generally comprise a sample holder configured to hold a plurality of localized light-emitting analytes each comprising a single one of a plurality of molecules to be analyzed, a light source configured to illuminate the sample holder, and an optical assembly configured to collect and detect light emitted from the source points.

[0046] FIG. **1** is a block diagram of an exemplary embodiment of a system **100** for detecting and analyzing light emitted from the plurality of light-emitting analytes. As shown in FIG. **1**, system **100** comprises a sample holder **110**, a light source **120**, and an optical assembly **130**. System **100** may further comprise a host computer system **140** (see also FIG. **18**) configured to analyze optical data detected by optical assembly **130**. System **100** may also comprise one or more digital signal processors (DSP) or field programmable gate arrays (FPGA) **150** coupled between optical assembly **130** and host computer system **140**. DSPs or FPGAs **150** can be used to execute algorithms for base determination, as explained in more detail below.

[0047] System **100** may optionally comprise an index-matching prism **108**. A space between the sample holder **110** and the optical assembly **130** may be filled with a fluid **104**. The utility of the index-matching prism **108** and the fluid **104** is discussed below. Although for reasons discussed below, it may be advantageous to direct the excitation light from the light source **120** to the sample holder **110** at an angle, as shown by the solid line **121** in FIG. **1**, system **100** is not limited to such use, and the excitation light can be directed via a dichroic filter **125** toward the sample holder along a normal N of the sample holder **110**, as shown by the dashed lines in FIG. **1**.

[0048] In exemplary embodiments of the present teaching, sample holder **110** is configured to support and confine the plurality of light-emitting analytes. For ease of discussion, each localized light-emitting analyte will hereafter be referred to as a dye or a "source point". In various embodiments, a dye or a source point comprises a single nucleic acid molecule, a fraction of a nucleic acid molecule, an oligonucleotide molecule, or a single nucleic acid polymerizing enzyme. The dye or source point may also comprise one or more other molecules, constituents, or reactants. The emitted light from the complex can be used to deduce the structure or properties of a target nucleic acid molecule.

[0049] In one exemplary embodiment, in applications employing nucleic acid sequencing, each source point is a complex of a single nucleic acid polymerizing enzyme, a target nucleic acid molecule, and at least one incorporated or incorporating fluorescence-labeled nucleotide analog. The source point is localized or spatially constrained in at least one dimension that is less than the wavelength of the excitation light. The fluorescent label on the nucleotide analog emits fluorescent light upon illumination by light source **120**. In exemplary embodiments of the present teaching, four different nucleotide analogs are labeled with four different fluorescent dyes each having a unique emission spectrum. The four different fluorescent dyes can also be associated with four different frequency bands each corresponding to a peak in emission intensity according to the respective spectrum. The four different frequency bands are hereafter referred to as first, second, third, and fourth frequency bands.

[0050] Thus, the time sequence of base incorporation can be observed by detecting fluorescent signals from sequentially incorporated nucleotide analogs associated with a source point. The fluorescent light signals from different source points on the sample holder **110** are substantially simultaneously collected and detected by optical assembly **130** and are analyzed by computer system **140** to determine the identities of the incorporated nucleic acid molecule in each of the source points. To reduce or eliminate interference between fluorescent signals associated with consecutive incorporation events on a same source point, after detection of an incorporation event, fluorescent label on the newly incorporated nucleotide can be bleached, cleaved or otherwise removed with a known technique. Photo-cleavable linkers may be utilized to facilitate efficient and consistent removal of the fluorescent labels.

[0051] In some embodiments, the source points are localized or spatially constrained at different locations on sample holder **110** by immobilizing the single nucleic acid molecule or the single nucleic acid polymerizing enzyme in each source point at one of the locations. This allows separate and substantially simultaneous detection of fluorescent emission from the plurality of source points. A conventional method or one of the methods discussed below can be used to immobilize the enzymes or the template nucleic acid molecules.

[0052] FIG. **2** is a block diagram of a top-down view of sample holder **110** according to exemplary embodiments. As shown in FIGS. **1** and **2**, in exemplary embodiments, sample holder **110** comprises a substrate **112** made of a material transparent to the excitation light from light source **120** and to the fluorescent emissions from the source points. A metallic film **114** is formed on a top surface of substrate **112**. Depending on specific applications, for reasons discussed below, metallic film **114** may extend to the side surfaces and edge portions of a bottom surface of the substrate **112**, as shown in FIG. **1**. Sample holder **110** may further comprise a sealer **115** and a cover **116** for evaporation control. A space **118** is formed between the cover **116** and the substrate **112**, which space serves as a sample chamber for holding a sample fluid that supplies at least one of the constituents or reactants in each source point. In various embodiments, in applications of

nucleic acid sequencing, the sample fluid comprises a fluorophore solution of different types of fluorescent-labeled nucleotides. Sample holder **110** may further comprise a fill hole **230** for filling the sample chamber **118** with the sample fluid and a drain hole **240** for draining the sample fluid from the sample chamber. Fill hole **230** and drain hole **240** are preferably located near two opposite corners of sample holder **110**, as shown in FIG. **2**, for more complete draining and washing away of sample fluid.

[0053] As shown in FIG. **2**, sample holder **110** is configured to hold a plurality of spatially separated and constrained source points **210** in a field of view **220** of the optical assembly **130**. The spatial separation and confinement of the source points **210** help in one aspect to detect light signals from the source points **210** separately and substantially simultaneously. Although FIG. **2** shows that the source points **210** on the sample holder **110** are arranged in an array having two rows and a number of columns, such arrangement is not necessary as long as the source points are sufficiently spaced from each other so that the light signals from them can be effectively resolved by optical assembly **130** When the source points are arranged in an array, the array may be perfect, meaning each array element site has an immobilized functional source point, or imperfect, meaning at least one array element site is missing a source point, has a source point that is not functional, or has multiple source points that are too close together to allow resolution by the optical assembly **130**.

[0054] In various embodiments, the metallic film **114** on the top surface of the substrate **112** has etched patterns forming cavities for housing the plurality of source points and separating the plurality of source points to allow resolution by the optical assembly **130**. In some embodiments, zero-mode waveguides, such as those described in Patent Application Number US 2003/0174992 by Levene et al, which is incorporated herein by reference, are formed in metallic film **114**, as shown in a cross-sectional view in FIG. **3**. Zero-mode waveguides are known in the art and can be created using a variety of materials and methods. As a specific, non-limiting example, substrate **112** is a fused silica substrate, metallic film **114** is an aluminum film formed on the fused silica substrate, and an array of holes **310** are formed by masking and plasma etching the aluminum film to create holes **310** in the aluminum film. Each hole **310** has a diameter that is substantially smaller than a wavelength of the excitation light from light source **120** and a depth that is sufficient to block transmission of the excitation light through the hole. Thus, each hole **310** acts as a zero-mode waveguide for the excitation light from light source **120**, allowing the excitation light, which comes to the waveguides from the substrate side, to penetrate only a bottom portion **312** of the hole **310**. At the same time, the zero-mode waveguides also serves to block light emitted or scattered from the sample fluid on the sample holder **110** except emissions coming from any light emitting agents immobilized in the bottom portions **312** of the waveguides or diffusing past the bottom portions **312** of the waveguides.

[0055] Thus, in some embodiments, to allow the detection and analysis of light emitted from the source points **210**, each source point **210** is immobilized in the bottom portion **312** of a zero-mode waveguide **310**, so that light emitted from the source point can escape the hole **310**, pass through substrate **112** and be collected by optical assembly **130**. Preferably, only one source point should be present in the bottom portion **310** of a hole **310** because it would be difficult for the optical assembly to distinguish the emitted light from more than one source point in a single hole **310** considering the size of the hole. Therefore, in the exemplary embodiment, holes **310** that either do not have any source point immobilized in the bottom portion **312** or have more than one source point immobilized in the bottom portion **312** do not contribute to the analysis and are considered as empty sites in an array of source points **210**.

[0056] For ease of discussion, the description hereafter will be illustrated in the context of nucleic acid sequencing, while the methods, systems and apparatus of the present teaching can be applied to other types of molecular analysis. Methods of immobilizing molecules involved in a genetic assay in waveguides **310** are described in detail in US Patent Application Number US 2003/0044781 by Korlach et al., which is incorporated herein by reference. Using the methods described by Korlach, some of the array of holes **310** can each contain a single DNA molecule or enzyme immobilized in the bottom portion **312**, while a large percentage of the holes may contain none or multiple molecules in each of them and are thus useless in the analysis.

[0057] FIG. **4** illustrates a flowchart of one embodiment of a method **400** for enriching the sample holder **110** with source points. Method **400** increases the efficiency and throughput of system **100** by maximizing the percentage of holes **310** that have in each of them a single source point in the bottom portion **312**. As shown in FIG. **4**, according to exemplary embodiments of the present teaching, method **400** comprises the following steps: step **410** in which uncovered portions of the substrate **112** are coated with streptavidin, step **420** in which a dilute solution including a plurality of molecules each being a nucleic acid molecule or a nucleic acid polymerizing enzyme is applied to the waveguides **310**. Each of the plurality of molecules has a photoactivatable biotin attached to it. The concentration of the plurality of molecules in the solution is selected to be lower than the optimal Poisson distribution so that when the solution is applied to the waveguides **310** on the sample holder **110**, most of the waveguides **310** would be populated by zero number of the molecules and that statistically few of the waveguides **310** would be occupied by more than one of the molecules.

[0058] Still referring to FIG. **4**, method **400** further comprises step **430** in which a first group of waveguides are identified as each being occupied by at least one nucleic acid molecule or a nucleic acid polymerizing enzyme. The first group of waveguides can be identified by using, for example, a simplified sequencing assay. Method **400** further comprises step **440** in which light is shown on each of the first group of waveguides. The light activates the biotin attachment in those waveguides and thus immobilizes the molecules using the biotin-streptavidin bound. Step **440** is followed by an optional step **450** in which the solution is removed from the sample holder by washing or inactivation, leaving only those molecules bonded to the bottom of the waveguides. Step **450** is followed by an optional step **460** in which another dilute solution of the biotin attached molecules are applied to the waveguides **310** on sample holder **110**. Method **400** further comprises step **470** in which an additional group of waveguides are identified as each being occupied by at least one nucleic acid molecule or a nucleic acid polymerizing enzyme. In one embodiment, the additional group of waveguides do not overlap with previously identified group (s) of waveguides. Method **400** further comprises step **480** in which light is shown on each of the additional group of

waveguides and thus immobilizes the nucleic acid molecules or enzymes in the additional group of waveguides. Method **400** then repeats steps **450**-**480** until most of the waveguides are populated by bound molecules. Note that steps **450** and **460** are optional because, instead of carrying out steps **450** and **460**, one can simply wait for a period of time to allow more of the plurality of molecules to diffuse into some of the waveguides.

[0059] Optionally, after populating the waveguides with polymerase molecules, a primer is attached to each polymerase molecule by a flexible linker. Attaching the primer to the polymerase molecule helps the analysis because the DNA template would be tethered and not float away, allowing subsequent synthesis to occur on the same template. In one aspect, this benefits the analysis by increasing read lengths and throughput. Longer read lengths help to simplify any fragment assembly problem.

[0060] In some embodiments, a method for enriching the sample holder involves the use of nanobeads. As shown in FIG. **5**, the sample fluid comprise nanobeads **510**, and the enzyme or nucleic acid molecule **520** is attached to a nanobead **510** by a cleavable linker **515**, in a manner that most of the nanobeads in the sample fluid each have at most one enzyme or nucleic acid molecule attached. The nanobeads are sized such that only one nanobead is likely to fit in a waveguide **310**. The enzyme or nucleic acid molecule **520** has a photoactivatable linker **525** that allows attachment of the composite including the nanobead, the nucleic acid molecule or enzyme, and the cleavable linker **515** to an attachment site **530** at the bottom of a waveguide **310**. The attachment is activated by shining light from the bottom of substrate **112**. Since only those nanobeads each having an enzyme or nucleic acid molecule with the photoactivatable linker can bind to the substrate **312**, and the presence of a nanobead in a waveguide **310** excludes other enzyme or nucleic acid molecules from diffusing into the same waveguide, there is no need to determine which waveguides are occupied by the nanobeads before shining light on the waveguides to activate the linkers **525**. The shining of light can be repeated later when more nanobeads with enzyme or nucleic acid molecules attached thereon diffuse into other waveguides. Thus, the nanobeads can be used to increase the number of waveguides each having a single nucleic acid molecule or enzyme attached therein. After binding, the nanobeads are removed from the enzyme or nucleic acid molecules by, for example, chemically cleaving the linkers **515** or dissolving the nanobeads.

[0061] In alternative embodiments of the present teaching, sample holder **110** comprises slots or channels to facilitate confining the plurality of source points **210** on the sample holder **110**. FIG. **6** illustrates a 3-dimensional view of a plurality of channels **610** formed in metallic film **114** on substrate **112**. As a non-limiting example, channels **610** are formed in an aluminum film over a fused silica substrate. Each channel **610** has a width w that is smaller than a wavelength associated with light source **120**. In exemplary embodiments of the present teaching, light from light source **120** is linearly polarized and the polarization direction is oriented with the electric field vector in the light wave along the width direction of the channels. Thus, only a bottom portion **612** in each channel **610** would be illuminated by the excitation light from light source **120**, as shown in FIG. **6**. Channels **610** can be formed using conventional techniques, such as conventional semiconductor processing or integrated circuit (IC) fabrication techniques.

[0062] Sample holder **110** with channels **610** formed thereon has multiple advantages over a sample holder with zero-mode waveguide holes **310** formed thereon. Because the fluorescent emissions are largely unpolarized, they would not be attenuated when they try to exit the channels **610** as much as when they try to exit holes **310** of sub-wavelength dimension. So, more emitted light from sample holder **110** can be collected and detected by optical assembly **130**, resulting in increased signal to noise ratio. In addition, each channel **610** can house a larger DNA template molecule if the DNA molecule is oriented parallel to the channel, as shown in a top-down view of the channel in FIG. **7**. This way, the polymerase can migrate down the template for a much longer distance without exiting the illuminated volume **612**. The DNA molecule can be tethered so that it can remain in one location while the polymerase, having a finite processivity, may fall off the template and be replaced by another polymerase. This can lead to longer read lengths and thus significantly simplified assembly processes, especially during denovo sequencing. Although FIG. **7** shows that channel **610** is closed at both ends **701** and **702**, the channels **610** on sample holder can be open on either or both ends by extending all the way to the edge(s) of the sample holder, as shown in FIG. **8**C below.

[0063] The polymerase or template molecules can be attached to sample holder **110** using conventional photoactivatable linkers. In exemplary embodiments of the present teaching, channels **610** may house more than one polymerase or template molecules attached to sample holder **110** by flexible linkers that are placed in the channels **610**. The molecules should be attached to the channels **610** in a resolvable fashion, meaning that they are sufficiently spaced from each other to allow efficient resolution of the emissions therefrom by the optical assembly **130**.

[0064] FIG. **8**A illustrates a method **800** for enriching the sample holder **110** with source points by attaching the polymerase or template molecules in channels **610** in a resolvable fashion according to exemplary embodiments of the present teaching. As shown in FIG. **8**A, method **800** comprises step **810** in which exposed portions of the top surface of the substrate **112** are coated with a photoactivatable linking substance such as PHOTOACTIVATABLE BIOTIN™ (PAB), step **820** in which a solution of enzyme or template molecules is applied to sample holder **110**, and step **830** in which the PAB is exposed to a pattern of light shone from the bottom surface of the substrate **112**. The pattern of light may be created by interference or refraction using grating or other conventional techniques and has interleaving lighted and dark areas in each channel **610**. The distance between two neighboring lighted areas is selected based on the resolution of the optical assembly **130** so that emissions from the two lighted areas can be separately and substantially simultaneously detected by the optical assembly **130**. The PAB in the lighted area will be activated causing the template molecules to be attached to the sample holder in those areas, while the PAB in the dark areas will not be activated so no template molecules will be attached to those areas. Method **800** further comprises step **840** in which sample holder **110** is washed to remove unbound template molecules, leaving the bound enzyme or nucleic acid molecules in each channel **610** and allowing the formation of optically resolvable source points **210** on sample holder **110**.

[0065] Referring to FIG. **8**B, instead of enzyme or template molecules, a solution of oligonucleotide (oligo) molecules can be applied to the substrate in step **820**, and additional

steps can be used to stretch an oligo along the bottom surface of each of a plurality of channels **610** on the sample holder **110**. As shown in FIG. **8**B, to attach an oligo to the bottom surface of a channel **610**, method **800** further comprises step **822** in which an end of the oligo is attached at one end **701** of the channel using a chemical linker, such as a biotin-streptavidin or PNA-PNA hybridization binding, where one part of the linker is attached to the end of the oligo and the other part of the linker is bound to the substrate. Method **800** further comprises step **824** in which the oligo is stretched along the channel and held to the bottom surface of the channel. Many conventional techniques of stretching DNA molecules can be used in step **824**, including but not limited to hydrodynamic, electrostatic, and magnetic manipulations. For example, the oligo molecules can be stretched using dielectrophoresis, in which the oligo is stretched by a direct current (DC) electric field or a high-frequency (e.g., 1 MHz) and high-density (e.g., 1 MV/m) alternating-current (AC) field applied between two electrodes.

[0066] In one embodiment, as shown in FIG. **8**C, the sample holder **110** can be placed in a container **880** for holding the fluid containing the oligonucleotides, an electrode **871** made of, for example, Indium Ti, is placed above the end **701** of the channel, and another electrode **872** made of the same or different conductive material as electrode **871** is placed in the container **880** below the bottom surface **612** of the channel **610** and near the other end **702** of the channel. A field is provided between the two electrodes **871** and **872** such that the oligo **710** is stretched along the channel **610** and held by the field along the bottom of the channel. Depending on the relative lengths of the oligo **710** and the channel **610**, the oligo **710** may extend beyond the channel toward the electrode **872**, if the channel is open at the end **702**. Electrode **872** may also be placed under the substrate **112**, as shown in FIG. **8**C.

[0067] With the electric field still on, step **830** is performed to further attach the oligo **710** so that the field can be removed later, preventing the field from interfering with sequencing operation afterwards. The oligo in each of the plurality of channels may be stretched and attached simultaneously using the same or different electrodes.

[0068] After binding the enzyme, oligonucleotide, or target nucleic acid molecules to the sample holder **110**, the sample holder **110** is placed in system **100**. A fluorophore solution comprising fluorescence labeled nucleotide analogs is applied to the sample holder **110**. In exemplary embodiments of the present teaching, the speed of chemistry of incorporation can be altered by changing the temperature, viscosity, and concentration of the fluorophore solution, and/or by modifying the base chemistry. For example, adding molecules such as dye molecules to the fluorophore solution has been found to slow the rate of base incorporation. In addition, the sample holder **110** in system **100** should ideally be under temperature control to insure consistency. The temperature could be changed during detection. For example, the temperature of the sample holder **110** can be reduced to slow down or stop incorporation activities until the rest of system **100** is ready to collect signals from the sample holder **110**, as discussed below.

[0069] To observe light emitted from the source points, excitation light from light source **120** is directed towards the substrate side of the sample holder **110**, and signals from fluorescing nucleotides are collected by optical assembly **130**. The confinement of the source points on sample holder **110** helps to distinguish the fluorescent signals emitted by incorporated nucleotides in the source points **210** from those emitted by freely diffusing fluorescent ligands.

[0070] As explained in more detail below, multiple methods can be used in exemplary embodiments for base determination. For example, color, signal strength, bleaching life, fluorescent lifetime, and incorporation time can be combined to gain better base discrimination. The consistency of these measurements can be used to predict a confidence value for the base determination. Confidence values can be used to sort or weight the data and to discard data of low quality, thus allowing automated consensus generation from large amount of data. This can improve the quality of the consensus as well as providing a measure of confidence.

[0071] Prior art systems, such as the one described by Levene et al., 2003 in "Zero-Mode Waveguides for Single-Molecule Analysis at High Concentrations," SCIENCE, Vol. 299: 682, which article is incorporated herein by reference, uses a confocal fluorescent set up. The confocal fluorescent set up has multiple shortcomings. First, the aluminum film reflects the excitation light directly back into the collection optics. The reflected excitation light is very intense compared to the fluorescent signals from incorporated nucleotides. To attenuate the reflected light, multiple filters are used, and each filter attenuates a significant percentage of the already weak fluorescent signals. Furthermore, the excitation light in the set up of Korlach and Levene, supra, can also excite fluorescence in the optics. This unwanted fluorescence could pass through the filters, increasing the background noise.

[0072] In exemplary embodiments of the present teachings, excitation light from light source **120** is directed to the source points in sample holder **110** in an off-axis manner such that reflected excitation light, or a significant amount of it, could not enter the optical assembly **130**. In some embodiments, where prism or wedge **108** is not provided, a light ray **901** from light source **120** is directed to sample holder **110** at an angle θ with respect to a normal direction N of substrate **112**, as shown in FIG. **9**. As the substrate **112** is made of a transparent material, such as fused silica, a relatively small first portion of the incident light **901** is reflected by the bottom surface **910** of substrate **112** and comes toward the optical assembly **103** as a first reflected light ray **912**, while a second portion of the incident light enters the substrate at a different angle θ' with respect to the normal N as a refracted light ray **914**. Angle θ' depends on angle θ and the refractive index n of the substrate **112**. The refracted light ray **914** impinges on the metallic film at the angle θ' and a relatively large portion of the refracted light ray **914** is likely to be reflected by the metallic film **114** and comes toward the bottom surface **910** of substrate **112** as light ray **916**. Light ray **916** when crossing the bottom surface **910** is refracted again and comes off the bottom surface **910** at the angle θ as light ray **918**. With the off-axis arrangement and a proper selection of the angle θ, little of the light ray **918** should enter the optical assembly **130** placed under sample holder **110**, as shown in FIG. **9**.

[0073] To eliminate or reduce reflection at the bottom surface **910** of substrate **112**, θ can be chosen to be within 10° of the Brewster's angle θ_B. Furthermore, to achieve zero or near zero reflection at the bottom surface **910** of substrate **112**, the light from the light source **120** is linearly polarized with the E vector in the light parallel to the plane of incidence, which is the plane containing the incident ray **901** and the normal N of substrate **112**. According to Brewster's Law, when the angle of incidence θ is equal to or near the Brewster's angle θ_B, the transmittance, i.e., the ratio of transmitted power in ray **914** to

the incident power in ray **901** across bottom surface **910** of substrate **112** should be one or near to one and the reflected power in ray **912** from surface **910** should be zero or near zero. Brewster's angle θ_B is given by:

$$\theta_B = \tan^{-1}\left(\frac{n_2}{n_1}\right) = \tan^{-1}\sqrt{\frac{\varepsilon_2}{\varepsilon_1}}$$

[0074] where n_1 and n_2 are the refractive indices of the respective media, i.e., air and substrate **112**, and ϵ_1 and ϵ_2 are their respective electric permittivity values.

[0075] In some embodiments, system **100** is configured to achieve total internal reflection so that a significant amount of the excitation light from light source **120** is recycled within substrate **112**, as shown in FIGS. **10**A-**10**C. Total internal reflection is a phenomenon that light incident upon a boundary from a denser medium to a less dense medium is completely reflected off the boundary. Since the light ray **916** reflected from metallic film **114** has to travel through the substrate **112** toward the boundary **1010** between the substrate **112** and air, it is possible to achieve total reflection such that the light ray **916** is recycled in the substrate **112**.

[0076] FIG. **10**A illustrates an optical arrangement for achieving total internal reflection according to exemplary embodiments of the present teaching. As shown in FIG. **10**A, prism **108** is ideally made of the same material as substrate **112** and is in direct or fluidic contact with the substrate. In exemplary embodiments, prism **108** is fused with substrate **112** at a first surface **1012** of the prism. Prism **108** has a second surface **1014** disposed at an angle α with respect to the first surface **1012**. In some embodiments, angle α is selected to be equal to the incident angle θ of light ray **901** from light source **120**. Thus, light ray **901** from light source **120** is directed toward the second surface **1012** of prism **108** along a normal direction of the second surface **1014** of the prism **108**. While a small portion of light ray **901** may be reflected by surface **1014**, the rest of light ray **901** enters substrate **112** without any change in direction because prism **108** and substrate **112** are ideally made of a same material and are fused together or optically coupled with each other with a fluid. A large portion of light ray **901** is reflected by metallic film **114** and comes off from the metallic film **114** as a light ray **1112**. Light ray **1112** impinges on the boundary **1010** between substrate **112** at angle equal to the angle θ with respect to the normal N of the substrate **112** from the inside of substrate **112**.

[0077] In exemplary embodiments of the present teaching, θ is selected to be equal or larger than a critical angle θ_τ such that light ray **1112** is totally reflected from boundary **1010** and comes back towards metallic film **114** as light ray **1114**. The above reflection from the metallic film **114** and the total reflection at the boundary **1010** are repeated for light ray **1114** and its derivatives, which are the reflected portion of light ray **1114** and reflected portion thereof and so on, as shown in FIG. **10**A. In exemplary embodiments, metallic film **114** is formed to extend to the side surfaces **1020** and in some embodiments to the edge portions **1030** of the bottom surface **910** of the substrate **112** so that light rays reflected from the metallic film **114** have little chance of escaping substrate **112** at the side surfaces **1020** but are recycled and used repeatedly as excitation light for the source points **210**, as shown in FIG. **10**A. According to Snell's Law, the critical angle θ_τ is determined by:

$$\theta_\tau = \sin^{-1}\left(\frac{n_1}{n_2}\right),$$

[0078] where n_1 and n_2 are the refractive indices of the respective media, i.e., air and substrate **112**, respectively.

[0079] In further embodiments, collection efficiency of optical assembly can be increased by using a fluid **104** having a refractive index between that of the air and that of the transparent material used to construct the substrate **112**. For example, when substrate **112** is made of fused silica having a refractive index of about 1.46, water can be used as the fluid **104** because it has a refractive index of 1.33, which is between the refractive index of air (~1) and that of fused silica (~1.46). The fluid **104** is placed between the substrate **112** and the optical assembly **130**. In the embodiments employing the fluid **104**, the critical angle is determined by:

$$\theta_\tau = \sin^{-1}\left(\frac{n_f}{n_2}\right),$$

[0080] where n_f is the refractive index of the fluid. The critical angle θ_τ is therefore increased by employing the fluid. With the increase in the critical angle θ_τ, the collection efficiency is increased because more emitted light from the source points is able to escape through the bottom surface **910** of the substrate **112** without going through total internal reflection, and can therefore be collected by the optical assembly **130**. The angle α of the prism **108** and the incident angle θ of the excitation light may be adjusted accordingly to allow total internal reflection of the excitation light to still occur in the presence of the fluid **104**.

[0081] In another exemplary embodiment, as shown in FIG. **10**B, total internal reflection is facilitated by directing the excitation light toward a side **1060** of the substrate **112**. Excitation light **1070** from the light source **120** is directed to a side surface **1062** at an angle θ_1 with respect to a normal N' of the side surface **1062**. A refracted portion **1072** of the excitation light **1070** leaves the side surface **1062** at an angle θ_2 that is dependent on the angle θ_1 according to the Snell's Law, and impinges on the aluminum film **114** on top of the substrate **112** at an angle $\theta_3=\alpha-\theta_2$, where α is the angle between the side surface **1062** and the bottom surface **1010** of the substrate **112**. After reflection from the aluminum film, a reflected portion **1074** of the excitation light impinges on the surface **1010** at an angle θ_4 that is equal to the angle θ_3. θ_2 and α can be selected such that θ_3 or θ_4 is equal to or larger than the critical angle θ_τ for total reflection at the bottom surface **1010** of the substrate **112**. For example, α in this case can be selected to be at or near 90° in order to create a large θ_3 or θ_4 angle. Thus, most of the refracted portion **1072** of the excitation light **1070** can be repeated used to illuminate the source points on the sample holder **110** before exiting the substrate **112**.

[0082] In another exemplary embodiment, as shown in FIG. **10**C, the side **1060** has a beveled surface **1062** forming an angle α with the bottom surface **910** of the substrate **112**, where α is less than 90° and larger or equal to the critical angle θ_τ. Excitation light **1070** from the light source **120** is

directed to the beveled surface **1062** along a normal N' of the beveled surface **1062**. A refracted portion **1072** of the excitation light **1070** leaves the beveled surface **1062** at an angle θ_2 that is dependent on the angle θ_1 according to the Snell's Law, and impinges on the aluminum film **114** on top of the substrate **112** at an angle $\theta_3=\alpha$. After reflection from the aluminum film, a reflected portion **1074** of the excitation light impinges on the surface **1010** at an angle θ_4 that is equal to the angle θ_3. With α being equal or larger than the critical angle θ_τ, θ_4 is also equal or larger than θ_τ and total reflection occurs at the bottom surface **1010** of the substrate **112**.

[0083] In another exemplary embodiment, as shown in FIG. **10**D, the side **1060** has a beveled surface **1062** forming an angle α with the bottom surface **910** of the substrate **112**, and excitation light **1070** from the light source **120** is directed to the beveled surface **1062** at an angle θ_1 with respect to a normal N' of the beveled surface **1062**. A refracted portion **1072** of the excitation light **1070** leaves the beveled surface **1062** at an angle θ_2 that is dependent on the angle θ_1 according to the Snell's Law, and impinges on the aluminum film **114** on top of the substrate **112** at an angle $\theta_3=\theta_2+\alpha$. After reflection from the aluminum film, a reflected portion **1074** of the excitation light impinges on the surface **1010** at an angle θ_4 that is equal to the angle θ_3. θ_2 and α can be selected such that θ_3 or θ_4 is equal to or larger than the critical angle θ_τ for total reflection at the bottom surface **1010** of the substrate **112**. Thus, most of the refracted portion **1072** of the excitation light **1070** can be repeated used to illuminate the source points on the sample holder **110** before exiting the substrate **112**.

[0084] In another exemplary embodiment, the excitation light **901** from the light source **120** is coupled into the substrate **112** through a grism, which is a prism and grating combination, or grating **1080** formed on or attached to a portion of the bottom surface **1010** of the substrate **112**, as shown in FIG **10**E. With the use of the grism or grating **1080**, the excitation light **901** can enter the substrate at an angle θ with respect to the normal N of the substrate that is equal or larger than the critical angle θ_τ, and after being reflected from the aluminum film **114**, would impinge on the bottom surface **1010** of the substrate **112** at the angle θ and be totally reflected from the bottom surface **1010** back into the substrate. Thus, the excitation light is recycled within the substrate, as shown in FIG. **10**C.

[0085] The arrangements in FIGS. **9** through **10**E are advantageous over conventional systems in part because, by placing the detector(s) and the optical assembly **130** under sample holder **110**, fluorescent signals can be collected through the bottom surface **910** of the sample holder **110** without the interference of reflected light from metallic film **114**. Furthermore, since the excitation light and the fluorescent signals entering the optical assembly **130** do not have a common light path, there is no need of heavy filtering to separate the excitation light entering the substrate **112** from the fluorescent signals exiting the substrate **112** through the bottom surface **910**.

[0086] In various embodiments, optical assembly **130** comprises at least one pixilated or multi-element detector configured to sense light signals landed thereon and a set of optical components configured to direct light emissions from the source points toward the multi-element detector(s). FIG. **11** illustrates a top view of a portion of the sample holder **110** showing a plurality of source points **210**. In exemplary embodiments, as shown in FIG. **12**, the pixilated or multi-element detector comprises a plurality of addressable light-sensing elements **1210** organized in an imaging plane **1220**, such as the x-y plane. The set of optical components is configured to direct light emissions from different source points toward different areas **1230** of the imaging plane **1220** so that light emissions from different source points **210** can be separately and substantially simultaneously detected.

[0087] Thus, as shown in FIG. **12**, light emissions from each source point **210** form an image of the source point in an area **1230** on the imaging plane **1220**. In exemplary embodiments, the set of optical components further comprises a light-dispersing setup configured to separate light emissions from the multiple source points **210** into multiple spectral components so that the detected light from each source point is spread out spectrally along the y axis and images **1230** represent spectrally resolved images of the source points **210**, as shown in FIG. **12**. When the light-dispersing setup is provided, enough separation between neighboring source points **210** on the sample holder **110** is provided to insure that the spectrally resolved images **1230** of the source points do not overlap with each other. In addition, sufficient gap g along the y-direction between an image **1230** of a source point and an image **1230** of a neighbor source point are provided to prevent overlap of data associated with the two source points due to cutoff filter tolerances.

[0088] The position of the images **1230** can be determined by a spatial calibration to associate each source point on the sample holder with an area **1230** on the image plane **1220**. The calibration can be done by using a dye solution or a light source that is not blocked by system filters. Such calibration, however, may not be required if there is no need to correspond the images **1230** with the source points **210**. In addition, tolerance should be allowed to insure that there is sufficient separation d between the areas **1230** and the edges of the image plane **1220**, and the separation should be controlled to allow detection of all of the source points **210** on the image plane **1220**. As a non-limiting example, the buffer zone d between a side **1232** of an areas **1230** facing an edge **1222** of the image plane **1220** is no more than 8 pixels wide.

[0089] Although FIG. **12** shows that the source point images **1230** each having a width ω of 2 pixels, a pitch between two neighboring images in the x-direction being 4 pixels, a gap g between two adjacent rows of images being 4 pixels, and a buffer distance d of the images **1230** from each edge of the image plane **1220** being 8 pixels, these numbers are shown as examples and can be different in different applications or are adjustable to suit different applications. Moreover, although FIG. **12** shows only two rows of source points **210** and two rows of images **1230** corresponding to the source points, in practice, there may be more or less rows of source points or images. Also, the source points **210** do not have to be arranged in rows and can be spread out on the sample holder in any order or even randomly as long as they are sufficiently separated so that their images **1230** do not overlap on the image plane **1220**.

[0090] In exemplary embodiments, the optical assembly **130** is similar to the one in the optical system disclosed in U.S. Pat. No. 6,690,467 B1 by Reel, which is incorporated herein by reference. As shown in FIG. **13**, as a non-limiting example, the optical assembly comprises a collection lens assembly **1310**, a reimaging lens assembly **1320**, and at least one CCD detector **1330** as the pixilated detector. The collection lens assembly **1310** comprises at least one collection lens configured to collect light emissions from the source points **210**. The reimaging assembly **1320** comprises at least one reim-

aging lens configured to focus the collected light emissions from different source points into different areas **1230** of the imaging plane **1220** of the detector **1330**.

[0091] The use of the collection lens assembly **1310** may also provides a substantially collimated region between the collection lens assembly **1310** and the reimaging lens assembly **1320**, which is suitable for insertion of a variety of optical devices such as a an aperture **1340**, a light-dispersing assembly **1350**, and/or a laser line filter **1360**. In exemplary embodiments, the light-dispersing assembly **1350** comprises at least one grating, prism, or grism configured to spread spectrally rays of light that pass through it. For example, a transmission grating deflects rays of light that strike thereon at an angle roughly proportional to the wavelength of the light. Thus, the collimated light emissions from the source points **210**, after going through the transmission gratings, are dispersed spectrally. With the spectral dispersion, a first light ray of a first wavelength and a second light ray of a second wavelength coming from a same source point **210** should arrive at the reimaging lens assembly **1320** at different angles with respect to an optical axis of the reimaging lens assembly **1320** and thus be focused onto different locations **1234** and **1236** of the area **1230** corresponding to the source point, as shown in FIG. **12**. Locations **1234** and **1236** are spaced apart from each other along the y-axis because of the spectral dispersion.

[0092] Instead of prism, grating, or grism in the light dispersing assembly **1350**, dichroic or bandpass filters can be used to separate the spectral components in the fluorescent signals from each source point. FIG. **14**A illustrates other embodiments of optical assembly **130**. As shown in FIG. **14**A, optical assembly **130** comprises a collection assembly **1310**, an imaging assembly comprising imaging lenses **1332-1**, **1332-2**, **1332-3**, and **1332-4** disposed at 90° angles with respect to each other, a plurality of CCD detectors **1330**, and a light dispersing assembly comprising dichroic or bandpass filters D**1**, D**2**, and D**3** placed at 90° angles with respect to each other. Each dichroic or bandpass filter is configured to allow passage of one of the first, second, and third bands of fluorescent signals, respectively, and to reflect all other frequencies. The plurality of CCD detectors **1330** comprises CCD detectors **1330-1**, **1330-2**, **1330-3**, and **1330-4**. CCD detector **1330-1** is placed behind dichroic filter D**1** to collect the first band of fluorescent signals, CCD detector **1330-2** is placed behind dichroic filter D**2** to collect the second band of fluorescent signals, CCD detector **1330-3** is placed behind dichroic filter D**3** to collect the third band of fluorescent signals, and CCD detector **1330-4** is placed in front of dichroic filter D**3** to collect the signals reflected therefrom, which should comprise the fourth band of fluorescent signals. Other filters (not shown) can be placed before CCD detectors **1330**, respectively, for improved frequency selection.

[0093] Alternatively, a dichroic or bandpass filter can be configured to reflect the first, second, third, or fourth band of fluorescent signals, and to allow passage of all other frequencies. It is also possible to combine bandpass, notch, lowpass and highpass filters in any combination that permits appropriate separation of the emission wavelengths. FIG. **14**B illustrates still other embodiments of optical assembly **130**. As shown in FIG. **14**B, optical assembly **130** comprises a collection lens assembly **1310**, a reimaging assembly comprising reimaging lenses **1320-1**, **1320-2**, **1320-3** and **1320-4**, and CCD detectors **1330-1**, **1330-2**, **1330-3**, and **1330-4** each behind a respective one of the reimaging lenses **1320-1**, **1320-2**, **1320-3** and **1320-4**. Optical assembly **130** further comprises a light dispersing assembly comprising dichroic or bandpass filters DF**1**, DF**2**, DF**3** and DF**4** placed in a row under collection assembly **1310** and each at an angle γ to an optical axis (shown by the dashed line) of the collection assembly **1310**. Dichroic or bandpass filters DF**1**, DF**2**, DF**3**, and DF**4** are each placed in front of a respective one of the reimaging lenses **1320-1**, **1320-2**, **1320-3** and **1320-4** and are configured to reflect the first, second, third and fourth bands of fluorescent signals, respectively, while allowing passage of signals of other frequencies. Other filters (not shown) can be placed before CCD detectors **1330**, respectively, for improved frequency selection.

[0094] Imaging lenses **1320-1**, **1320-2**, **1320-3** and **1320-4** can be separate lenses or sections of a single lens, CCD detectors **1330-1**, **1330-2**, **1330-3**, and **1330-4** in FIG. **14**B can be separate CCD detectors or sections of a single CCD detector. Although FIG. **14**B shows that the dichroic or bandpass filters DF**1**, DF**2**, DF**3** and DF**4** are at a roughly 45° angle with respect to the optical axis of the collection assembly **1310**, such placement is not necessary and the angle γ can be larger or smaller than 45°. FIG. **14**C illustrates an exemplary configuration of the optical assembly **130** when the angle γ is close to 90° so that the collimated light emissions from a source point would impinge on the dichroic or bandpass filters DF**1**, DF**2**, DF**3** and DF**4** at a small incident angle β and be imaged by the reimaging lens assembly **1320** onto the CCD detector(s) **1330**.

[0095] The CCD assembly **1330** comprises at least one charge-coupled device (CCD) array, such as a regular CCD array, a complimentary metal-oxide-semiconductor (CMOS) array, an electron-multiplying CCD (EMCCD) array, an intensified CCD (ICCD) array, or an electron-bombarded CCD (EBCCD) array. A CCD array is advantageous over other multi-element detectors, such as an array of avalanche photodiode (APD) based detectors or photomultiplier tube (PMT) based detectors, because the number of elements in a CCD array is much higher, as the size of a CCD pixel in the CCD array can be as small as 3 μm or even smaller. Therefore, signals from different source points can be differentiated by detecting them using different groups of elements in the CCD array, as discussed above. A CCD array can be much less costly than an APD or PMT array.

[0096] To amplify the low light signals from fluorescing labels on the incorporated bases above background noises in CCD arrays, a high-sensitivity CCD-based device such as EMCCD, ICCD, or EB-CCD, is used in exemplary embodiments. Due to fast base incorporation rates of DNA molecules, in addition to sensitivity, the speed of reading data out from a CCD detector is also important because it is associated with the ability to capture event data and to readout the data out over a short period of time to allow the next event to be observed. Through careful design of a readout scheme, a CCD array can be made to be fast enough to resolve fluorescent emissions from two consecutive incorporation events associated with a same source point. Moreover, a CCD with multiple outputs or taps can be used to increase the CCD readout speed. For example, a CCD with 4 taps can allow a 4-times increase in readout speed, which allows images of more source points to be read for increased throughput.

[0097] To further improve the readout speed, a frame transfer CCD (FTCCD) array **1500**, as illustrated in FIG. **15**A, is used in detector **1330** according to exemplary embodiments of the present teaching. The FTCCD array **1500** allows data readout operations to be performed concurrently with data

collection operations. As shown in FIG. **15**A, CCD array **1500** comprises a dark area **1510**, an image area **1520**, a masked storage area **1530**, and a horizontal register **1540**. The image area **1520** of CCD array **1500** is where light signals are detected and is constructed as a two-dimensional array of light-sensitive elements or pixels. Storage area **1530** comprises an array of storage elements covered with an opaque mask to provide temporary storage for an image frame transferred from the image area. The signal that is accumulated in each pixel in image area **1520** is read out by a process of parallel transfer in the negative y-direction shown in the figure, whereby charges in each horizontal row within image area **1520** and storage area **1530** are transferred to the next row and so forth until ultimately they reach the horizontal register **1540**, which is a serial readout register that allows charges to be transferred in the x-direction to an output node (not shown), from which they are read out. While data from storage array **1530** is read, image area **1520** is available to collect a next round of light signals.

[0098] Dark area **1510** is a region of excess pixels. Because these pixels are not illuminated, they do not have to be cleared during each readout. Usually, the combination of dark area **1510** and image areas **1520** maps directly onto the storage area **1530**. In one embodiment, image area **1520** occupies a small fraction (e.g. 1/10) of the combination so that source point data can be read out at, for example, 10 times the normal frame rate. In exemplary embodiments, CCD array **1500** is kept cool at about 80° C. below zero so that minimal dark current charges are generated. In certain embodiments, the dark area is eliminated when CCD **1500** is custom built to have just the right amount of rows in the image area **1520**.

[0099] In some embodiments, an interline CCD or a combined interline and frame transfer CCD may be employed. FIG. **15**B illustrates an interline CCD array **1550** in CCD detector **1330** according to exemplary embodiments of the present teaching. As shown in FIG. **15**B, interline CCD array **1550** comprises separate image regions **1560** and CCD storage regions **1570**. CCD storage regions **1570** are protected with a mask structure and positioned alongside respective ones of image regions **1560** such that CCD storage regions **1570** and image regions **1560** together form an alternating parallel array. Image regions **1560** may comprise circuit elements such as photodiodes for capturing the images of the source points while CCD storage regions **1570** shift previously acquired images in a parallel fashion towards a horizontal register **1590**. The horizontal register **1590** then sequentially shifts image information from each CCD storage region to an output amplifier or other processing circuits (not shown) as a serial data stream. The readout process is repeated until data from all of the CCD storage regions **1570** are transferred to the output amplifier.

[0100] Where an actual two-dimensional image is desired from the CCD, the image data in a digital format is reconstructed to yield the final image. Where the data is to be used for non-pictorial or non-imaging applications, the relevant pixel data may be identified and processed according to its intended purpose. One advantage of the interline CCDs is their ability to operate without a shutter or synchronized strobe, allowing for an increase in device speed and faster frame rates. An interline CCD array can be used to eliminate blurring or image smear, which is a common problem with frame-transfer CCDs, by effectively doing horizontal shifts directly from the image regions to the respective ones of the storage regions.

[0101] In exemplary embodiments, readout speed is further improved by limiting the number of source points to be imaged on the CCD so that the number of data rows to be read are minimized. The number of data rows to be read may also be minimized by binning vertically (in the y-direction), especially when the source points **210** and thus the images of the source points **1230** are in an array so that the positions of the images can be fairly accurately predicted, as shown in FIG. **12**. With the fluorescent signals dispersed spectrally over a range of pixels along the y-direction, the pixels can be binned in the y-direction to capture informative wavelength groupings for base determination. For example, as shown in FIG. **12**, each source point image **1230** may comprise four interested wavelength groups C**1**, C**2**, C**3**, and C**4** interleaved with uninterested wavelength groups U**1**, U**2**, and U**3**. Data in each of the interested wavelength groups can be binned, while data in the uninterested wavelength groups can be cleared without being read, as discussed below. Binning can also be done in the x-direction for further increase in readout speed because shifting is often faster than reading. With a CCD array, binning can be done on-chip in a conventional manner that does not introduce noise.

[0102] The CCD array in detector **1330** may also be made to allow clearing of the horizontal register **1540** or **1590**. This can speed up readouts if desired data is separated by rows of unneeded pixels. FIG. **16**A illustrates examples of four normalized fluorescent spectra associated with the four different fluorescent labels used to label respective ones of four different nucleotides. The four spectra have peak regions **1610**, **1620**, **1630**, and **1640** corresponding to respective ones of the first, second, third and fourth frequency bands. Only four fluorescent frequency bands need to be collected by detector **1330**. Data can be over-determined, however, to comprise more than the spectrum of data associated with an incorporation event. With the light dispersing setup shown in FIG. **13**, a continuous portion of a spectrum of data is spread along the y-direction, as shown in FIG. **16**B. FIG. **16**C illustrates the over-determined data with the most informative wavelengths corresponding to the four frequency bands in gray and the less informative wavelength ranges shown in white. Data rows in the less informative wavelength range between two gray bands is referred to as a band gap bg, which may not be needed and can be cleared without being read out for increased readout speed. The width and position of each informative frequency band in FIG. **16**C can be optimized for best signal to noise (S/N) ratio after multicomponenting, as explained in more detail below.

[0103] While the frequency bands in FIGS. **12**, **16**B, and **16**C are shown to spread along the y-direction, this is not essential and orienting the frequency bands in a different direction on the CCD may be advantageous in some cases.

[0104] FIG. **17**A is a flow chart of a method **1700** for reading out image data associated with a plurality of source points according to exemplary embodiments of the present teaching. As a non-limiting example, method **1700** is described in the context of a frame transfer EMCCD array of 512×512 pixels in the image area that allows clearing of the horizontal register. Nevertheless, the method can be readily adapted to other types of CCD array. As shown in FIG. **17**A, method **1700** comprises step **1710** in which the pixels in the image area are shifted down by a frame having, for example, 128 rows. So, 128 rows of pixels are shifted from the image area into the masked storage area. These pixels are not read out for a number of (e.g., 4) frames (512/128=4). Pixels from

the dark area will be shifted down to collect the next images, but the provision of a dark area is not necessary because the CCD is kept cool (−80° C.) so minimal dark current charge is generated. Method **1700** further comprises step **1720** in which a first number of (e.g., 8) buffer rows in a frame is shifted down to the register and cleared. The number 8 is arbitrarily chosen. This number should correspond to the distance d shown in FIG. **12**. Method **1700** further comprises step **1730** in which the pixels associated with the first frequency band is vertically binned and shifted. As a non-limiting example, 4 rows of pixels are associated with a frequency band, and these rows are binned and read out as one row. Method **1700** further comprises step **1740** in which at least some of the rows in the band gap bg before the next frequency band are cleared. Steps **1730** and step **1740** are thereafter repeated for each of the other frequency bands. Method **1700** further comprises step **1750** in which at least some of the buffer rows in the gap g between two rows of images **1230** are shifted and cleared. Steps **1730** through **1750** are repeated for each row of source points when the source points are arranged in an array.

[0105] FIG. **17**B is a spreadsheet for estimating the throughput for system **100** using a frame transfer EMCCD as detector **1330** according to exemplary embodiments. As shown in FIG. **17**B, for a frame transfer CCD having 512×512 pixels in the image area, a normal read out time of 1×10^{-7} second, a vertical shift time of 4×10^{-7} second, and a horizontal register clear time of 5×10^{-6} second, using method **1700**, the time to read out emission data from 4 rows of source points is about 0.00096 seconds, resulting in a readout speed higher than 1000 Hz, which is much faster than the normal readout speed of 30 Hz for the CCD. FIG. **17**C is a spreadsheet for estimating the throughput for system **100** using a 1004×1002 EMCCD as detector **1330** according to exemplary embodiments. As shown in FIG. **17**C, the time for reading out emission data from 4 rows of source points in this case is about 0.00113 seconds, resulting in a readout speed close to 1000 Hz.

[0106] In exemplary embodiments of the present teaching, optical data collected by detector **1330** is sent to computer system **140** and optionally or additionally DSP or FPGA **150** for base determination. FIG. **18** is a block diagram of computer system **140** according to exemplary embodiments of the present teaching. As shown in FIG. **18**, computer system **140** comprises a central processing unit (CPU) **1810**, a memory unit **1820**, a data input port **1830**, and a user interface **1840**, interconnected by one or more buses **1850**. Memory unit **1820** preferably stores operating system software **1822** and other software programs including a program **1824** for base determination. Memory unit **1820** further comprises a data storage unit **1826** for storing optical data collected by detector **1330** and sent to computer system **140** through data input port **1830**. Program **1824** comprises coded instructions, which, when executed by CPU **1810**, cause computer system **140** to carry out methods for detecting light emissions from multiple source points as described above and/or methods for base determination based on the detected data according to exemplary embodiments of the present teaching, as explained in more detail below.

[0107] With the illumination of excitation light, a labeled and incorporated nucleotide should fluoresce by emitting photons from an associated source point. The spectrum of the photons collected at detector **130** from this single incorporation event should be a collection of photons with different energies or frequencies. When the number of collected photons is large, the spectrum should resemble the normal dye spectrum corresponding to the fluorescent dye used to label the incorporated nucleotide. The spectrum will vary, however, due to the small number of photons that are typically collected by detector **1330** from the single incorporation event in each collection time period.

[0108] For example, a fluorescing dye may emit 10,000 photons over a 10 micro second period, and about 4% of the 10,000 photons may be detected by detector **1330** in ten time bins each corresponding to, for example, a one micro second period. Thus, roughly 40 photons may be collected in each time bin. Plotted spectrally over 10 spectral bins, the 40 expected photons might spread out like the histogram shown in FIG. **19**. Because of the small number of photons, the distribution in FIG. **19** may not match the normal dye spectrum corresponding to the incorporated nucleotide, and the mismatch may lead to a chance of incorrect base determination.

[0109] In exemplary embodiments, to avoid a base determination problem caused by a small number of photons from a single incorporation event, the present teaching comprises a method **2000** for base determination illustrated by the flowchart in FIG. **20**. As shown in FIG. **20**, method **2000** comprises step **2010** in which data from all spectral bins collected in each of a plurality of consecutive time bins are copied and combined to form composite light data. Method **2000** further comprises step **2020** in which the composite data from the plurality of consecutive time bins are used to determine an incorporation time interval T.

[0110] FIG. **21** is a plot of the composite data over a plurality of 24 time bins showing the number of photons detected in each time bin. As shown in FIG. **21**, during time bins **1**, **2**, **3**, **4**, and **5**, and time bins **16**, **17**, **18**, and **19**, the composite data indicate small numbers of photons coming from a background of diffusion events, trial events, and substrate fluorescence. In other time bins, the numbers of photons are significantly larger, resulting in large rises **2110** and **2120** above the background noises. Rises **2110** and **2120** indicate incorporation events. The incorporation time T for the incorporation event corresponding to rise **2110** can be determined by measuring the width of rise **2110**, as shown in FIG. **21**.

[0111] Since most of the photons detected during the incorporation time interval T are from a single incorporation event, for each color bin, data associated with the same spectral bin but collected in different time bins in the incorporation time interval can be combined, resulting in increased data points for the spectral bin. The increase in the number of data points leads to an improved multicomponenting process, which is used to convert color data to dye composition. Thus, method **2000** further comprises step **2030** in which data associated with each spectral bin or frequency band of interest but collected in different time bins in the incorporation time interval T are combined, and step **2040** in which the combined data is used in a conventional multicomponenting process to determine a dominant dye, which is used to determine the base being incorporated. Method **2000** further comprises step **2050** in which the residuals of the multicomponenting process is used to determine a confidence level.

[0112] Method **2000** for improving the signal to noise ratio by combining data from multiple time bins may be coded as a computer program and executed by computer system **140**. Alternatively, since the same algorithm in method **2000** is executed a large number of times, hardware solutions such as

field program gate arrays (FPGA) or digital signal processors (DSP) **150** and the like can be used to reduce the computation load and data stream size. The FPGAs or DSPs **150** could be integrated in detector(s) **1330**, between detector **1330** and computer system **140**, as shown in FIG. **1**, or installed into computer system **140**.

[0113] The foregoing descriptions of specific embodiments of the present teaching have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the teaching to the precise forms disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the teaching and its practical application, to thereby enable others skilled in the art to best use the teaching and various embodiments with various modifications as are suited to the particular use contemplated. It is intended that the scope of the teaching be defined by the claims appended hereto and their equivalents.

What is claimed is:

1. A method of identifying nucleotides in a nucleic acid sequence, comprising:

providing a plurality of polymerization complexes within a plurality of confined reaction environment, wherein each complex comprises a polymerase enzyme, and a template nucleic acid;

contacting the plurality of complexes with a plurality of types of nucleotide analogs labeled with distinguishable fluorescent labels under conditions suitable for polymerization;

transmitting fluorescent signals associated with incorporation of a nucleotide analog to a detector, wherein location of the fluorescent signals on the detector is indicative of an individual confined reaction environment and a type of nucleotide incorporated; and

identifying a nucleotide in a nucleic acid sequence based upon the type of nucleotide incorporated and the confined reaction environment.

2. The method of claim **1**, wherein the transmitting step comprises collecting fluorescent signals from the plurality of confined reaction environments and passing the fluorescent signals through an optical train that directs different spectral components of the fluorescent signals to different locations on the detector.

3. The method of claim **2**, wherein the optical train comprises a dispersive optical element selected from a prism and an optical grating.

4. The method of claim **1**, wherein the confined reaction environments comprise zero mode waveguides.

5. The method of claim **1**, wherein fluorescent signals associated with incorporation of a plurality of nucleotide analogs in a complex are transmitted to the detector, and a plurality of nucleotides in the nucleic acid sequence are identified.

6. The method of claim **1**, wherein the optical train comprises at least a first prism to direct spectrally different fluorescent signals to different locations on the detector.

7. A method of identifying nucleotides incorporated in polymerase mediated, template dependent nucleic acid synthesis reactions, comprising:

providing a plurality template nucleic acid/polymerase complexes on a substrate;

contacting the plurality of complexes with a plurality of nucleotide analogs having spectrally distinct fluorescent labels, wherein incorporation of nucleotides produces characteristic incorporation signals;

directing incorporation signals to a single detector, wherein a location of a signal on the detector is indicative of a complex incorporating the nucleotide and a type of nucleotide incorporated; and

identifying the nucleotide incorporated into a template mediated nucleic acid synthesis reaction from the location of the signal on the detector.

8. The method of claim **7**, wherein the directing step comprises passing optical signals through dispersive optical element to separate signals from the spectrally distinct labels and image the signals onto different regions of the signal detector.

9. The method for claim **7**, wherein the complexes are individually optically resolvable.

10. The method of claim **7**, wherein the plurality of complexes are optically confined.

11. The method of claim **10**, wherein the plurality of complexes are disposed within an array of zero mode waveguides.

12. A method of analyzing a reaction, comprising:

providing a first reactant immobilized on a substrate;

contacting the first reactant with a second reactant and a third reactant, each bearing a spectrally distinct label;

directing signals from the spectrally distinct labels that are characteristic of interaction of the first reactant with one of the second and third reactants to a location on a first detector, wherein the location on the detector is indicative of the first reactant location on the substrate and the second or third reactant with which the first reactant interacted; and

identifying the first reactant and the second or third reactant with which the first reactant interacted.

13. The method of claim **12**, wherein the first reactant comprises at least a first nucleic acid.

14. The method of claim **13**, wherein the first reactant further comprises a polymerase enzyme.

15. The method of claim **13**, wherein the second and third reactants comprise second and third nucleic acids, respectively.

16. The method of claim **14**, wherein the second and third reactants comprise first and second nucleotide analogs, respectively.

17. The method of claim **15**, wherein the second and third nucleic acids each comprise a spectrally distinct fluorescent label.

18. The method of claim **16**, wherein the first and second nucleotide analogs each comprises a spectrally distinct fluorescent label.

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