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(54) Title: NANOPORE-BASED ANALYSIS OF COMPOUNDS USING MOBILE FRET PAIRS

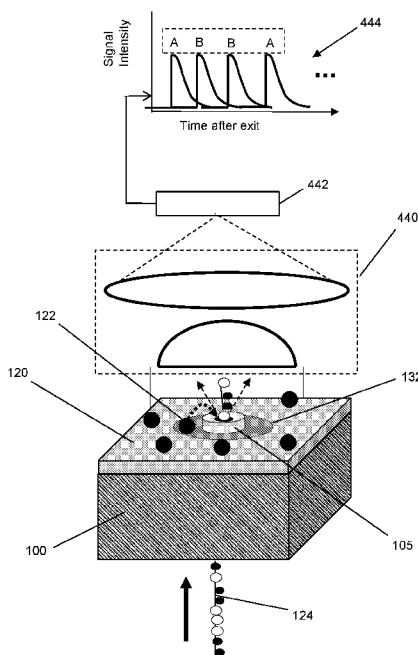


Fig. 4A

(57) Abstract: The invention is directed to devices and methods for optically based nanopore analysis which employ FRET signaling wherein at least one member of a FRET pair is mobile within a lipid bilayer containing one or more nanopores. In some embodiments, mobile FRET donors are constrained to a lipid bilayer so that they may continuously diffuse into and within a FRET distance of acceptor-labeled analytes entering or exiting a nanopore so that bleached or degraded FRET donors are continuously replaced.

**Declarations under Rule 4.17:**

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
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NANOPORE-BASED ANALYSIS OF COMPOUNDS USING MOBILE FRET PAIRS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority to U.S. Provisional Application No. 62/173,350, filed 9 June 2015, which is incorporated by reference herein in its entirety.

BACKGROUND

[0002] DNA sequencing technologies developed over the last decade have revolutionized the biological sciences, e.g. Lerner et al, *The Auk*, 127: 4-15 (2010); Metzker, *Nature Review Genetics*, 11: 31-46 (2010); Holt et al, *Genome Research*, 18: 839-846 (2008); and have the potential to revolutionize many aspects of medical practice in coming years, e.g. Voelkerding et al, *Clinical Chemistry*, 55: 641-658 (2009); Anderson et al, *Genes*, 1: 38-69 (2010); Freeman et al, *Genome Research*, 19: 1817-1824 (2009); Tucker et al, *Am. J. Human Genet.*, 85: 142-154 (2009). However, to realize such potential there are still a host of challenges that must be addressed, including reduction of per-run sequencing cost, simplification of sample preparation, reduction of run time, increasing sequence read lengths, improving data analysis, and the like, e.g. Baker, *Nature Methods*, 7: 495-498 (2010); Kircher et al, *Bioessays*, 32: 524-536 (2010); Turner et al, *Annual Review of Genomics and Human Genetics*, 10: 263-284 (2009). Single molecule sequencing using nanopores may address some of these challenges, e.g., Maitra et al, *Electrophoresis*, 33: 3418-3428 (2012); Venkatesan et al, *Nature Nanotechnology*, 6: 615-624 (2011); however, this approach has its own set of technical difficulties, such as, reliable nanopore fabrication, control of DNA translocation rates, nucleotide discrimination, detection of electrical signals from large arrays of nanopore sensors, and the like, e.g. Branton et al, *Nature Biotechnology*, 26(10): 1146-1153 (2008); Venkatesan et al (cited above).

[0003] Optical detection of nucleotides has been proposed as a potential solution to some of the technical difficulties in the field of nanopore sequencing, e.g. Huber, U.S. patent 8,771,491; Russell, U.S. patent 6,528,258; Pittaro, U.S. patent publication 2005/0095599; Joyce, U.S. patent publication 2006/0019259; Chan, US. patent 6,355,420; McNally et al, *Nano Lett.*, 10(6): 2237-2244 (2010); and the like. Several promising approaches to optical detection exploit fluorescence resonance energy transfer (FRET) for generation of localized analyte-specific optical signals at a nanopore, which can be distinguished from signals at other nanopores. Unfortunately, some of the best candidates for FRET donors require difficult design trade-offs. In particular, quantum dots as donors have favorable

1 stability, but have size and structural properties that make them difficult to position on nanopore arrays.
2 Organic FRET donors have convenient sizes and chemistries available for direct attachments to, for
3 example, protein nanopores, but they lack chemical stability for prolonged use and soon become
4 “bleached” and inoperable.

5 **[0004]** In view of the above, it would be advantageous to nanopore sensor technology in general
6 and its particular applications, such as optically based nanopore sequencing and analyte detection, if
7 methods and devices were available for addressing the limitations imposed by FRET donors.

8 SUMMARY OF THE INVENTION

10 **[0005]** The present invention is directed to devices and methods for efficient optical detection and
11 analysis of analytes, such as polymers (including polynucleotides) using nanopore arrays.

12 **[0006]** In one aspect, the invention is directed to devices for detecting an analyte comprising the
13 following elements: (a) a solid phase membrane separating a first chamber and a second chamber, the
14 solid phase membrane having at least one aperture connecting the first chamber and the second
15 chamber, (b) a lipid bilayer disposed on at least one surface of the solid phase membrane, the lipid
16 bilayer comprising a concentration of donors of a fluorescence resonance energy transfer (FRET) pair,
17 the donors being mobile within the lipid bilayer; and (c) a nanopore immobilized in the aperture, the
18 nanopore having a bore with an entrance and an exit and the nanopore interacting with the lipid layer to
19 form a seal with the solid phase membrane in the aperture so that fluid communication between the
20 first chamber and the second chamber occurs solely through the bore of the nanopore; wherein the
21 concentration of donors in the lipid bilayer is selected so that whenever an analyte labeled with an
22 acceptor of the FRET pair exits or enters the bore of the nanopore with a predetermined likelihood at
23 least one donor is within a FRET distance of the acceptor.

24 **[0007]** In some embodiments, the nanopore is a protein nanopore. In some embodiments,
25 concentration of donors is selected so that an expected frequency of donors coming within a FRET
26 distance of an entrance or an exit of said bore (depending on which surface of the solid phase
27 membrane the lipid bilayer is disposed) is equal to or greater than a frequency with which the acceptors
28 enter or exit said bore, respectively. In other embodiments, an expected frequency of donors coming
29 within a FRET distance of an entrance or exit of said bore is equal to or greater than ten times the
30 frequency with which acceptors enter or exit said bore, respectively.

31 **[0008]** In another aspect the invention is directed to methods of determining a nucleotide sequence
32 of a polynucleotide comprising the steps of: (a) providing a device comprising: (i) a solid phase
33 membrane separating a first chamber and a second chamber, the solid phase membrane having at least
34 one aperture connecting the first chamber and the second chamber, and the solid phase membrane

1 having a lipid bilayer on at least one surface; (ii) a protein nanopore immobilized in the aperture, the
2 protein nanopore having a bore, the protein nanopore contacting and extending through the lipid bilayer
3 so that the first chamber and the second chamber are in fluid communication through the bore; and (iii)
4 donors of one or more FRET pairs diffusably disposed in the lipid bilayer; (b) translocating the
5 polynucleotide through the protein nanopore so that acceptor labels attached to the polynucleotide pass
6 sequentially therethrough and so that whenever an acceptor label exits the bore of the protein nanopore
7 at least one donor with a predetermined likelihood is within a FRET distance of such acceptor label;
8 and (c) determining a nucleotide sequence of the polynucleotide by a sequence of FRET interactions
9 between donors and acceptor labels exiting the bore or the protein nanopore. In some embodiments,
10 donors are capable of FRET only within a predetermined proximity of an exit of a bore of a nanopore.
11 In some embodiments, donors are ion-sensitive dyes that are capable of FRET only when a
12 predetermined ion is present at a predetermined concentration.

13 **[0009]** The present invention is exemplified in a number of implementations and applications,
14 some of which are summarized below and throughout the specification.

16 BRIEF DESCRIPTION OF THE DRAWINGS

17 **[0010]** Figs. 1A-1B illustrates a design of a hybrid nanopore configured for FRET detection using a
18 donor attached directly to the stem of a protein nanopore.

19 **[0011]** Figs. 1C-1F illustrate various embodiments of a hybrid nanopore employing mobile FRET
20 donors in a lipid bilayer.

21 **[0012]** Figs. 2A, 2B and 2C illustrate embodiment where one or more nanopores, e.g. protein
22 nanopores, may occupy a single aperture of a solid phase membrane.

23 **[0013]** Figs. 3A-3B further illustrate embodiments of a hybrid nanopore employing mobile FRET
24 donors in a lipid bilayer wherein the mobile donors are ion-sensitive dyes (calcium ion shown) that are
25 capable of FRET only in the presence of a predetermined concentration of predetermined ion.

26 **[0014]** Fig. 3C illustrates an embodiment wherein donors are lipophilic and are delivered to a lipid
27 bilayer adjacent to a nanopore by a carrier molecule. After illumination FRET donors, which may be
28 bleached, diffuse away from the nanopore in the lipid bilayer.

29 **[0015]** Fig. 4A illustrates the operation of a device of the invention.

30 **[0016]** Fig. 4B illustrates a surface of a solid phase membrane on which a lipid bilayer containing a
31 single type of mobile donor (122) is disposed.

32 **[0017]** Fig. 4C illustrates a surface of a solid phase membrane on which a lipid bilayer containing
33 two types of mobile donors (122 and 123) is disposed.

DETAILED DESCRIPTION OF THE INVENTION

[0018] While the invention is amenable to various modifications and alternative forms, specifics thereof have been shown by way of example in the drawings and will be described in detail. It should be understood, however, that the intention is not to limit the invention to the particular embodiments described. On the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention. For example, particular nanopore types and numbers, particular labels, FRET pairs, detection schemes, fabrication approaches of the invention are shown for purposes of illustration. It should be appreciated, however, that the disclosure is not intended to be limiting in this respect, as other types of nanopores, arrays of nanopores, and other fabrication technologies may be utilized to implement various aspects of the systems discussed herein. Guidance for aspects of the invention is found in many available references and treatises well known to those with ordinary skill in the art, including, for example, Cao, *Nanostructures & Nanomaterials* (Imperial College Press, 2004); Levinson, *Principles of Lithography*, Second Edition (SPIE Press, 2005); Doering and Nishi, Editors, *Handbook of Semiconductor Manufacturing Technology*, Second Edition (CRC Press, 2007); Sawyer et al, *Electrochemistry for Chemists*, 2nd edition (Wiley Interscience, 1995); Bard and Faulkner, *Electrochemical Methods: Fundamentals and Applications*, 2nd edition (Wiley, 2000); Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd edition (Springer, 2006); Hermanson, *Bioconjugate Techniques*, Second Edition (Academic Press, 2008); and the like, which relevant parts are hereby incorporated by reference.

[0019] In part the invention addresses the problem of donors becoming bleached or degraded upon extended illumination in the field of FRET-based detection of labeled analytes using nanopores. In one aspect, multiple donors are made available for FRET interactions at each nanopore by disposing nanopores in a lipid bilayer containing donors that are constrained to the lipid bilayer but are otherwise capable of diffusing freely within the lipid bilayer. Thus, each nanopore (which is in a fixed position) is replenished continuously with donors as new donors diffuse to within a FRET distance of the nanopore. As illustrated in Figs. 1C-1F, a lipid bilayer may be disposed on a cis(-) side or a trans(+) of a solid phase membrane containing nanopores. In some embodiments hybrid nanopores are employed that comprise solid phase membranes with one or more orifices in which protein nanopores are disposed. In such embodiments, in addition to lipid bilayers disposed on either cis(-) or trans(+) sides (or surfaces) of solid phase membranes, protein nanopores may have different orientations within a lipid bilayer; namely, for protein nanopores having stem and cap structures along the axis of a nanopore's bore, or lumen, the cap may be oriented toward the solid phase membrane, as illustrated in Fig. 1C, or the cap may be oriented away from the solid phase membrane, as illustrated in Fig. 1E.

[0020] In a typical configuration, illustrated in Figs. 1A-1B, protein nanopore (104) may be immobilized in aperture, or orifice, (102) on the cis(-) side of solid phase membrane (100), which may include coating (106), e.g. a lipophilic or hydrophobic coating, to facilitate placement of protein nanopore (104). The cis(-) side is defined in this configuration as the side of solid phase membrane (100) from which nucleic acid analytes enter a nanopore, for example, under the influence of an electrical field (although this is not meant to limit devices and methods of the invention to the use of electric fields to translocate analytes through nanopores). The trans(+) side is defined in this configuration as the side of solid phase membrane (100) to which nucleic acid analytes exit a nanopore. In typical embodiments, single stranded nucleic acid analytes are disposed on the cis(-) side of solid phase membrane (100) in an electrolyte solution under conditions, e.g. pH, that give the single stranded nucleic acids a net negative charge, so that upon the application of electric field, E (101), the single stranded nucleic acid analytes are driven through nanopores in solid phase membrane (100).

[0021] Illustrated in the Figs. 1A and 1B is an exemplary protein nanopore, such as hemolysin, which has a cap end (103) and stem end (105), for example, as described in Song et al, Science, 274: 1859-1866 (1996). In this configuration, because of steric constraints of orifice (102), donor (108) attached to stem end (105) of protein nanopore (104) is limited to an organic dye, or a similarly sized molecule that is not sterically excluded from orifice (102). Upon translocation of nucleic acid analyte (110) through protein nanopore (104), acceptor-labeled monomers of analyte (110) move within a FRET distance of donor (108), which excites adjacent acceptors and generates signal (114) whenever donor (108) is illuminated by excitation beam (112). If donor (108) is a conventional organic dye, it may be rapidly degraded or bleached by continuous or repeated excitation, thereby limiting the application of the illustrated configuration for analyzing long and/or multiple nucleic acid strands.

[0022] In some embodiments, the invention addresses this problem by disposing nanopore (104), for example, a protein nanopore, into orifice (102) on the trans(+) side of solid phase membrane (100) with its stem end (105) embedded in lipid bilayer (120) disposed on the trans(+) surface of solid phase membrane (100), as illustrated in Fig. 1C. Lipid bilayer (120) provides a medium for containing donors (122) that are lipophilic or that contain a lipophilic moiety that constrains them spatially to lipid bilayer (120), but otherwise allows them to move within lipid bilayer (120). Donors (122) may be detergent-like, that is, having a lipophilic portion and a hydrophilic portion so that donors (122) are partially immersed in lipid bilayer (120)(as illustrated in Fig. 1D), or donors (122) may be completely or predominantly lipophilic so that they are fully immersed in lipid bilayer (120)(not shown). Donors may be hydrophilic and directly attached to the hydrophilic part of a lipid bilayer, but allows them to move within the lipid bilayer through the lipid component they are attached to. In some embodiments, donors (122) move randomly by diffusion within lipid bilayer (120). The rate of such movement may

be adjusted by adding components, such as lipophilic polymers, or other compounds, such as cholesterol, to lipid bilayer (120) that inhibit movement of donors (122) or by providing donors (122) with higher or lower molecular weights to reduce the rate of movement or to increase the rate of movement, as desired, respectively. In some embodiments, more than one kind of donor (122) may be provided. For example, donors (122) may include donors that have different absorption bands or that have different emission bands. Such differing donors may provide a wider selection of FRET pairs for use in the invention. In some embodiments, donors (122) include a plurality of different kinds of donor, each having a different and substantially non-overlapping emission band. In accordance with the invention, donors are excited only within an illumination zone. Because donors are mobile within lipid bilayer (120), donors that degrade or are bleached diffuse away from end (105) of nanopore (104) and are replenished by “fresh” donors (i.e. capable of participating in a FRET interaction with an acceptor) that diffuse into the illumination region. An illumination zone may be an area encompassing one or more nanopores or it may be generated by intermittent illumination. That is, in the latter case, an illumination zone may encompass the entire surface of solid phase membrane (100) and nanopores contained therein, but an excitation beam is directed to it periodically so that there are alternating intervals of excitation and non-excitation (or darkness). In some embodiments, degraded or bleached donors are replenished essentially continuously by selecting a concentration of donors in lipid bilayer (120) sufficiently high so that new donors enter the FRET zone at a frequency equivalent to or greater than the frequency by which new acceptors enter the FRET zone, for example, at a frequency 5 times greater, or 10 times greater, or 100 times greater than the frequency by which new acceptors enter the FRET zone. In alternative embodiments, degraded or bleached donors are replenished within a FRET zone by providing “dark” intervals when no excitation beam is present and during which new donors may diffuse into the FRET zone; that is, degraded and bleached donors are replenished by intermittently illuminating illumination zone (130) with an excitation beam, so that during non-illuminated periods donors are replenished by diffusion.

[0023] As illustrated in Figs. 1E and 1F, in some embodiments, the degradation and bleaching problem may be addressed by disposing nanopore (104), for example, a protein nanopore, into orifice or aperture (102) on the cis(-) side of solid phase membrane (100) with its stem end (105) embedded in lipid bilayer (120) disposed on the cis(-) surface of solid phase membrane (100). As above, mobile donors constrained to lipid bilayer (120) continually replenish donors in a FRET zone by diffusion. Operation of such embodiments is similar to those of Figs. 1C and 1D. Typically, solid phase membrane (100) is transparent or nearly transparent so that similar, if not identical, illumination and detection schemes may be used in both embodiments.

1 [0024] One of ordinary skill in the art would recognize and appreciate that while the above
2 examples illustrate the invention with a protein nanopore, other embodiments may include non-protein
3 nanopores for aligning acceptor-labeled polymer analytes for excitation with mobile donors in a lipid
4 bilayer.

5 [0025] The operation of the above embodiments of the invention is illustrated in Figs. 1D and 4A.
6 Fig. 1D illustrates polymer analyte (124) whose monomers are labeled with two different acceptor
7 molecules (126, white; and 128, black) translocating through protein nanopore (104) disposed in solid
8 phase membrane (100) in accordance with the invention. Excitation beam (112) illuminates a region
9 around stem end (105) of protein nanopore (104) (referred to herein as an illumination zone, shown in
10 Fig. 1D as (130)). Adjacent to stem end (105) there is a FRET zone (132) within which a FRET
11 interaction takes place between a donor and an acceptor whenever a donor (122) is in FRET zone (132)
12 and an acceptor label of polymer analyte (124) is in FRET zone (132). As a result of such FRET
13 interaction, FRET signal (114) is produced, which is detected.

14 [0026] These concepts are further illustrated in Fig. 4A. Excitation of donors (122) may be
15 accomplished using a total internal reflectance fluorescence (TIRF) microscope system. Such a system
16 may also serve as a detection system, as illustrated diagrammatically by (440). Labels on polymer
17 analyte (124) may be excited directly or indirectly using FRET donor-acceptor pairs. Detector (442)
18 collects optical signals and convert them into values that can be displayed, such as the curves (444).
19 An objective of the analytical systems such as illustrated in Fig. 4A is to use the optical signals
20 generated by the optical labels to identify a sequence of monomers of polymer analyte (124). For
21 example, in the single nanopore case, base (or monomer) calls can be made successfully in a 2-label
22 system; that is, A, B (i.e. "not-A"), B and A (444).

23 [0027] In some embodiments, a concentration of donors (122), diffusion coefficient, and operating
24 conditions are selected so that the expected frequency of donors entering a FRET zone is equal to or
25 greater than the frequency of acceptors entering the FRET zone. Such concentration will depend on the
26 mobility of donors (122), the rate of translocation of polymer analyte (124), the spacing of acceptor
27 labels on polymer analyte (124), the nature of the distribution of donors (122) in lipid bilayer (120),
28 and like factors well-known to those of ordinary skill in the art. In some embodiments, donors will
29 have an expected occupancy time in a FRET zone which depends on the above-listed factors. In some
30 embodiments, the above-listed factors are selected so that donor occupancy time is greater than
31 acceptor expected occupancy time in the FRET zone due to translocation speed. In other embodiments,
32 the above-listed factors are selected so that donor occupancy time is at least three times greater than
33 acceptor expected occupancy time; in other embodiments; the above-listed factors are selected so that
34 donor occupancy time is at least ten times greater than acceptor expected occupancy time; in other

embodiments; the above-listed factors are selected so that donor occupancy time is at least twenty times greater than acceptor expected occupancy time. In other embodiments, the above-listed factors are selected so that donor occupancy time is less than the expected bleaching time of the donor.

[0028] Fig. 4B provides an additional view of a surface of a solid phase membrane (450) with lipid bilayer (120) and mobile donors (122) immersed in, or constrained thereto. For example, lipid bilayer (120) may be on a trans surface of membrane (450) when analytes are negatively charged polynucleotides. Also shown is a FRET zone (132) defined by radius (445) in a proximity of nanopore exit (105). Fig. 4C illustrates a similar view of an embodiment wherein more than one mobile donors (452 and 453) are constrained to, and mobile in, lipid bilayer (120). In some embodiments, a plurality of different kinds of mobile donors may be used that correspond to different FRET pairs, so that different analytes may be detected by interactions between their own FRET pairs. In other embodiments, each mobile donor may be capable of a FRET interaction with one or more acceptors on one or more analytes. For example, an embodiment may comprise two mobile donors, say, X and Y, where in a device for determining nucleotide sequences, donor X may have a FRET interaction with acceptors on nucleotides A and G, and donor Y may have a FRET interaction with acceptors on nucleotides C and T. In some embodiments, there may be a plurality of mobile donors; in other embodiments, such plurality may be in the range of from 2 to 5 mobile donors; in still other embodiments, such plurality may be in the range of from 2 to 3 mobile donors.

[0029] In some embodiments, as noted further below, cross-sectional dimensions (200) of apertures, such as (102) in Figs. 2A-2C may be considerably larger than that of a protein nanopore, such as illustrated by (104) in Fig. 2A. In such embodiments, protein nanopore (104) may diffuse freely with the boundaries (202) of the cross section of aperture (102). With such larger aperture cross sectional areas more than one protein nanopore may be disposed in a single aperture, as illustrated in figures 2B and 2C by (104a and 104b). In some embodiments, a plurality of protein nanopores in a single aperture is advantageous, since the duty cycles of apertures may be effectively increased since there would be reduced wait times for the next analyte to be processed by an aperture. The concentration of protein nanopores and the concentration of analytes may be selected to optimize processing throughput, especially, for example, when analytes are polynucleotides.

[0030] In some embodiments, as exemplified in Figs. 3A-3C, donors are employed that are capable of FRET only within a predetermined proximity of a nanopore. In some embodiments, a predetermined proximity may be the same or greater than a dimension of a FRET zone; in other embodiments, a predetermined proximity may be less than the distance or half the distance to an adjacent nanopore. In some embodiments, a predetermined proximity is a radial distance from an exit or entrance of a bore of a nanopore which is less than 1 μm ; or which is less than 500 nm. Such

embodiments are advantageous in that background fluorescence from donors outside the proximity of apertures is reduced or eliminated. One such embodiment is illustrated for a protein nanopore (104 in Fig. 3A) in lipid bilayer (120) spanning an aperture (102) in solid phase membrane (100). In this embodiment, ion-sensitive donors (302-FRET-capable shown in white, and 304-FRET-inert, shown in black) are employed that are capable of FRET only under conditions of an ion concentration that exceeds a predetermined value. Locally elevated concentrations of a desired ion, such as calcium, Ca^{++} , may be created at an entrance or exit of a nanopore with initial conditions where a first chamber (310) (on one side of solid phase membrane (100)) has a concentration of ions below the predetermined value, for example, zero or substantially zero concentration, and a second chamber (320) has a concentration of ions above the predetermined value. Guidance in establishing and measuring such gradients is found in Anderson et al, ACS Nano, 8(11): 11836-11845 (2014), which is incorporated herein by reference. In some embodiments, first chamber (310) and second chamber (320) may correspond to a cis and trans orientation of electrical field (101), respectively. Average or bulk ion concentrations of the chambers may be maintained by using chelator compounds, e.g. EDTA for Ca^{++} . However, diffusion of ions from chamber two to chamber one will occur through nanopores, e.g. (104), or by the addition of ion-selective ionophores to aperture (102), so that locally elevated concentrations of ions (300) are created in the proximities of the exits of nanopores (104). A predetermined concentration of ions in chamber two is selected so that an elevated local concentration (300) renders lipid-bound ion-sensitive donors (304) capable of FRET whenever such donor is within a FRET distance (132) of a nanopore exit. Exemplary ion-sensitive donors include calcium ion-sensitive Fluor-4 or related dyes derivatized with a lipophilic anchor moiety using conventional techniques. One of ordinary skill in the art would understand that a gradient of any chemical activator of a fluorescent donor may be used in accordance with the above embodiments of the invention. Other chemical gradients include, but are not limited to, pH, electron accepting compounds or oxidizing agents, electron donating compounds or reducing agents, or developers or activators of fluorescent leuco dyes, and the like.

[0031] Fig. 3C illustrates another embodiment in which donor fluorescence is minimized outside a proximity of apertures. Lipid-sensitive and FRET-capable dyes (122a) are delivered from first chamber (310) by carrier (350) to lipid bilayer (120) in the proximity of nanopore (104), for example, defined in this example by the cross-sectional area of aperture (102). In the proximity of nanopore (104), dyes (122a) are illuminated by excitation beam (112) and undergo FRET with an acceptor within FRET distance (132), which generates fluorescent signal (114). Dye (122a), which may be bleached and inert after a FRET event (122b) diffuses out of proximity of aperture (102). Exemplary carriers (350) include cyclodextrin and like molecules.

1 [0032] The FRET distance (and therefore “FRET zone”) around a nanopore, may be defined
2 operationally such as the distance from a nanopore within which a FRET interaction occurs with
3 ninety-nine percent probability, or other selected value, for example, ninety percent, or ninety-five
4 percent.

6 Nanopores and Nanopore Arrays

7 [0033] Nanopores used with the invention may comprise solid-state nanopores, protein nanopores, or
8 hybrid nanopores comprising protein nanopores or organic nanotubes such as carbon nanotubes,
9 configured in a solid-state membrane, or like framework. In some embodiments, functions and
10 properties of nanopores include (i) constraining analytes, particularly polymer analytes, to pass through
11 a detection zone in sequence, or in other words, so that monomers pass a detection zone one at a time,
12 or in single file, (ii) compatibility with a translocating means (if one is used), that is, whatever method
13 is used to drive an analyte through a nanopore, such as an electric field, and optionally in some
14 embodiments, (iii) suppression of fluorescent signals within the lumen, or bore, of the nanopore. In
15 some embodiments, nanopores are used in connection with the methods and devices of the invention in
16 the form of arrays, either regular arrays, such as rectilinear arrays of a plurality nanopores in a planar
17 support or membrane, or random arrays, for example, where a plurality of nanopores are spaced in
18 accordance with a Poisson distribution in a planar support or membrane, or an array of clusters of
19 nanopores, which may be disposed regularly on a planar surface.

20 [0034] Solid phase membranes from which nanopores and/or nanopore arrays are constructed may be
21 fabricated in a variety of materials including but not limited to, silicon nitride (Si_3N_4), silicon dioxide
22 (SiO_2), Hafnium oxide (HfO_2), Titanium oxide (TiO_2), Aluminum oxide (Al_2O_3) or combinations
23 thereof and the like. The fabrication of nanopores and/or nanopore arrays and their operation (e.g.
24 sample handling systems, detection systems, and the like) for analytical applications, such as DNA
25 sequencing, are disclosed in the following exemplary references which are incorporated by reference:
26 Russell, U.S. patent 6,528,258; Feier, U.S. patent 4,161,690; Ling, U.S. patent 7,678,562; Hu et al,
27 U.S. patent 7,397,232; Golovchenko et al, U.S. patent 6,464,842; Chu et al, U.S. patent 5,798,042;
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2 Nature Nanotechnology, 2: 209-215 (2007); Storm et al, Nature Materials, 2: 537-540 (2003); Wu et al,
3 Electrophoresis, 29(13): 2754-2759 (2008); Nakane et al, Electrophoresis, 23: 2592-2601 (2002); Zhe
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5 (2004); Jagtiani et al, J. Micromech. Microeng., 16: 1530-1539 (2006); Nakane et al, J. Phys. Condens.
6 Matter, 15 R1365-R1393 (2003); DeBlois et al, Rev. Sci. Instruments, 41(7): 909-916 (1970); Clarke et
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8 and the like.

9 **[0035]** Briefly, in one aspect, a 1-50 nm channel is formed through a substrate, usually a membrane,
10 through which an analyte, such as DNA, is induced to translocate. The solid-state approach of
11 generating nanopores offers robustness and durability as well as the ability to tune the size and shape of
12 the nanopore, the ability to fabricate high-density arrays of nanopores on a wafer scale, superior
13 mechanical, chemical and thermal characteristics compared with systems solely employing lipid bilayer
14 supports, and the possibility of integrating with electronic or optical readout techniques. Biological
15 nanopores on the other hand provide reproducible narrow bores, or lumens, especially in the 1-10
16 nanometer range, as well as techniques for tailoring the physical and/or chemical properties of the
17 nanopore and for directly or indirectly attaching groups or elements, such as lipophilic anchoring
18 groups, fluorescent labels, which may be FRET donors or acceptors, or the like, by conventional
19 protein engineering methods. Protein nanopores typically rely on delicate lipid bilayers for mechanical
20 support, and the fabrication of solid-state nanopores with precise dimensions remains challenging.
21 Combining solid-state nanopores with a biological nanopore overcomes some of these shortcomings,
22 especially the precision of a biological pore protein with the stability of a solid state nanopore. For
23 optical read out techniques lipid hybrid nanopores provide a precise location of the nanopores by
24 deploying regularly spaced apertures which simplifies the data acquisition greatly by constraining the
25 lateral diffusion of the protein nanopores to the cross sections of the apertures (as illustrated, for
26 example, in Fig. 2C).

27 **[0036]** In some embodiments, a hydrophilic coating is optional in that the surface of the solid phase
28 membrane is sufficiently hydrophilic itself so that a lipid bilayer adheres to it stably. The at least one
29 aperture will have an inner surface, or wall, connected to, or contiguous with the surfaces of the solid
30 phase membrane. In some embodiments, the at least one aperture will be a plurality of apertures, and
31 the plurality of apertures may be arranged as a regular array, such as a rectilinear array of apertures, the
32 spacing of which depending in part on the number and kind of FRET pairs employed and the optical
33 detection system used. In particular, in some embodiments, a plurality of apertures is arranged in an
34 array of clusters of apertures. Each of the apertures has a diameter, which in some embodiments is

1 such that a protein nanopore is substantially immobilized therein. In some embodiments, substantially
2 immobilized means that a protein nanopore may move no more than 5 nm in the plane of the solid
3 phase membrane relative to the wall of the aperture. In another embodiment, substantially immobilized
4 means that a protein nanopore may move no more than 35 nm in the plane of the solid phase membrane
5 relative to the wall of the aperture. In yet another embodiment, substantially immobilized means that a
6 protein nanopore may move no more than 55 nm in the plane of the solid phase membrane relative to
7 the wall of the aperture. In yet another embodiment, substantially immobilized means that a protein
8 nanopore may move no more than 100 nm in the plane of the solid phase membrane relative to the wall
9 of the aperture. In yet another embodiment, substantially immobilized means that a protein nanopore
10 may move no more than 200 nm in the plane of the solid phase membrane relative to the wall of the
11 aperture. The protein nanopores each have a bore, or passage, or lumen, which permits fluid
12 communication between the first and second chambers when the protein nanopore is immobilized in an
13 aperture. Generally, the bore is coaxially aligned with the aperture. One function of the hydrophilic
14 layer is to provide a surface to retain a lipid bilayer which can span one or a multitude of apertures. The
15 hydrophilic layer can be on one or on both sides of the solid phase membrane. Such lipid bilayers, in
16 turn, permit disposition and immobilization of a protein nanopore within an aperture in a functional
17 conformation and in a manner that forms a seal with the wall of the aperture. In some embodiments,
18 such seal also prevents electrical current passing between the first and second chambers around the
19 protein nanopore. In some embodiments, charged analytes are disposed in an electrolyte solution in the
20 first chamber and are translocated through the bore(s) of the protein nanopore(s) into an electrolytic
21 solution in the second chamber by establishing an electrical field across the solid phase membrane. For
22 convenience of manufacture, in some embodiments the hydrophilic coating will be on one or both
23 surfaces of the solid phase membrane and the wall(s) of the aperture(s). Hydrophilic surfaces can be
24 generated by chemically modifying the solid support membrane by direct deposition of a layer of
25 hydrophilic reagents. Alternatively, the solid surface can be treated by a plasma which renders the
26 surface hydrophilic. In another embodiment a harsh acidic or basic treatment can render a surface such
27 as SiN or SiO₂ hydrophilic. By incorporating certain groups in the hydrophilic layer that specifically
28 bind to the lipids in the bilayer a so called tethered lipid bilayer with increased stability can be
29 generated. All of the above techniques are within the scope of this invention.

30 **[0037]** In some embodiments, the solid phase membrane may be treated with a low energy ion
31 beam to bleach its autofluorescence, e.g. as described in Huber et al, U.S. patent publication
32 2013/0203050, which is incorporated herein by reference.

33 **[0038]** In some embodiments, the present invention employs a hybrid nanopore, particularly for
34 optical-based nanopore sequencing of polynucleotides. Such embodiments comprise a solid-state

1 orifice, or aperture, into which a protein biosensor, such as a protein nanopore or any other
2 transmembrane protein is stably inserted.

3 **[0039]** Once stable hybrid nanopores have formed, single stranded, fluorescently labeled (or acceptor
4 labeled) DNA is added to the cis chamber. The applied electric field forces the negatively charged
5 ssDNA to translocate through the hybrid nanopore during which the labeled nucleotides get in close
6 vicinity of a donor fluorophore immersed in the lipid bilayer.

7 **[0040]** Solid state, or synthetic, nanopores may be prepared in a variety of ways, as exemplified in the
8 references cited above. In some embodiments a helium ion microscope may be used to drill the
9 synthetic nanopores in a variety of materials, e.g. as disclosed by Yang et al, Nanotechnology, 22:
10 285310 (2011), which is incorporated herein by reference. A chip that supports one or more regions of
11 a thin-film material, e.g. silicon nitride, that has been processed to be a free-standing membrane is
12 introduced to the helium ion microscope (HIM) chamber. HIM motor controls are used to bring a free-
13 standing membrane into the path of the ion beam while the microscope is set for low magnification.
14 Beam parameters including focus and stigmation are adjusted at a region adjacent to the free-standing
15 membrane, but on the solid substrate. Once the parameters have been properly fixed, the chip position
16 is moved such that the free-standing membrane region is centered on the ion beam scan region and the
17 beam is blanked. The HIM field of view is set to a dimension (in μm) that is sufficient to contain the
18 entire anticipated nanopore pattern and sufficient to be useful in future optical readout (i.e. dependent
19 on optical magnification, camera resolution, etc.). The ion beam is then rastered once through the entire
20 field of view at a pixel dwell time that results in a total ion dose sufficient to remove all or most of the
21 membrane autofluorescence. The field of view is then set to the proper value (smaller than that used
22 above) to perform lithographically-defined milling of either a single nanopore or an array of nanopores.
23 The pixel dwell time of the pattern is set to result in nanopores of one or more predetermined
24 diameters, determined through the use of a calibration sample prior to sample processing. This entire
25 process is repeated for each desired region on a single chip and/or for each chip introduced into the
26 HIM chamber.

27 **[0041]** In some embodiments, the solid-state substrate may be modified to generate active sites on the
28 surface to make it more suitable for a given application. Such modifications may be of covalent or non-
29 covalent nature. A covalent surface modification includes a silanization step where an organosilane
30 compound binds to silanol groups on the solid surface. For instance, the alkoxy groups of an
31 alkoxysilane are hydrolyzed to form silanol-containing species. Reaction of these silanes involves four
32 steps. Initially, hydrolysis of the labile groups occurs. Condensation to oligomers follows. The
33 oligomers then hydrogen bond with hydroxyl groups of the substrate. Finally, during drying or curing,
34 a covalent linkage is formed with the substrate with concomitant loss of water. For covalent attachment

organosilanes with active side groups may be employed. Such side groups consist of, but are not limited to epoxy side chain, aldehydes, isocyanates, isothiocyanates, azides or alkynes (click chemistry) to name a few. For someone skilled in the art it is obvious that multiple ways of covalently attaching a lipid bilayer to a surface to form a tethered lipid bilayer are possible. For instance, certain side groups on an organosilane may need to be activated before being capable of binding or integrating into a supported lipid bilayer. Another way of attaching a lipid bilayer to the solid surface may be achieved through affinity binding by having one affinity partner attached to the lipid moiety part of the lipid bilayer and the second affinity partner being located on the solid surface. Such affinity pairs consist of the group of, but are not limited to biotin-streptavidin, antigen-antibody and aptamers and the corresponding target molecules. In a preferred embodiment the surface modification of the solid state nanopore includes treatment with an organosilane that renders the surface hydrophilic. To form a lipid bilayer on a solid state membrane a vesicle fusion approach is the most common way of generating stable supported lipid bilayers. Lipids are dried *in vacuo* to remove any solvent (usually Chloroform) and the dried lipid is then rehydrated in an aqueous buffer solution such as 10mM MES pH 6.8, 150mM KCl, 2mM CaCl₂. Unilaminar vesicles are extruded through a 100-1000nm filter and applied to a hydrophilic surface where they burst and form a supported lipid bilayer.

Labeling Polymer Analytes

[0042] As mentioned above, in some embodiments, polymer analytes, such as DNA, may be labeled using “click chemistry,” e.g. using commercially available kits (such as “Click-It” from Life Technologies, Carlsbad, CA). Click chemistry in general refers to a synthetic process in which two molecules are linked together by a highly efficient chemical reaction, one which is essentially irreversible, in which the yield is nearly 100%, and which produces few or no reaction byproducts.. More recently, the meaning has come to refer to the cyclization reaction of a substituted alkyne with a substituted azide to form a 1,2,3-triazole bearing the two substituents. When catalyzed by copper at room temperature the reaction is known as the Huisgen cycloaddition, and it fully satisfies the requirements for click chemistry in that no other chemical functionality on the two molecules is affected during the reaction. Thus the coupling reaction has found broad application in bioconjugate chemistry, for example, in dye labeling of DNA or proteins, where many amine, hydroxy, or thiol groups may be found. The key requirement is that an alkyne group and an azide can easily be introduced into the molecules to be coupled. For example, in the coupling of a fluorescent dye to a DNA oligonucleotide, the azide group is typically introduced synthetically into the dye, while the alkyne group is incorporated into the DNA during oligonucleotide synthesis. Upon mixing in the presence of Cu⁺ the two components are quickly coupled to form the triazole, in this case bearing the

oligonucleotide as one substituent and the dye as the other. Another more recent advance provides the alkyne component within a strained ring structure. In this case the reaction with an azide does not require the copper catalyst, being driven by release of the ring strain energy as the triazole is formed. This is better known as the copper-free click reaction. Guidance for applying click chemistry to methods of the invention may be found in the following references which are incorporated by reference: Rostovtsev VV, Green LG, Fokin, Valery V, Sharpless KB (2002). "A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes". *Angewandte Chemie International Edition* **41** (14): 2596–2599. Moses JE and Moorhouse AD (2007). "The growing applications of click chemistry". *Chem. Soc. Rev.* **36** (8): 1249–1262.

[0043] A combination of 1, 2, 3 or 4 nucleotides in a nucleic acid strand may be exchanged with their labeled counterpart. The various combinations of labeled nucleotides can be sequenced in parallel, e.g., labeling a source nucleic acid or DNA with combinations of 2 labeled nucleotides in addition to the four single labeled samples, which will result in a total of 10 differently labeled sample nucleic acid molecules or DNAs (G, A, T, C, GA, GT, GC, AT, AC, TC). The resulting sequence pattern may allow for a more accurate sequence alignment due to overlapping nucleotide positions in the redundant sequence read-out. Guidance for labeling polynucleotides for optically based nanopore analysis is found in the following references which are incorporated by reference: Goodman et al, U.S. patent 5,945,312; Jett et al, U.S. patent 5,405,747; Muchlegger et al, U.S. patent publication US2004/0214221; Giller et al, *Nucleic Acids Research*, **31**(10): 2630-2635 (2003); Tasara et al, *Nucleic Acids Research*, **31**(10): 2636-2646 (2003); Augustin et al, *J. Biotechnology*, **86**: 289-301 (2001); Brakmann, *Current Pharmaceutical Biotechnology*, **5**(1): 119-126 (2004); and the like.

[0044] A method for sequencing a polymer, such as a nucleic acid molecule includes providing a nanopore or pore protein (or a synthetic pore) inserted in a membrane or membrane-like structure or other substrate. An electric field is applied to the nanopore which forces the labeled nucleic acid polymer through the nanopore, while an external monochromatic or other light source may be used to illuminate the nanopore, thereby exciting donors. As, after or before labeled nucleotides of the nucleic acid pass through, exit or enter the nanopore, energy is transferred from an adjacent donor to a nucleotide acceptor-label, which results in emission of lower energy radiation. The nucleotide label radiation is then detected by a confocal microscope setup or other optical detection system or light microscopy system capable of single molecule detection known to people having ordinary skill in the art. Examples of such detection systems include but are not limited to confocal microscopy, epifluorescent microscopy and total internal reflection fluorescent (TIRF) microscopy. Other polymers (e.g., proteins and polymers other than nucleic acids) having labeled monomers may also be sequenced according to the methods described herein. In some embodiments, fluorescent labels or donor

1 molecules are excited in a TIRF system with an evanescent wave, sometimes referred to herein as
2 “evanescent wave excitation.” Guidance for application of TIRF systems to nanopore-based devices is
3 found in the following references which are incorporated by reference: Soni et al, Review of Scientific
4 Instruments, 81: 014301 (2010); and U.S. patent publication 2012/0135410.

5 **[0045]** Each acceptor labeled monomer (e.g., nucleotide) of a polymer (e.g., nucleic acid) can interact
6 sequentially with a donor positioned next to the exit of a nanopore or channel through which the
7 polymer is translocated. The interaction between the donor and acceptor labels may take place outside
8 of the nanopore channel or opening, e.g., after the acceptor labeled monomer exits the nanopore. The
9 interaction may take place within or partially within the nanopore channel or opening, e.g., while the
10 acceptor labeled monomer passes through, or exits the nanopore.

11 **[0046]** In some embodiments, when one of the four nucleotides of a nucleic acid is labeled, the time-
12 dependent signal arising from the single nucleotide label emission is converted into a sequence
13 corresponding to the positions of the labeled nucleotides in the nucleic acid sequence. The process is
14 then repeated for each of the four nucleotides in separate samples and the four partial sequences are
15 then aligned to assemble an entire nucleic acid sequence.

16 **[0047]** In some embodiments, when multi-color labeled nucleic acid (DNA) sequences are analyzed,
17 the energy transfer from one or more donor labels to each of the four distinct acceptor labels that may
18 exist on a nucleic acid molecule may result in light emission at four distinct wavelengths or colors
19 (each associated with one of the four nucleotides) which allows for a direct sequence read-out.

20 21 Exemplary Lipophilic Donors

22 **[0048]** As mentioned above, a feature of the invention is the use of donors or FRET pairs that are
23 constrained to and mobile within a lipid bilayer, either by complete immersion or by way of a lipophilic
24 anchor moiety. In some embodiments, donors (referred to herein as “mobile donors”) are constrained
25 and mobile within a lipid bilayer. Such mobile donors may be hydrophobic and immersed in the lipid
26 portion of a lipid bilayer or mobile donors may be amphiphilic or hydrophilic and constrained to a lipid
27 bilayer by a lipophilic anchor moiety. Exemplary donors include, but are not limited to, fluorescein
28 dyes with lipophilic anchor moieties, rhodamine dyes with lipophilic anchor moieties, Cy3 dyes with
29 lipophilic anchor moieties, Alexa dyes with lipophilic anchor moieties, quantum dots derivatized with
30 lipophilic anchor moieties, nanodiamonds derivatized with lipophilic anchor moieties, and the like. A
31 wide selection of suitable lipophilic dyes, or membrane probes, are available commercially, e.g.
32 Molecular Probes, Inc. or Sigma-Aldrich Co.

33 34 Translocation Speed

1 **[0049]** An obstacle associated with nanopore based sequencing approaches has been the high
2 translocation velocity of nucleic acid through a nanopore (~500,000 – 1,000,000 nucleotides/sec) which
3 doesn't allow for direct sequence readout due to the limited bandwidth of the recording equipment. A
4 way of slowing down the nucleic acid translocation with two different nanopore proteins was recently
5 shown by Cherf et al. (Nat Biotechnol. 2012 Feb 14; 30(4):344-8) and Manrao et al. (Nat Biotechnol.
6 2012 Mar 25; 30(4):349-53) and are incorporated herein by reference. Both groups used a DNA
7 polymerase to synthesize a complementary strand from a target template which resulted in the step-
8 wise translocation of the template DNA through the nanopore. Hence, the synthesis speed of the
9 nucleic acid polymerase (10-500 nucleotides/sec) determined the translocation speed of the DNA and
10 since it's roughly 3-4 orders of magnitude slower than direct nucleic acid translocation the analysis of
11 single nucleotides became feasible. However, the polymerase-aided translocation requires significant
12 sample preparation to generate a binding site for the polymerase and the nucleic acid synthesis has to
13 be blocked in bulk and can only start once the nucleic acid-polymerase complex is captured by the
14 nanopore protein. This results in a rather complex set-up which might prevent the implementation in a
15 commercial setting. Furthermore, fluctuation in polymerase synthesis reactions such as a stalled
16 polymerization as well as the dissociation of the polymerase from the nucleic acid may hamper the
17 sequence read-out resulting in a high error rate and reduced read-length, respectively. Optical nanopore
18 sequencing as described in this application uses a different way of slowing down the DNA
19 translocation. A target nucleic acid is enzymatically copied by incorporating fluorescent modified
20 nucleotides. The resulting labeled nucleic acid has an increased nominal diameter which results in a
21 decreased translocation velocity when pulled through a nanopore. The preferred translocation rate for
22 optical sequencing lies in the range of 1-1000 nucleotides per second with a more preferred range of
23 200-800 nucleotides per second and a most preferred translocation rate of 200-600 nucleotides per
24 second.

25 **[0050]** Alternatively, translocation speed of a polynucleotide, especially a single stranded
26 polynucleotide, may be controlled by employing a nanopore dimensioned so that adducts and/or labels,
27 e.g. organic dyes attached to bases, inhibit but do not prevent polynucleotide translocation. A
28 translocation speed may be selected by attaching labels and/or adducts at a predetermined density.
29 Such labels and/or adducts may have regular spaced attachments, e.g. every third nucleotide or the like,
30 or they may have random, or pseudorandom attachments, e.g. every C may be labeled. In some
31 embodiments, a selected number of different nucleotides may be labeled, e.g. every A and C, or every
32 A and G, or every A and T, or every C, or the like, that results in an average translocation speed. Such
33 average speed may be decreased by attaching adducts to unlabeled nucleotides. Adducts include any
34 molecule, usually an organic molecule, that may be attached to a nucleotide using conventional

1 chemistries. Typically adducts have a molecular weight in the same range as common organic dyes,
2 e.g. fluorescein, Cy3, or the like. Adducts may or may not be capable of generating signals, that is,
3 serving as a label. In some embodiments, adducts and/or labels are attached to bases of nucleotides. In
4 other embodiments, labels and/or adducts may be attached to linkages between nucleosides in a
5 polynucleotide. In one aspect, a method of controlling translocation velocity of a single stranded
6 polynucleotide through a nanopore comprises the step of attaching adducts to the polynucleotide at a
7 density, wherein translocation velocity of the single stranded polynucleotide monotonically decreases
8 with a larger number of adducts attached, or with the density of adducts attached. In some
9 embodiments, not every kind of nucleotide of a polynucleotide is labeled. For example, four different
10 sets of a polynucleotide may be produced where nucleotides of each set are labeled with the same
11 molecule, e.g. a fluorescent organic dye acceptor, but in each set a different kind of nucleotide will be
12 labeled. Thus, in set 1 only A's may be labeled; in set 2 only C's may be labeled; in set 3 only G's may
13 be labeled; and so on. After such labeling, the four sets of polynucleotides may then be analyzed
14 separately in accordance with the invention and a nucleotide sequence of the polynucleotide
15 determined from the data generated in the four analyses. In such embodiments, and similar
16 embodiments, e.g. two labels are used, where some of the nucleotides of a polynucleotide are not
17 labeled, translocation speed through a nanopore will be affected by the distribution of label along the
18 polynucleotide. To prevent such variability in translocation speed, in some embodiments, nucleotides
19 that are not labeled with an acceptor or donor for generating signals to determine nucleotide sequence,
20 may be modified by attaching a non-signal-producing adduct that has substantially the same effect on
21 translocation speed as the signal-producing labels.

Nanopore Sequencing

24 **[0051]** In some embodiments, the invention is directed to the use of nanopores and fluorescence
25 resonance energy transfer (FRET) to sequentially identify monomers of polymer analytes, such as a
26 polynucleotide. Such analysis of polymer analytes may be carried out on single polymer analytes or on
27 pluralities of polymer analytes in parallel at the same time, for example, using an array of nanopores.

28 **[0052]** In some embodiments, methods of determining a sequence of labeled monomers of a
29 polymer comprise steps of: (a) providing a device comprising: (i) a solid phase membrane separating
30 a first chamber and a second chamber, the solid phase membrane having at least one aperture
31 connecting the first chamber and the second chamber, and the solid phase membrane having a lipid
32 bilayer on at least one surface; (ii) a protein nanopore immobilized in the aperture, the protein nanopore
33 having a bore, the protein nanopore contacting and extending through the lipid bilayer so that the first
34 chamber and the second chamber are in fluid communication through the bore; and (iii) donors of one

1 or more FRET pairs diffusably disposed in the lipid bilayer; (b) translocating the polynucleotide
2 through the protein nanopore so that acceptor labels attached to the polynucleotide passes sequentially
3 therethrough and so that whenever an acceptor label exits the bore of the protein nanopore at least one
4 donor with a predetermined likelihood is within a FRET distance of such acceptor label; and (c)
5 determining a nucleotide sequence of the polynucleotide by a sequence of FRET interactions between
6 donors and acceptor labels exiting the bore of the protein nanopore. In some embodiments, such
7 predetermined likelihood is a likelihood of at least ninety percent; in other embodiments, such
8 predetermined likelihood is a likelihood of at least ninety-nine percent; in other embodiments, such
9 predetermined likelihood is a likelihood of at least 99.9 percent.

10 **[0053]** A likelihood that a FRET interaction will occur when an acceptor exits (or enters—
11 depending on embodiment) a nanopore depends in part on whether an excited donor happens to be
12 within a FRET distance of the acceptor at the time of exit (or during the time interval when the
13 acceptor move through a FRET zone (“FRET interval”). Thus, the likelihood of a FRET interaction
14 occurring depends on the likelihood of an excited donor being in the FRET zone or entering the FRET
15 zone during a FRET interval of a translocating acceptor. The latter likelihood depends on the
16 concentration and mobility of donors in the lipid bilayer. Both of these quantities are design selections
17 of a practitioner of the invention. A higher concentration and greater mobility leads to donors entering
18 a FRET zone more frequently; and conversely, lower concentration and lower mobility leads to donors
19 entering a FRET zone at a lower frequency. In some embodiments, these parameters are selected so
20 that the frequency of new (i.e. non-degraded, non-bleached) donors entering a FRET zone is equal to or
21 greater than the frequency with which acceptors translocate the same FRET zone. In other
22 embodiments, such as where donors and FRET zones are intermittently illuminated with alternating
23 light and dark cycles, lower concentrations and mobilities may be selected and durations of dark cycles
24 selected to permit replenishment of donors in FRET zones.

25 **[0054]** In other embodiments, devices and methods of the invention may use the property of
26 protein nanopores to dampen or suppress fluorescence of translocating fluorescently labeled polymer,
27 such as a fluorescently labeled polynucleotide. In such embodiments, monomers are labeled with
28 fluorescent labels that are capable of at least three states while attached to a target polymer: (i) A
29 quenched state wherein fluorescence of an attached fluorescent label is quenched by a fluorescent label
30 on an immediately adjacent monomer; for example, a fluorescent label attached to a polymer in
31 accordance with the invention is quenched when the labeled polymer is free in an aqueous solution. (ii)
32 A sterically constrained state wherein a labeled polymer is translocating through a nanopore such that
33 the free-solution movements or alignments of an attached fluorescent label is disrupted or limited so
34 that there is little or no detectable signal generated from the fluorescent label. (iii) A transition state

wherein a fluorescent label attached to a polymer transitions from the sterically constrained state to the quenched state as the fluorescent label exits the nanopore (during a “transition interval”) while the polymer translocates through the nanopore. In part, this embodiment is an application of the discovery that during the transition interval a fluorescent label is capable of generating a detectable fluorescent signal. Without the intention of being limited by any theory underlying this discovery, it is believed that the fluorescent signal generated during the transition interval is due to a freely rotatable dipole. In both, the sterically constrained state as well as the quenched state the dipoles are limited in their rotational freedom thereby reducing or limiting the number of emitted photons. In some embodiments, the translocating labeled polymer is a polynucleotide, usually a single stranded polynucleotide, such as, DNA or RNA, but especially DNA. In some embodiments, the invention includes a method for determining a nucleotide sequence of a polynucleotide by recording signals generated by attached fluorescent labels as they exit a nanopore one at a time as a polynucleotide translocates the nanopore. Upon exit, each attached fluorescent label transitions during a transition interval from a constrained state in the nanopore to a quenched state on the polynucleotide in free solution. As mentioned above, during this transition interval or period the fluorescent label is capable of emitting a detectable fluorescent signal indicative of the nucleotide it is attached to.

[0055] In some embodiments, a nucleotide sequence of a target polynucleotide is determined by carrying out four separate reactions in which copies of the target polynucleotide have each of its four different kinds of nucleotide (A, C, G and T) labeled with a single fluorescent label. In a variant of such embodiments, a nucleotide sequence of a target polynucleotide is determined by carrying out four separate reactions in which copies of the target polynucleotide have each of its four different kinds of nucleotide (A, C, G and T) labeled with one fluorescent label while at the same time the other nucleotides on the same target polynucleotide are labeled with a second fluorescent label. For example, if a first fluorescent label is attached to A’s of the target polynucleotide in a first reaction, then a second fluorescent label is attached to C’s, G’s and T’s (i.e. to the “not-A” nucleotides) of the target polynucleotides in the first reaction. Likewise, in continuance of the example, in a second reaction, the first label is attached to C’s of the target polynucleotide and the second fluorescent label is attached to A’s, G’s and T’s (i.e. to the “not-C” nucleotides) of the target polynucleotide. And so on, for nucleotides G and T.

[0056] The same labeling scheme may be expressed in terms of conventional terminology for subsets of nucleotide types; thus, in the above example, in a first reaction, a first fluorescent label is attached to A’s and a second fluorescent label is attached to B’s; in a second reaction, a first fluorescent label is attached to C’s and a second fluorescent label is attached to D’s; in a third reaction, a first

1 fluorescent label is attached to G's and a second fluorescent label is attached to H's; and in a fourth
2 reaction, a first fluorescent label is attached to T's and a second fluorescent label is attached to V's.

3 **[0057]** In some embodiments, a feature of the invention is the labeling of substantially all
4 monomers of a polymer analyte with fluorescent dyes or labels that are members of a mutually
5 quenching set. Such sets of fluorescent dyes have the following properties: (i) each member quenches
6 fluorescence of every member (for example, by FRET or by static or contact mechanisms), and (ii)
7 each member generates a distinct fluorescent signal when excited and when in a non-quenching state.
8 That is, if a mutually quenching set consists of two dyes, D1 and D2, then (i) D1 is self-quenched (e.g.
9 by contact quenching with another D1 molecule) and it is quenched by D2 (e.g. by contact quenching)
10 and (ii) D2 is self-quenched (e.g. by contact quenching with another D2 molecule) and it is quenched
11 by D1 (e.g. by contact quenching). Guidance for selecting fluorescent dyes or labels for mutually
12 quenching sets may be found in the following references, which are incorporated herein by reference:
13 Johansson, *Methods in Molecular Biology*, 335: 17-29 (2006); Marras et al, *Nucleic Acids Research*,
14 30: e122 (2002); and the like. Exemplary mutually quenching sets of fluorescent dyes, or labels, may
15 be selected from rhodamine dyes, fluorescein dyes and cyanine dyes. In one embodiment, a mutually
16 quenching set may comprise the rhodamine dye, TAMRA, and the fluorescein dye, FAM. In another
17 embodiment, mutually quenching sets of fluorescent dyes may be formed by selecting two or more
18 dyes from the group consisting of Oregon Green 488, Fluorescein-EX, fluorescein isothiocyanate,
19 Rhodamine Red-X, Lissamine rhodamine B, Calcein, Fluorescein, Rhodamine, one or more BODIPY
20 dyes, Texas Red, Oregon Green 514, and one or more Alexa Fluors. Representative BODIPY dyes
21 include BODIPY FL, BODIPY R6G, BODIPY TMR, BODIPY 581/591, BODIPY TR, BODIPY
22 630/650 and BODIPY 650/665. Representative Alexa Fluors include Alexa Fluor 350, 405, 430, 488,
23 500, 514, 532, 546, 555, 568, 594, 610, 633, 635, 647, 660, 680, 700, 750 and 790.

24 **[0058]** A wide range of embodiments of the above may be implemented depending on the type of
25 analytes being detected, the types of donors and acceptors employed, the physical arrangement of the
26 nanopores, donors and acceptors, whether analytes are labeled with donors or with acceptors, and the
27 like. In one embodiment, analytes measured by the invention are acceptor-labeled polymers, especially
28 acceptor-labeled polynucleotides. In one species of the latter embodiment, different nucleotides of a
29 polynucleotide analyte are labeled with one or more different kinds of acceptors, so that a nucleotide
30 sequence of the polynucleotide may be determined from measuring FRET signals generated as it
31 translocates through a nanopore. In another embodiment, analytes measured by the invention are
32 donor-labeled polymers, especially donor-labeled polynucleotides. The sequence of the polynucleotide
33 may be determined from measuring FRET signals as it translocates through a nanopore. In yet another
34 embodiment of the present invention, at least one of the four nucleotides of a polynucleotide analyte is

1 labeled with a member of a FRET pair. The positions of the labeled nucleotides in the polynucleotide
2 are determined by translocating the labeled polynucleotide through a labeled nanopore and measuring
3 FRET events. By labeling the remaining nucleotides of the same polynucleotide sample and
4 subsequently translocating said samples through a labeled nanopore, sub-sequences of the
5 polynucleotide are generated. Such sub-sequences can be re-aligned resulting in a full sequence of the
6 polynucleotide.

7 **[0059]** In another embodiment the invention is directed to a device for analyzing one or more labeled
8 polymer analytes, such as a device for determining a nucleotide sequence of one or more labeled
9 polynucleotide analytes, such device comprising the following elements: (a) a solid phase membrane
10 separating a first chamber and a second chamber, the solid phase membrane having an array of
11 nanopores each fluidly connecting the first chamber and the second chamber through a bore or lumen,
12 the bore or lumen having a cross-sectional dimension such that labels of a labeled polymer
13 translocating therethrough are sterically constrained so that detectable signals are not generated, and so
14 that the labels of adjacent monomers of the labeled polymer are self-quenching; (b) an excitation
15 source for exciting each label when it exits each nanopore and enters the second chamber so that a
16 signal is generated indicative of a monomer to which the label is attached; and (c) a detector for
17 collecting at least a portion of the signal generated by each excited label; and (d) identifying the
18 monomer to which the excited label is attached by the collected signal whenever emitted from a
19 sequence-able nanopore; and wherein the array of nanopores is an array of clusters of nanopores.

20 **[0060]** In another embodiment, the invention is directed to a system for analyzing polymers
21 comprising monomers that are substantially all labeled with a mutually quenching dye set and a
22 nanopore device for sequentially detecting optical signals from the dyes of the mutually quenching dye
23 set which are attached to the polymer. Such an embodiment for determining a sequence of a
24 polynucleotide may comprise the following elements: (a) a solid phase membrane separating a first
25 chamber and a second chamber, the solid phase membrane having an array of apertures each
26 connecting the first chamber and the second chamber, and having a hydrophilic coating on at least one
27 surface; (b) a lipid bilayer disposed on the hydrophilic coating; (c) protein nanopores immobilized in
28 the aperture-spanning lipid bilayer, the protein nanopores each having a bore with an exit, and the
29 protein nanopores interacting with the lipid bilayer to form a seal with the solid phase membrane in the
30 apertures so that fluid communication between the first chamber and the second chamber occurs solely
31 through the bore of the protein nanopore, and the protein nanopores each being cross-sectionally
32 dimensioned so that nucleotides of the polynucleotide pass through the exit of the bore in sequence
33 and so that fluorescent labels attached to the polynucleotide are sterically constrained; and (d) a first
34 member of the FRET pair attached to the solid phase membrane or the protein nanopore, so that

1 whenever nucleotides of the polynucleotide emerge from the bore, a plurality of the nucleotides are
2 within a FRET distance of the first member of the FRET pair; and wherein the array of apertures is an
3 array of clusters of apertures.

4 **[0061]** This disclosure is not intended to be limited to the scope of the particular forms set forth,
5 but is intended to cover alternatives, modifications, and equivalents of the variations described herein.
6 Further, the scope of the disclosure fully encompasses other variations that may become obvious to
7 those skilled in the art in view of this disclosure. The scope of the present invention is limited only by
8 the appended claims.

10 Definitions

11 **[0062]** “Evanescent field” means a non-propagating electromagnetic field; that is, it is an
12 electromagnetic field in which the average value of the Poynting vector is zero.

13 **[0063]** “FRET” or “Förster, or fluorescence, resonant energy transfer” means a non-radiative
14 dipole-dipole energy transfer mechanism from a donor to acceptor fluorophore. The efficiency of
15 FRET may be dependent upon the distance between donor and acceptor as well as the properties of the
16 fluorophores (Stryer, L., Annu Rev Biochem. 47 (1978): 819–846). “FRET distance” means a distance
17 between a FRET donor and a FRET acceptor over which a FRET interaction can take place and a
18 detectable FRET signal produced by the FRET acceptor.

19 **[0064]** “Nanopore” means any opening positioned in a substrate that allows the passage of analytes
20 through the substrate in a predetermined or discernable order, or in the case of polymer analytes,
21 passage of their monomeric units through the substrate in a predetermined or discernable order. In the
22 latter case, a predetermined or discernable order may be the primary sequence of monomeric units in
23 the polymer. Examples of nanopores include proteinaceous or protein based nanopores, synthetic or
24 solid state nanopores, and hybrid nanopores comprising a solid state nanopore having a protein
25 nanopore embedded therein. A nanopore may have an inner diameter of 1-10 nm or 1-5 nm or 1-3 nm.
26 Examples of protein nanopores include but are not limited to, alpha-hemolysin, voltage-dependent
27 mitochondrial porin (VDAC), OmpF, OmpC, MspA and LamB (maltoporin), e.g. disclosed in Rhee,
28 M. et al., Trends in Biotechnology, 25(4) (2007): 174-181; Bayley et al (cited above); Gundlach et al,
29 U.S. patent publication 2012/0055792; and the like, which are incorporated herein by reference. Any
30 protein pore that allows the translocation of single nucleic acid molecules may be employed. A
31 nanopore protein may be labeled at a specific site on the exterior of the pore, or at a specific site on the
32 exterior of one or more monomer units making up the pore forming protein. Pore proteins are chosen
33 from a group of proteins such as, but not limited to, alpha-hemolysin, MspA, voltage-dependent
34 mitochondrial porin (VDAC), Anthrax porin, OmpF, OmpC and LamB (maltoporin).. A synthetic

nanopore, or solid-state nanopore, may be created in various forms of solid substrates, examples of which include but are not limited to silicones (e.g. Si_3N_4 , SiO_2), metals, metal oxides (e.g. Al_2O_3) plastics, glass, semiconductor material, and combinations thereof. A synthetic nanopore may be more stable than a biological protein pore positioned in a lipid bilayer membrane. A synthetic nanopore may also be created by using a carbon nanotube embedded in a suitable substrate such as but not limited to polymerized epoxy. Carbon nanotubes can have uniform and well-defined chemical and structural properties. Various sized carbon nanotubes can be obtained, ranging from one to hundreds of nanometers. The surface charge of a carbon nanotube is known to be about zero, and as a result, electrophoretic transport of a nucleic acid through the nanopore becomes simple and predictable (Ito, T. et al., Chem. Commun. 12 (2003): 1482-83). The substrate surface of a synthetic nanopore may be chemically modified to allow for covalent attachment of the protein pore or to render the surface properties suitable for optical nanopore sequencing. Such surface modifications can be covalent or non-covalent. Most covalent modification include an organosilane deposition for which the most common protocols are described: 1) Deposition from aqueous alcohol. This is the most facile method for preparing silylated surfaces. A 95% ethanol-5% water solution is adjusted to pH 4.5-5.5 with acetic acid. Silane is added with stirring to yield a 2% final concentration. After hydrolysis and silanol group formation the substrate is added for 2-5min. After rinsed free of excess materials by dipping briefly in ethanol. Cure of the silane layer is for 5-10min at 110 degrees Celsius. 2) Vapor Phase Deposition. Silanes can be applied to substrates under dry aprotic conditions by chemical vapor deposition methods. These methods favor monolayer deposition. In closed chamber designs, substrates are heated to sufficient temperature to achieve 5mm vapor pressure. Alternatively, vacuum can be applied until silane evaporation is observed. 3) Spin-on deposition. Spin-on applications can be made under hydrolytic conditions which favor maximum functionalization and polylayer deposition or dry conditions which favor monolayer deposition.

[0065] “Polynucleotide” or “oligonucleotide” are used interchangeably and each mean a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include PNAs, phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill

1 would understand that oligonucleotides or polynucleotides in those instances would not contain certain
2 analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions.
3 Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually
4 referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or
5 oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it
6 will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes
7 deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine,
8 "U" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context.
9 Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed
10 in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually
11 polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine,
12 deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by
13 phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g.
14 including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that
15 where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity,
16 e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for
17 the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill,
18 especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition
19 (Cold Spring Harbor Laboratory, New York, 1989), and like references. Likewise, the oligonucleotide
20 and polynucleotide may refer to either a single stranded form or a double stranded form (i.e. duplexes
21 of an oligonucleotide or polynucleotide and its respective complement). It will be clear to one of
22 ordinary skill which form or whether both forms are intended from the context of the terms usage.

23 **[0066]** "Sequence determination", "sequencing" or "determining a nucleotide sequence" or like
24 terms in reference to polynucleotides includes determination of partial as well as full sequence
25 information of the polynucleotide. That is, the terms include sequences of subsets of the full set of four
26 natural nucleotides, A, C, G and T, such as, for example, a sequence of just A's and C's of a target
27 polynucleotide. That is, the terms include the determination of the identities, ordering, and locations of
28 one, two, three or all of the four types of nucleotides within a target polynucleotide. In some
29 embodiments, the terms include the determination of the identities, ordering, and locations of two, three
30 or all of the four types of nucleotides within a target polynucleotide. In some embodiments sequence
31 determination may be accomplished by identifying the ordering and locations of a single type of
32 nucleotide, e.g. cytosines, within the target polynucleotide "catcgcc . . ." so that its sequence is
33 represented as a binary code, e.g. "100101 . . ." representing "c-(not c)(not c)c-(not c)-c . . ." and the
34 like. In some embodiments, the terms may also include subsequences of a target polynucleotide that

- 1 serve as a fingerprint for the target polynucleotide; that is, subsequences that uniquely identify a target
- 2 polynucleotide within a set of polynucleotides, e.g. all different RNA sequences expressed by a cell.

CLAIMS

What is claimed is:

1. A device for detecting an analyte, the device comprising:

a solid phase membrane separating a first chamber and a second chamber, the solid phase membrane having at least one aperture connecting the first chamber and the second chamber

a lipid bilayer disposed on at least one surface of the solid phase membrane, the lipid bilayer comprising a concentration of donors of a fluorescence resonance energy transfer (FRET) pair, the donors being mobile within the lipid bilayer; and

at least one nanopore immobilized in the aperture, each nanopore having a bore with an entrance and an exit and the nanopore interacting with the lipid layer so that fluid communication between the first chamber and the second chamber occurs solely through one or more bores of the at least one nanopore;

wherein the concentration of donors in or on the lipid bilayer is selected so that whenever an analyte labeled with an acceptor of the FRET pair exits or enters the bore of the nanopore with a predetermined likelihood at least one donor is within a FRET distance of the acceptor.

2. The device of claim 1 wherein said nanopore is a protein nanopore.

3. The device of claim 2 wherein said concentration of donors in said lipid bilayer is selected so that an expected nearest neighbor distance between said bore of said nanopore and said at least one donor is less than or equal to said FRET distance.

4. The device of claim 1 wherein said concentration of donors is selected so that an expected frequency of donors coming within a FRET distance of said entrance or said exit of said bore is equal to or greater than a frequency with which said acceptors enter or exit said bore, respectively.

5. The device of claim 4 wherein said concentration of donors is selected so that an expected frequency of donors coming within a FRET distance of said entrance or said exit of said bore is at least ten times greater than a frequency with which said acceptors enter or exit said bore, respectively.

6. The device of claim 1 wherein said lipid bilayer is disposed on a trans surface of said solid phase membrane and said exit of said bore is located on the trans surface of said solid phase membrane and

1 wherein said concentration of said donors is selected so that whenever said analyte labeled with said
2 acceptor exits said bore with said predetermined likelihood at least one donor is within said FRET
3 distance of said acceptor.

4
5 7. The device of claim 1 wherein said at least one nanopore is a single nanopore.

6
7 8. The device of claim 7 wherein said single nanopore is a protein nanopore.

8
9 9. The device of claim 1 wherein said donors in said lipid bilayer are capable of FRET only in a
10 proximity of said at least nanopore.

11
12 10. The device of claim 9 wherein said proximity is an area encompassing a radial distance of at most
13 1 μm from said at least one nanopore.

14
15 11. The device of claim 9 wherein said donors in said lipid bilayer are substantially non-fluorescent
16 outside a proximity of said at least one nanopore.

17
18 12. The device of claim 9 wherein said donors are activator-sensitive dyes that are capable of FRET
19 only in the presence of a predetermined concentration of a chemical activator.

20
21 13. The device of claim 12 wherein said first chamber has substantially a zero concentration of
22 chemical activator and said second chamber has a concentration of chemical activator equal to or
23 greater than the predetermined concentration and wherein the chemical activators of the second
24 chamber diffuse through said bores of said nanopores and create at said exit of each of said nanopores a
25 concentration gradient in the proximity thereof.

26
27 14. The device of claim 13 where said concentration of chemical activator in said second chamber is
28 selected so that said concentration of said chemical activator within a FRET distance of at least one of
29 said exits renders said donors capable of FRET within said FRET distance.

30
31 15. The device of claim 14 wherein said chemical activator is a selected ion, pH, an oxidizing agent, or
32 a reducing agent.

16. The device of claim 15 wherein said chemical activator is a selected ion and said activator-sensitive dye is an ion-sensitive dye.

17. A method of determining a nucleotide sequence of a polynucleotide, the method comprising the steps of:

(a) providing a device comprising:

(i) a solid phase membrane separating a first chamber and a second chamber, the solid phase membrane having at least one aperture connecting the first chamber and the second chamber, and the solid phase membrane having a lipid bilayer on at least one surface;

(ii) a protein nanopore immobilized in the aperture, the protein nanopore having a bore, the protein nanopore contacting and extending through the lipid bilayer so that the first chamber and the second chamber are in fluid communication through the bore; and

(iii) donors of one or more FRET pairs diffusably disposed in or on the lipid bilayer;

(b) translocating the polynucleotide through the protein nanopore so that acceptor labels attached to the polynucleotide passes sequentially therethrough and so that whenever an acceptor label exits the bore of the protein nanopore at least one donor with a predetermined likelihood is within a FRET distance of such acceptor label; and

(c) determining a nucleotide sequence of the polynucleotide by a sequence of FRET interactions between donors and acceptor labels exiting the bore or the protein nanopore.

18. The method of claim 17 wherein said donors are disposed in said lipid bilayer at a concentration selected so that a frequency of said acceptor labels exiting said bore of said protein nanopore is less than or equal to a frequency of said donors coming within a FRET distance of said exit of said bore.

19. A method of determining a nucleotide sequence of a polynucleotide, the method comprising the steps of:

translocating a polynucleotide through a protein nanopore, the polynucleotide having monomers labeled with acceptors of one or more fluorescent resonance energy transfer (FRET) pairs and the protein nanopore being immobilized in an aperture through a solid phase membrane, wherein a surface of the solid phase membrane has a lipid bilayer containing concentrations of donors movably disposed therein and the protein nanopore has a bore and contacts and extends through the lipid bilayer so that the first chamber and the second chamber are in fluid communication through the bore, and wherein the concentrations of donors are selected so that whenever a monomer of the polynucleotide

1 having an acceptor attached traverses the bore, such acceptor passes within a FRET distance of at least
2 one donor of its FRET pair to generate a FRET interaction; and

3 determining a nucleotide sequence of the polynucleotide by a sequence of FRET interactions.
4

5 20. The method of claim 19 wherein said monomers are labeled with at least two FRET pairs
6 comprising at least two different acceptors that generate distinguishable FRET signals.
7

8 21. The method of claim 20 wherein said at least two FRET pairs comprise at least two FRET donors.
9

10 22. The method of claim 19 wherein concentrations of said donors and said translocation speeds are
11 selected so that so that whenever said monomer labeled with an acceptor of a FRET pair exits or enters
12 the bore of said nanopore at least one donor is within a FRET distance of the acceptor with a likelihood
13 of at least ninety-nine percent.
14
15

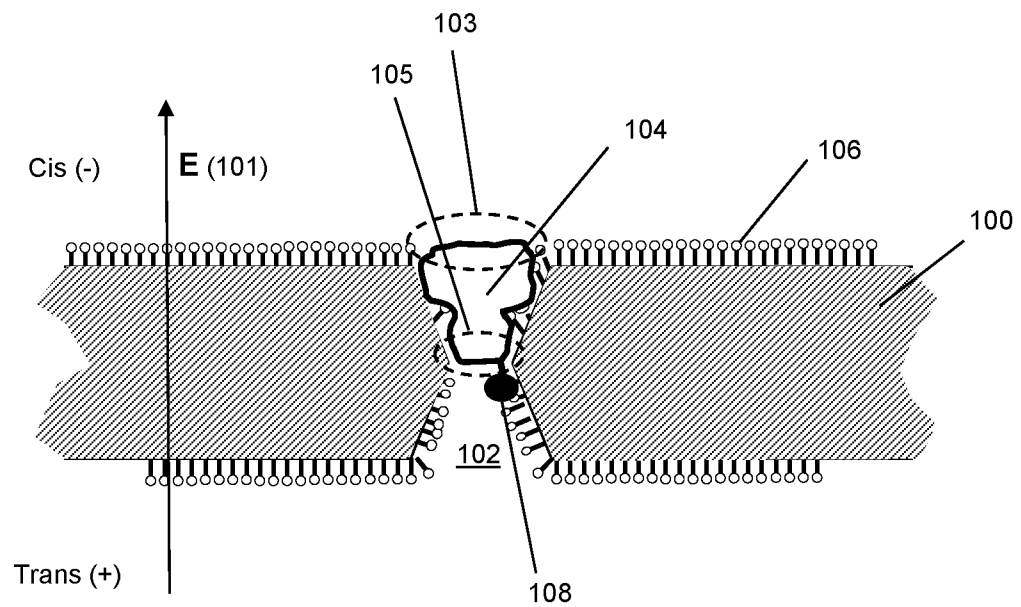


Fig. 1A

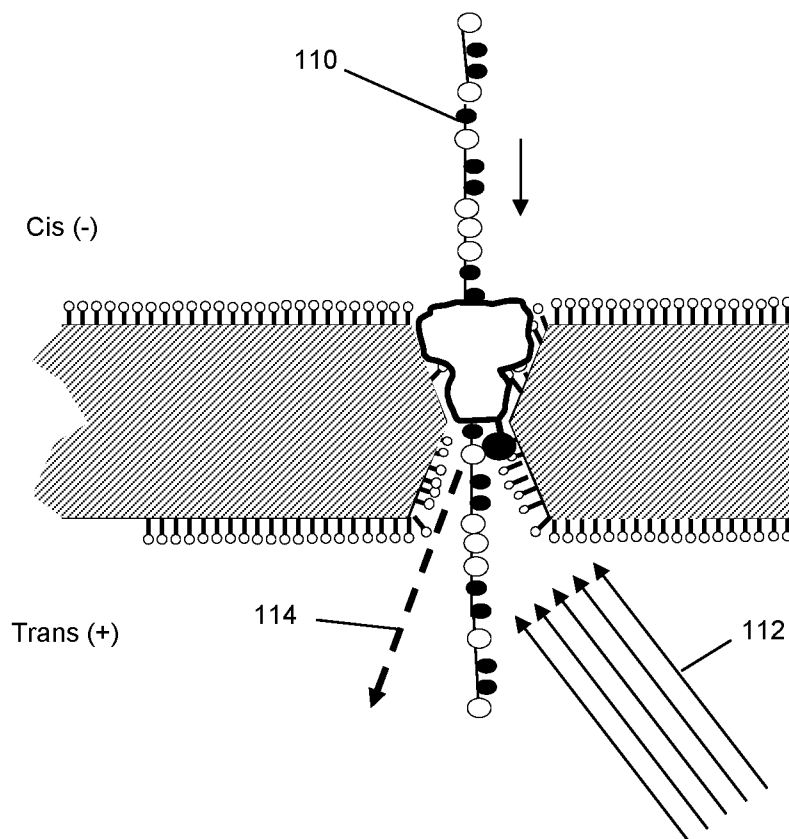


Fig. 1B

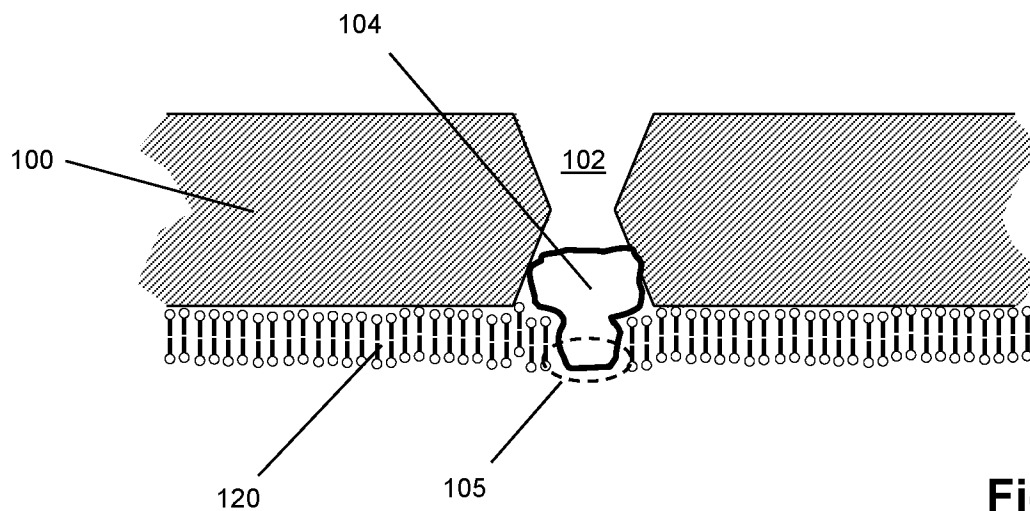


Fig. 1C

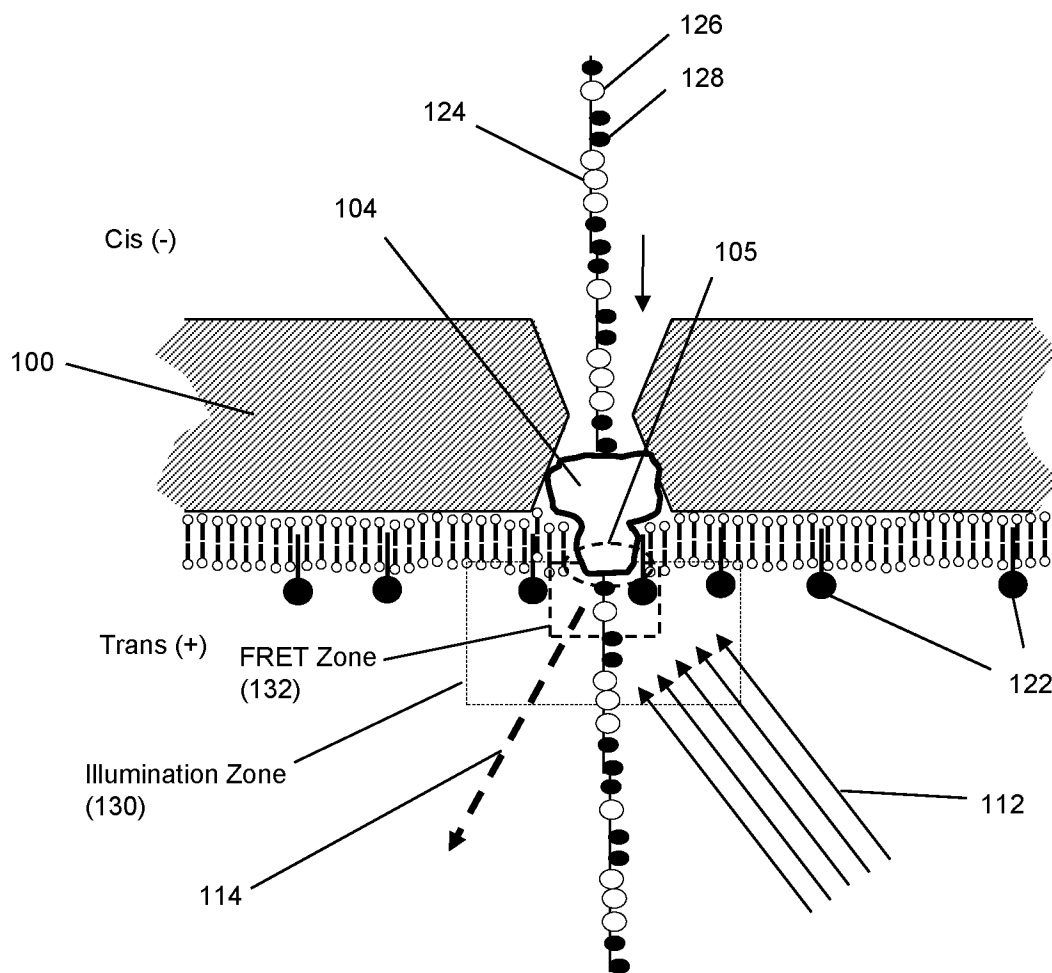


Fig. 1D

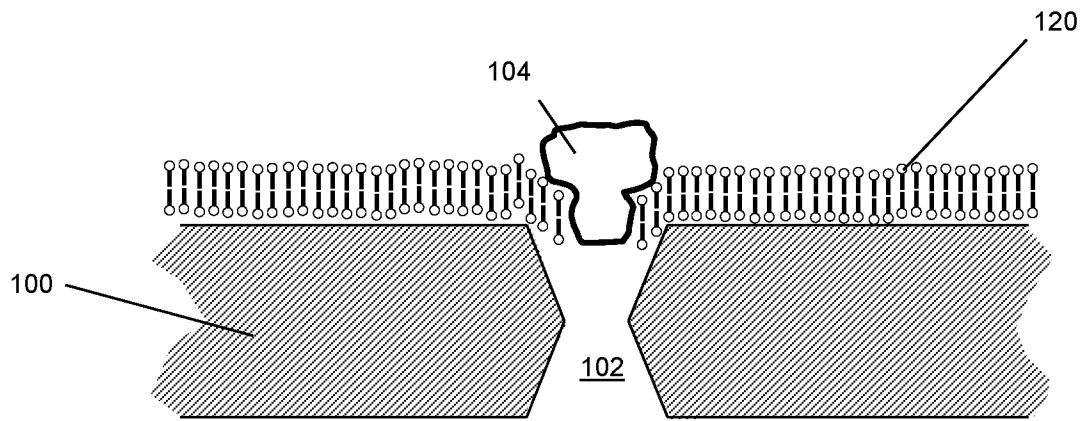


Fig. 1E

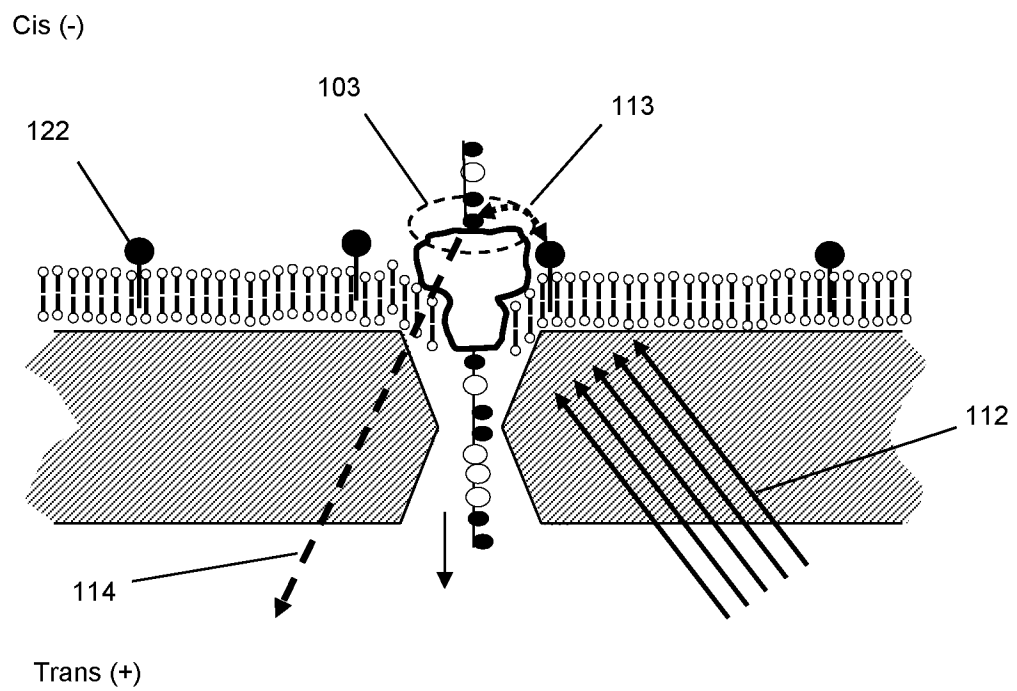


Fig. 1F

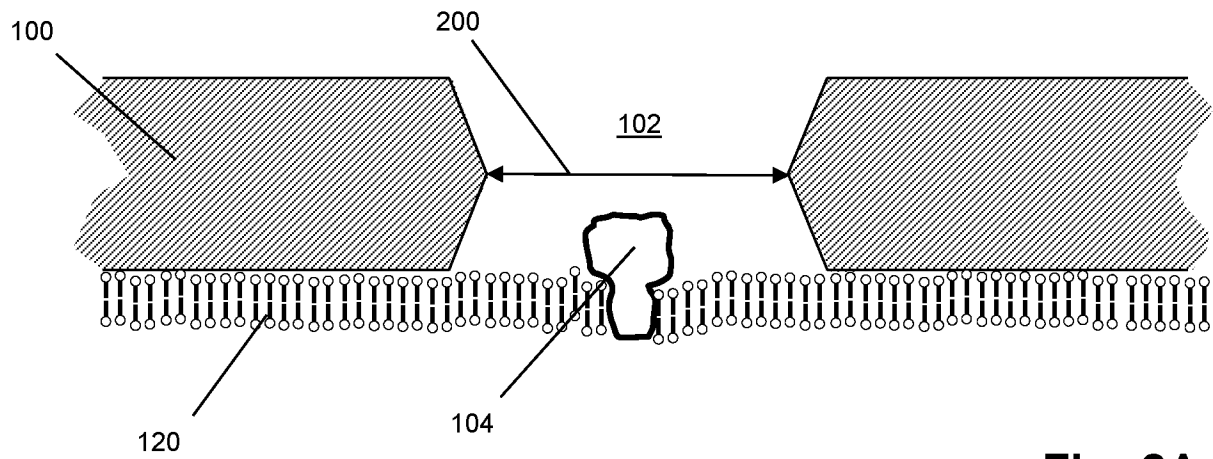


Fig. 2A

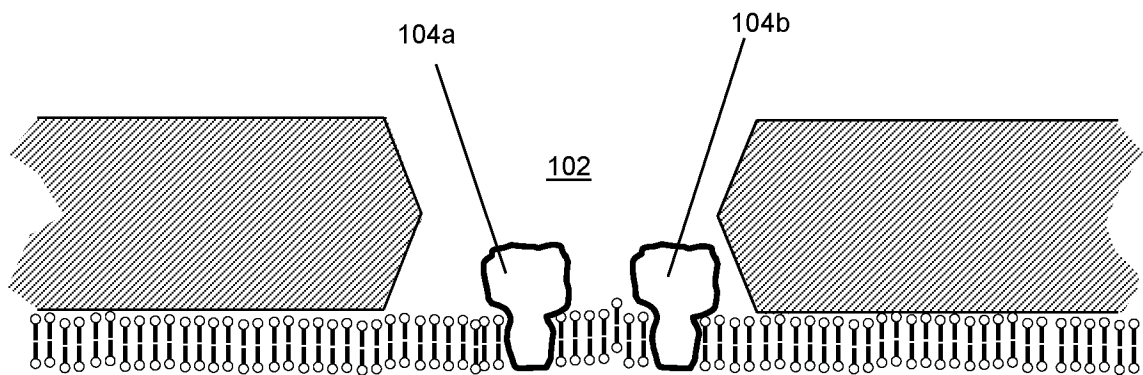
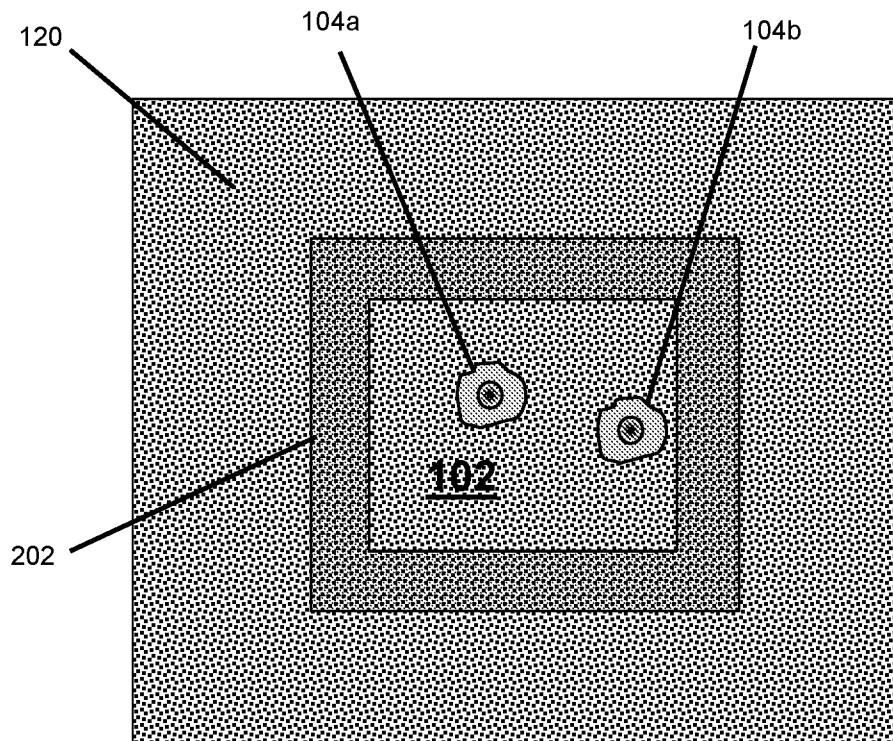


Fig. 2B



Bottom View

Fig. 2C

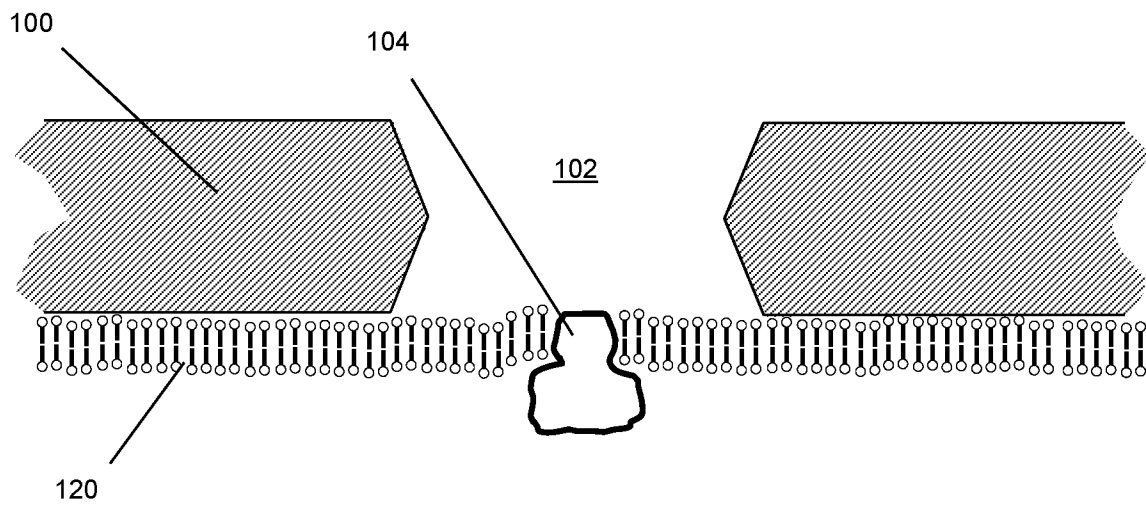


Fig. 3A

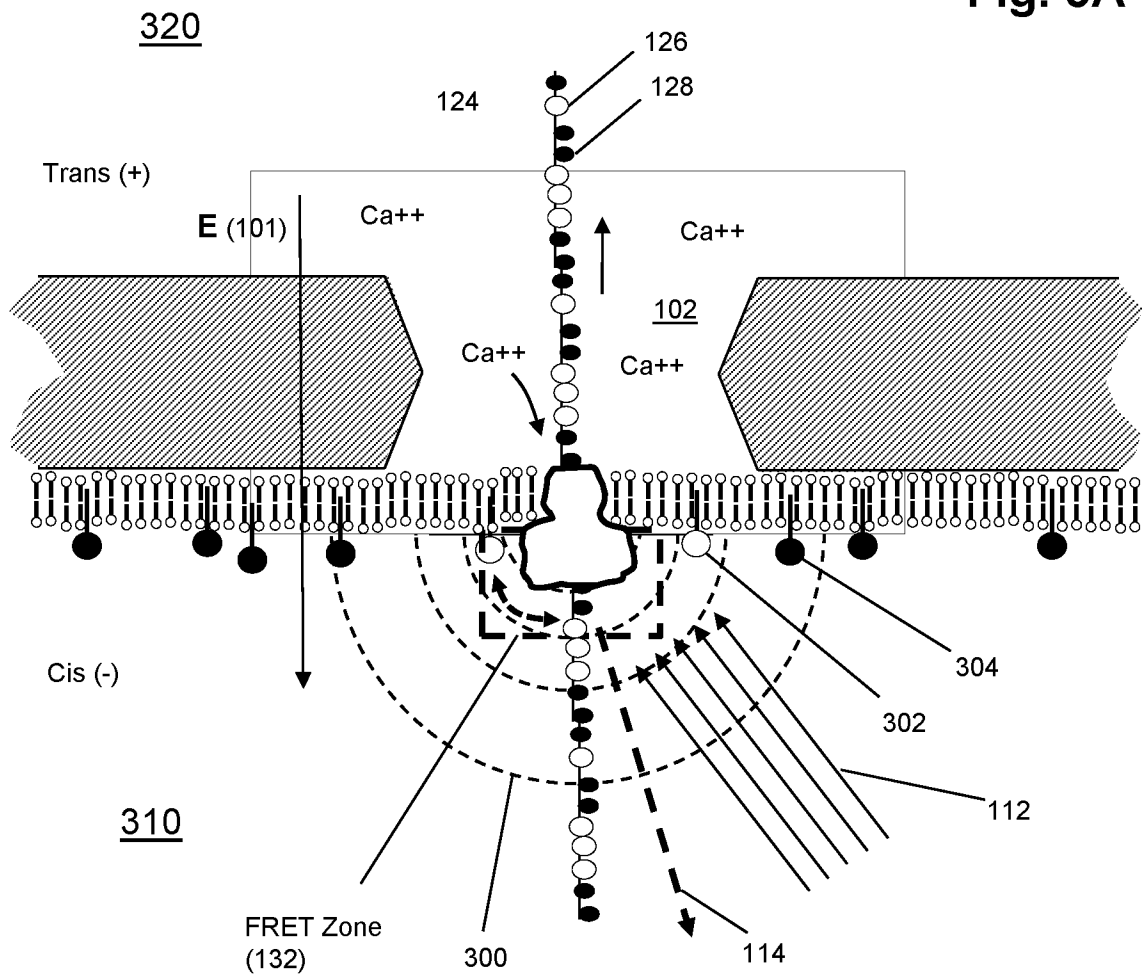


Fig. 3B

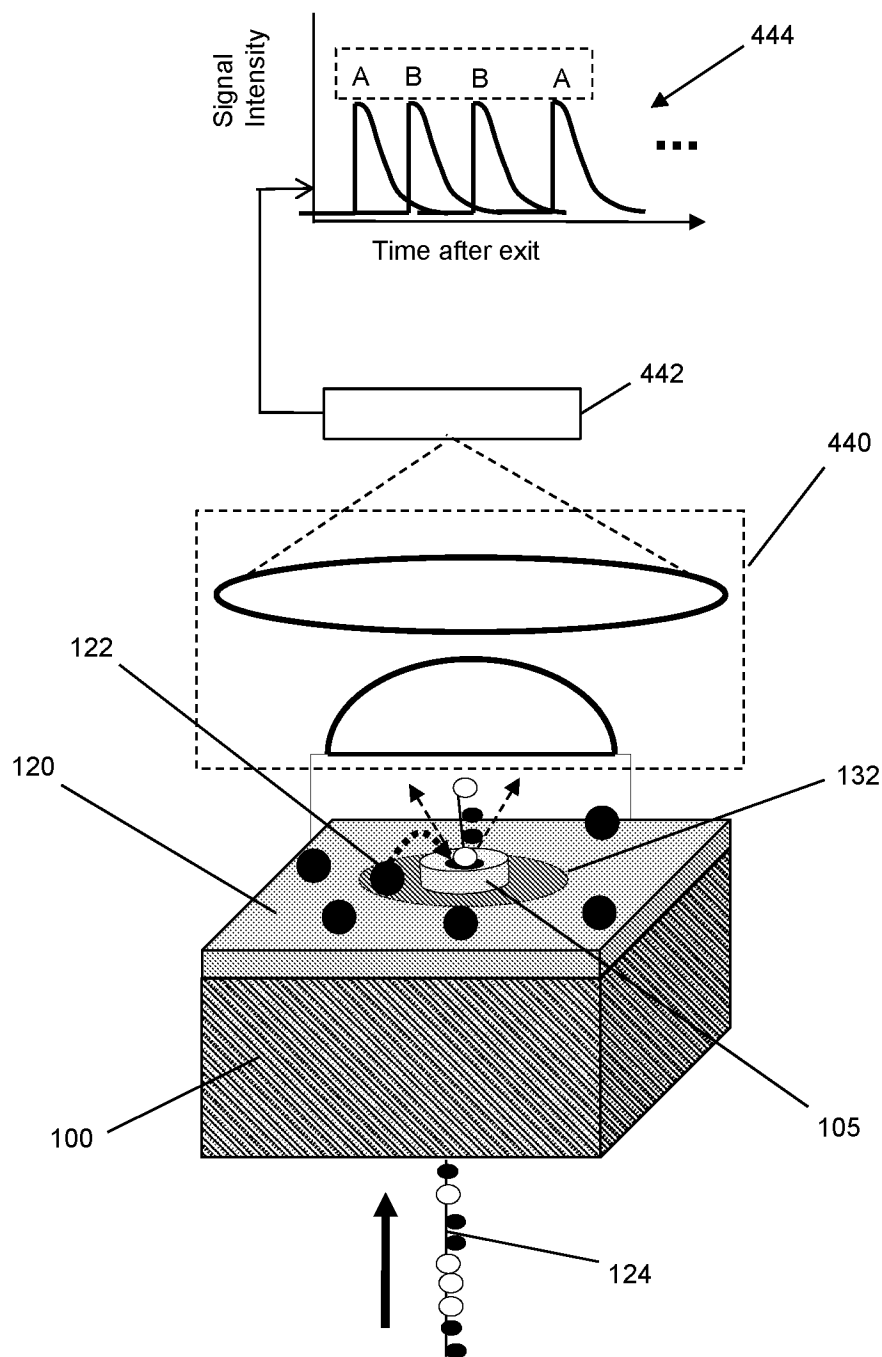


Fig. 4A

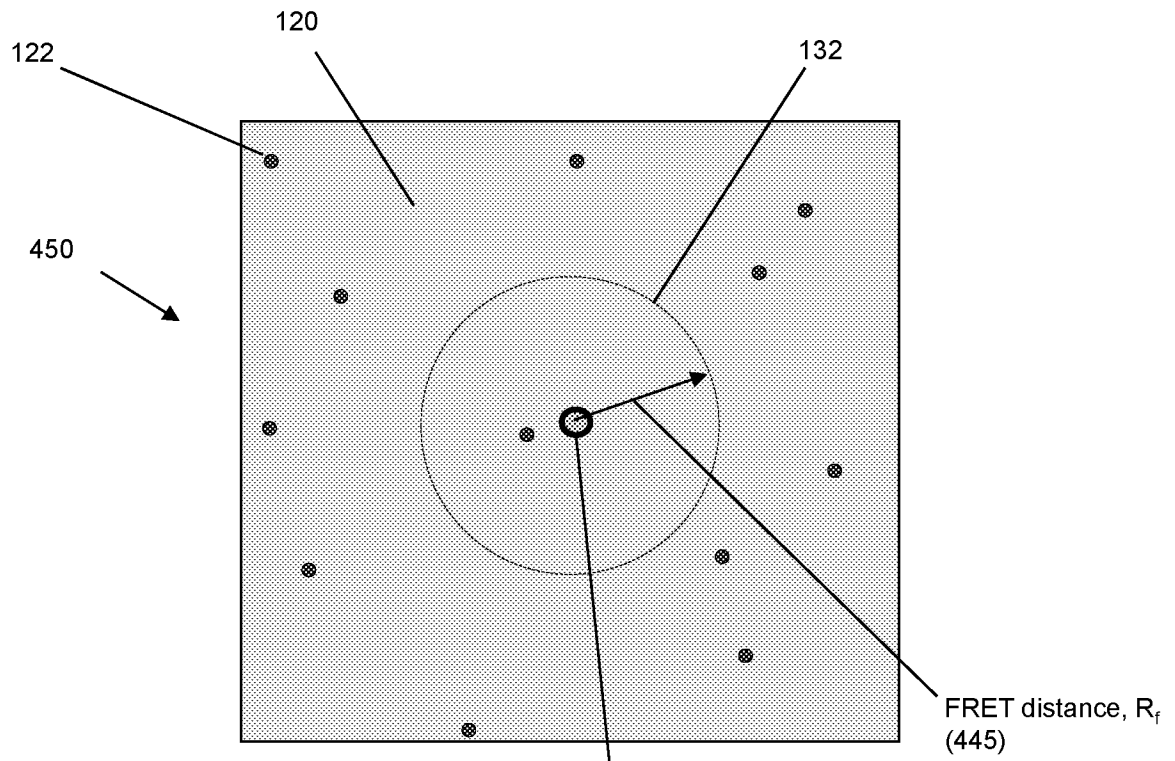


Fig. 4B

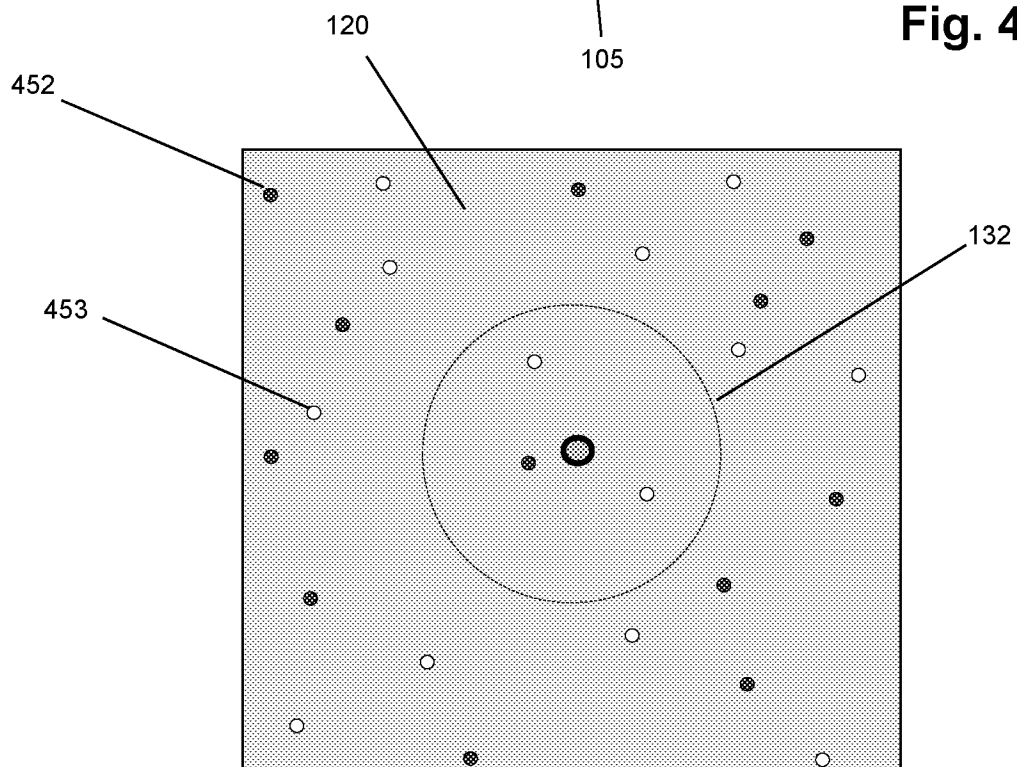


Fig. 4C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/36048

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 21/64, 27/447, 33/58; C12Q 1/68 (2016.01)

CPC - G01N 21/6428, 33/48721, 33/582; C12Q 1/68, 1/6818, 1/6869

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): G01N 21/64, 27/447, 33/58; C12Q 1/68 (2016.01)

CPC: G01N 21/6428, 33/48721, 33/582; C12Q 1/68, 1/6818, 1/6869

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

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

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); IP.com; Google/Google Scholar; EBSCO; analyte, polynucleotide, nucleic acid, DNA, RNA, lipid bilayer, nanopore, nanochannel, protein nanopore, fluorescence resonance energy transfer, donor, acceptor, bore, chamber, concentration, amount, label, pair, FRET distance, diffusion, mobile, proximity, activator, sensitive, dye

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2014/066902 A1 (QUANTAPORE, INC.) 01 May 2014; abstract; paragraphs [0008], [0017], [0078]-[0079], [0093]; figures 2C, 3B; claims 1, 3, 11, 14, 19-20	1-22
Y	US 2001/0018194 A1 (TERPETSCHNIG, EA et al.) 30 August 2001; paragraphs [0094], [0101]	1-22
Y	US 6,528,258 B1 (RUSSELL, TS) 04 March 2003; abstract; column 5, lines 32-35	10
Y	(ANDERSON, BN, et al.) Probing solid-state nanopores with light for the detection of unlabeled analytes. ACS nano. 2014. Vol. 8. No. 11; abstract; page 11836	11-16
A	WO 2014/190322 A2 (QUANTAPORE, INC.) 27 November 2014; entire document	1-22
A	US 8,771,491 B2 (HUBER, M) 08 July 2014; entire document	1-22
A	EP 2,728,355 A1 (TECHNISCHE UNIVERSITAT DRESDEN) 07 May 2014; see English translation; entire document	1-22
A	(STRYER, L et al.) "Diffusion-enhanced fluorescence energy transfer." Annual review of biophysics and bioengineering. 1982. Vol. 11. No. 1; pages 203-222	1-22

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

02 August 2016 (02.08.2016)

Date of mailing of the international search report

31 AUG 2016

Name and mailing address of the ISA/

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