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UNITED STATES DISTRICT COURT

NORTHERN DISTRICT OF CALIFORNIA

SAN JOSE DIVISION

SARTORIUS BIOANALYTICAL INSTRUMENTS, INC., **Plaintiff,** **v.** **GATOR BIO, INC. and HONG TAN,** **Defendants.**	Case No. **COMPLAINT** **JURY DEMAND**

1. Sartorius Bioanalytical Instruments, Inc. ("Sartorius" or "Plaintiff"), by and through its attorneys, submits this Complaint against Gator Bio, Inc. ("Gator Bio") and Hong Tan (collectively with Gator Bio, "Defendants") and alleges as follows:

NATURE OF SUIT

2. This is a civil action arising out of Gator Bio and Hong Tan's patent infringement in violation of the Patent Laws of the United States, 35 U.S.C. § 1 *et seq*.

3. Defendants' accused products infringe one or more claims of U.S. Patent No. 7,394,547 (the "'547 Patent"), U.S. Patent No. 7,445,887 (the "'887 Patent"), U.S. Patent No. 7,728,982 (the "'982 Patent"), and U.S. Patent No. 8,305,585 (the "'585 Patent") (collectively, the "Asserted Patents").

PARTIES

I. Sartorius

4. Sartorius Bioanalytical Instruments, Inc. is a Delaware corporation, and its principal place of business is located at 565 Johnson Avenue, Bohemia, NY 11716.

5. Sartorius is an international pharmaceutical and laboratory equipment supply company with offices around the world. Sartorius's industry segments cover bioprocess solutions and lab products and services.

6. Sartorius produces and sells, among many other products, the Octet® Bio-Layer Interferometry Label-Free Detection Systems ("Octet®") for biomolecular interactions analysis, which allow for real-time, label-free analysis for the determination of kinetics, affinity and antibody and protein quantitation by measuring optical interference.

7. Sartorius owns the Asserted Patents.

II. Gator Bio and Hong Tan

8. Upon information and belief, Gator Bio is a California corporation, and its principal place of business is located at 2454 Embarcadero Way, Palo Alto, CA 94303.

9. Upon information and belief, Hong Tan is an individual who resides at 1031 Prouty Way, San Jose, CA 95129, and serves as President and CEO of Gator Bio.

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10. Upon information and belief, Gator Bio and Hong Tan sell the Gator Bioanalysis System line of devices that uses bio-layer interferometry technology for label-free, "real-time analysis of biomolecular interactions providing information on affinity, kinetics, concentration, and epitope binning, etc."[1]

JURISDICTION AND VENUE

11. This action arises under the Patent Laws of the United States of America, 35 U.S.C. § 1 *et seq.*, including 35 U.S.C. § 271. This Court has federal-question subject-matter jurisdiction under 28 U.S.C. §§ 1331 and 1338(a) because this is a civil action arising under the Patent Act.

12. This Court has personal jurisdiction over Defendant Gator Bio via general and specific jurisdiction. This Court has general personal jurisdiction over Defendant Gator Bio because it is organized under the laws of California and has its principal place of business in Palo Alto, California. This Court has specific personal jurisdiction over Defendant Gator Bio because it has systematic and continuous contacts with the State of California, including because it continues to infringe the Asserted Patents by making, using, selling, offering to sell and/or importing infringing products in the Northern District of California.

13. This Court has personal jurisdiction over Defendant Hong Tan via general and specific jurisdiction. This Court has general personal jurisdiction over Defendant Hong Tan because he resides in California. This Court has specific personal jurisdiction over Defendant Hong Tan because he has systematic and continuous contacts with the State of California, including because he serves as the President/CEO of Gator Bio, a company organized under the laws of the State of California, and he continues to infringe the Asserted Patents by making, using, selling, offering to sell and/or importing infringing products in the Northern District of California.

[1] Cision PR Newswire, Gator Bio, Inventors of biolayer interferometry technology launch a next gen platform and novel biosensors https://www.prnewswire.com/news-releases/inventors-of-biolayer-interferometry-technology-launch-a-next-gen-platform-and-novel-biosensors-301376466.html (last visited Mar. 4, 2022).

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14. Venue is proper in this District under 28 U.S.C. § 1400(b). Defendant Gator Bio "resides" in this District as it is organized under the laws of California and has its principal place of business in Palo Alto, California. Defendant Gator Bio also has "committed acts of infringement and has a regular and established place of business" in this District by making, using, selling, offering to sell and/or importing products that infringe the Asserted Patents in this District, and having a physical place in the District, which is a regular and established place of business, and is the place of the Defendant. Defendant Hong Tan "resides" and/or has "committed acts of infringement and has a regular and established place of business" in this District by making, using, selling, offering to sell and/or importing products that infringe the Asserted Patents in this District, and having a physical place in the District, which is a regular and established place of business, and is the place of the Defendant.

INTRADISTRICT ASSIGNMENT

15. This action substantially arises in the County of Santa Clara, as that is the location of Gator Bio's principal place of business of and Hon Tan's place of residence. Thus, under Civil L.R. 3-2(d), this action is be assigned to the San Jose Division.

FACTUAL BACKGROUND

I. Bio-layer Interferometry

16. Bio-layer Interferometry ("BLI") is a technique for analyzing the interaction between two different types of biological molecules. It is an optical analytical technique that analyzes the interference pattern of white light reflected from two surfaces on the tip of a biosensor: an external biolayer coated with an immobilized ligand, and an internal reference optical layer. A white light is streamed constantly along the biosensor fiber to reflect from either the internal reference optical layer or the biolayer. The resulting interference pattern of the reflected light is measured by a photodetector. Once the baseline measurements are determined, the coated biolayer is submerged in a buffer containing the second biological molecule, an analyte. The binding of the ligand to the analyte changes its optical thickness and thus alternates the phase of the reflected light. This effect causes a wavelength shift in the interference pattern, which correlates directly to the distance between the two surfaces. After binding, or association,

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the biosensors are submerged in buffer without analyte and dissociation occurs. The interference pattern is measured constantly, allowing one to collect particular data, including monitor binding specificity, rates of association and dissociation, or concentration regarding the molecules of interest in real time.

II. FortéBio, Inc.

17. Upon information and belief, Hong Tan founded FortéBio, Inc. ("FortéBio") in 2001, where he served as Chief Executive Officer ("CEO") and Chief Technology Officer ("CTO") and helped develop BLI technology. FortéBio developed and sold the Octet® line of BLI technology. Hong Tan left FortéBio in 2008.

18. Pall Corporation acquired FortéBio in 2012. Danaher Corporation then acquired Pall Corporation in 2015. Finally, Sartorius acquired selected life sciences businesses of Danaher Corporation in 2020, including the portion of Danaher Corporation that was once FortéBio. Sartorius continues to sell the Octet® line of BLI technology.

III. Gator Bio and Hong Tan

19. Upon information and belief, shortly after leaving FortéBio in 2008, Hong Tan began working at Access Medical Systems, Ltd., where he currently serves as the CEO.

20. Upon information and belief, on July 14, 2017, the company Probe Life, Inc. incorporated in the State of California.

21. Upon information and belief, on August 15, 2017, Hong Tan filed a Statement of Information for Probe Life, Inc. with the Secretary of State of the State of California listing himself as President/CEO, Secretary, Chief Financial Officer ("CFO"), and Director of Probe Life, Inc.

22. Upon information and belief, on April 1, 2020, by and through its President/CEO and Secretary, Hong Tan, Probe Life, Inc. changed its name to "Gator Bio, Inc." Hong Tan currently remains President/CEO of Gator Bio.

23. Upon information and belief, Robert Zuk is the CTO of Gator Bio.

24. Upon information and belief, Access Medical Systems, Ltd. is the parent company to ET Healthcare and Gator Bio.

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IV. U.S. Patent No. 7,394,547

25. On July 1, 2008, the United States Patent and Trademark Office ("USPTO") duly and legally issued the '547 Patent, entitled "Fiber-optic assay apparatus based on phase-shift interferometry." A copy of the '547 Patent is attached as Exhibit A.

26. The named inventors on the '547 Patent are: Hong Tan, Duan Jun Chen, Yushan Tan, and Krista Leah Witte.

27. Plaintiff owns all right, title, and interest in the '547 Patent.

28. The '547 Patent claims an assembly for use in detecting an analyte in a sample based on interference and a method for detecting analyte in a sample.

29. Upon information and belief, Defendant Gator Bio, through its President/CEO Hong Tan, had knowledge of the '547 Patent on July 14, 2017.

30. Upon information and belief, Defendant Hong Tan had knowledge of the '547 Patent as of the date of its issuance, July 1, 2008.

V. U.S. Patent No. 7,445,887

31. On November 4, 2008, the USPTO duly and legally issued the '887 Patent, entitled "Enzyme activity measurements using bio-layer interferometry." A copy of the '887 Patent is attached as Exhibit B.

32. The named inventors on the '887 Patent are: Robert Zuk, Sae Choo, Weilei Ma, and Krista Witte.

33. Plaintiff owns all right, title, and interest in the '887 Patent.

34. The '887 Patent claims a method for assaying enzyme activity.

35. Upon information and belief, Defendant Gator Bio, through its President/CEO Hong Tan and its CTO Robert Zuk, had knowledge of the '887 Patent on July 14, 2017.

36. Upon information and belief, Defendant Hong Tan had knowledge of the '887 Patent as of the date of its issuance, November 4, 2008.

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VI. U.S. Patent No. 7,728,982

37. On June 1, 2010, the USPTO duly and legally issued the '982 Patent, entitled "Fiber-optic assay apparatus based on phase-shift interferometry." A copy of the '982 Patent is attached as Exhibit C.

38. The named inventors on the '982 Patent are: Hong Tan, Yushan Tan, Duan Jun Chen, and Krista Leah Witte.

39. Plaintiff owns all right, title, and interest in the '982 Patent.

40. The '982 Patent claims an optical assembly for use in detecting an analyte in a sample based on interference and a method for detecting analyte in a sample.

41. Upon information and belief, Defendant Gator Bio through its President/CEO Hong Tan, had knowledge of the '982 Patent on July 14, 2017.

42. Upon information and belief, Defendant Hong Tan had knowledge of the '982 Patent as of the date of its issuance, June 1, 2010.

VII. U.S. Patent No. 8,305,585

43. On November 6, 2012, the USPTO duly and legally issued the '585 Patent, entitled "Fiber-optic assay apparatus based on phase-shift interferometry." A copy of the '585 Patent is attached as Exhibit D.

44. The named inventors on the '547 Patent are: Hong Tan, Yushan Tan, Duan Jun Chen, and Krista Leah Witte.

45. Plaintiff owns all right, title, and interest in the '585 Patent.

46. The '585 Patent claims an optical assembly for use in detecting an analyte in a sample based on interference.

47. Upon information and belief, Defendant Gator Bio, through its President/CEO Hong Tan, had knowledge of the '585 Patent on July 14, 2017.

48. Upon information and belief, Defendant Hong Tan had knowledge of the '585 Patent as of the date of its issuance, November 6, 2012.

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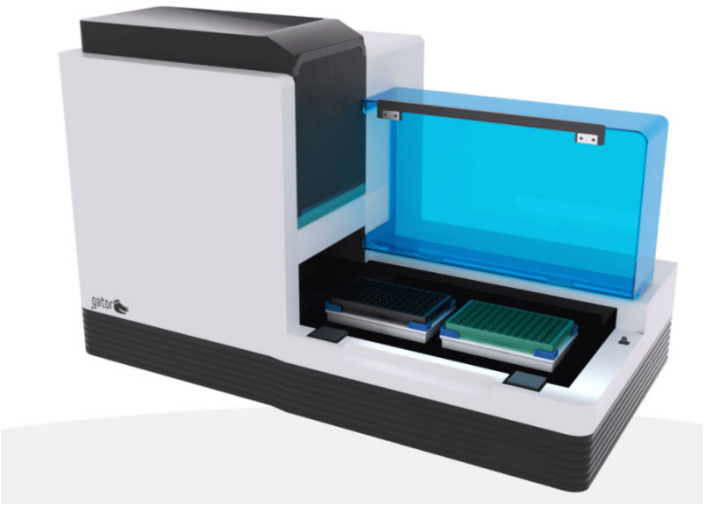
VIII. The Gator Bio Accused Products

49. Upon information and belief, Defendants Gator Bio and Hong Tan sell the Gator Bioanalysis System line of devices that "use[] biolayer interferometry for label-free, real-time measurement of biomolecules."[2] The GatorPrime and GatorPlus are sold under the Gator Bioanalysis System line of devices.

50. Upon information and belief, Defendants Gator Bio and Hong Tan launched the GatorPrime around March 2019.[3]

51. Upon information and belief, the GatorPrime allows for applications relating to quantitation, kinetics, and epitope binning and is based on BLI technology. The GatorPrime allows from 1–168 samples per run and 10 x 10 epitope binning. The GatorPrime is shown below.



52. Upon information and belief, the GatorPlus also allows for applications in quantitation, kinetics, and epitope binning and is based on BLI technology. The GatorPlus allows from 1–456 samples per run and 16 x 16 epitope binning. The GatorPlus is shown below:

[2] Products, Gator Bio, https://www.gatorbio.com/products (last visited Mar. 4, 2022).
[3] Cision PR Newswire, Gator Bio, Inventors of biolayer interferometry technology launch a next gen platform and novel biosensors https://www.prnewswire.com/news-releases/inventors-of-biolayer-interferometry-technology-launch-a-next-gen-platform-and-novel-biosensors-301376466.html (last visited Mar. 4, 2022).

53. Upon information and belief, the Gator Bioanalysis System line of devices infringes the Asserted Patents.

54. Upon information and belief, Gator Bio sells a line of Gator Biosensor Probes designed for use with the Gator Bioanalysis System line of devices.[4] This line of Gator Biosensor Probes includes the: Gator™ AAVX, Gator™ Flex SA, Gator™ Protein A, Gator™ Protein G, Gator™ Protein L, Gator™ Anti-Mouse Fc, Gator™ Anti-Human Fc, Gator™ Anti-His, Gator™ Amine-Reactive, Gator™ Streptavidin, Gator™ Aminopropylsilane (APS), Gator™ Small Molecule, Antibody, and Protein (SMAP), and Gator™ Ni-NTA.

55. Upon information and belief, Defendants Gator Bio and Hong Tan design, engineer, and manufacture the Gator Bioanalysis System line of devices and Gator Biosensor Probes either directly or through intermediaries (including distributors, retailers, and others), ship, distribute, offer for sale, sell, and advertise the infringing devices in the United States (including in this District) and abroad.

FIRST CAUSE OF ACTION

INFRINGEMENT OF U.S. PATENT NO. 7,394,547

56. Plaintiff restates and realleges the foregoing allegations as if fully stated herein.

[4] Gator Bio, Biosensors and Reagents, https://www.gatorbio.com/products/biosensors-and-reagents (last visited Mar. 4, 2022).

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57. Defendants' Gator Bioanalysis System line of devices, including, but not limited to, the representative GatorPrime device, infringes at least independent claim 1 of the '547 Patent, which recites:

> **1**. An assembly for use in detecting an analyte in a sample based on interference, comprising:
> an optical fiber having a tip;
> a first optical element adapted for coupling to a light source through a mechanical coupling that engages the first optical element with the fiber and provides an air gap between the first optical element and the fiber; and
> second optical element attached to the first optical element, the second optical element commensurate in size with the fiber tip and adapted for coupling to the first optical element,
> said second optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface separated from the first reflecting surface by the transparent material, said first and second reflecting surfaces separated by at least 50 nm, wherein said first reflecting surface comprises a layer of analyte binding molecules, and an interference between light reflected into the fiber from said first and second reflecting surfaces varies as analyte in the sample binds to the analyte binding molecules.

58. Upon information and belief, the GatorPrime is an assembly for use in detecting an analyte in a sample based solution.[5]

59. Upon information and belief, the GatorPrime has an optical fiber that has a tip[6] as shown in Figure 1 below:

[5] Technology, Gator Bio, https://www.gatorbio.com/technology ("Biolayer interferometry (BLI) is a label free biomolecular detection method created by Gator Bio co-founder, Hong Tan. In biolayer interferometry, biomolecular interactions are detected by measuring the interference pattern of white light reflected from the surface of a biosensor.") (last visited Mar. 4, 2022); Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 8:28 "Bio layer interferometry or BLI is part of the label free systems that has many other biophysical techniques where label free meaning you do not have any dye or fluorophore or any kind of special tags that you need to conjugate to your molecule of interest in order to see binding by using a fluorescence or chemiluminescence or anything all you do in this um technique is we are using uh white light and that's all.").

[6] UF ICBR, Seminar and Demonstration with Gator Bio BLI, YouTube (Oct. 21, 2021), https://www.youtube.com/watch?v=Myc2YUHkiMQ (at 26:29 "The optical fibers are shining through the probes.").

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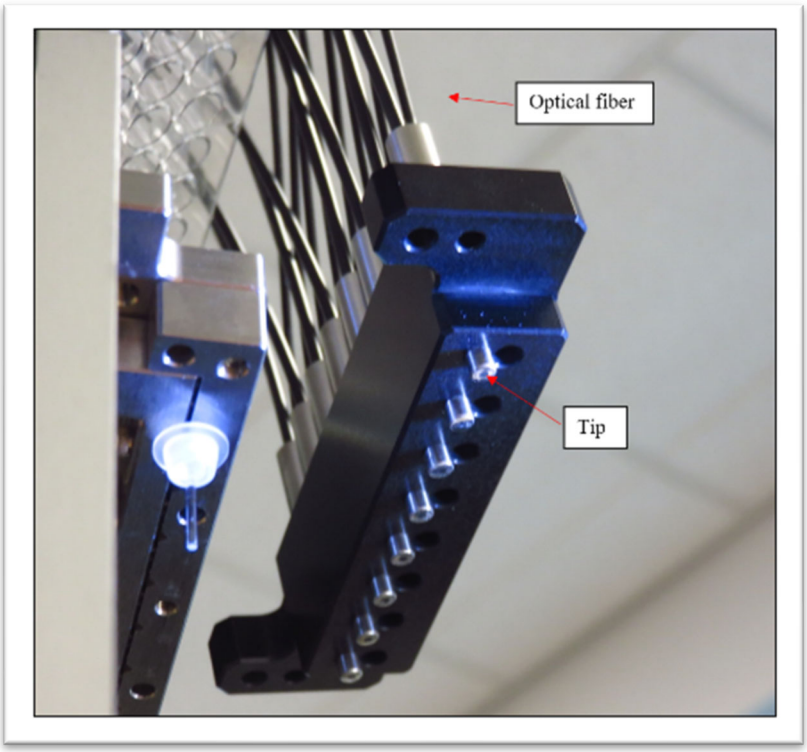

(Figure 1).

60. Upon information and belief, the GatorPrime has a first optical element adapted for coupling to a light source. Upon further information and belief, the first optical element is located beneath the incident light source,[7] as shown in Figure 2 below:

[7] The optical elements are referred to as layers. Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 9:55 "This um biosensor has two layers one is the optical layer and this is the surface layer.").

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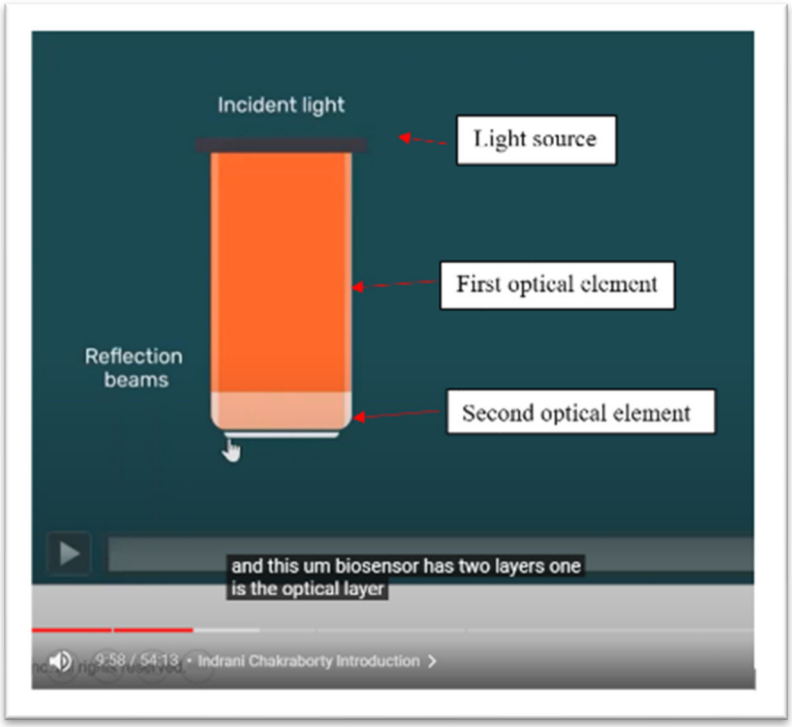

(Figure 2).

61. Upon information and belief, in the GatorPrime, there is a mechanical coupling that engages the first optical element with the fiber and provides an air gap between the first optical element and the fiber.

62. Upon information and belief, the GatorPrime has a second optical element attached to the first optical element, the second optical element commensurate in size with the fiber tip and adapted for coupling to the first optical element. Upon further information and belief, the second optical element is located beneath the first optical element,[8] as shown in Figure 2.

63. Upon information and belief, in the GatorPrime the second optical element comprises a transparent material.

[8] The optical elements are referred to as layers. Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 9:55 "This um biosensor has two layers one is the optical layer and this is the surface layer.").

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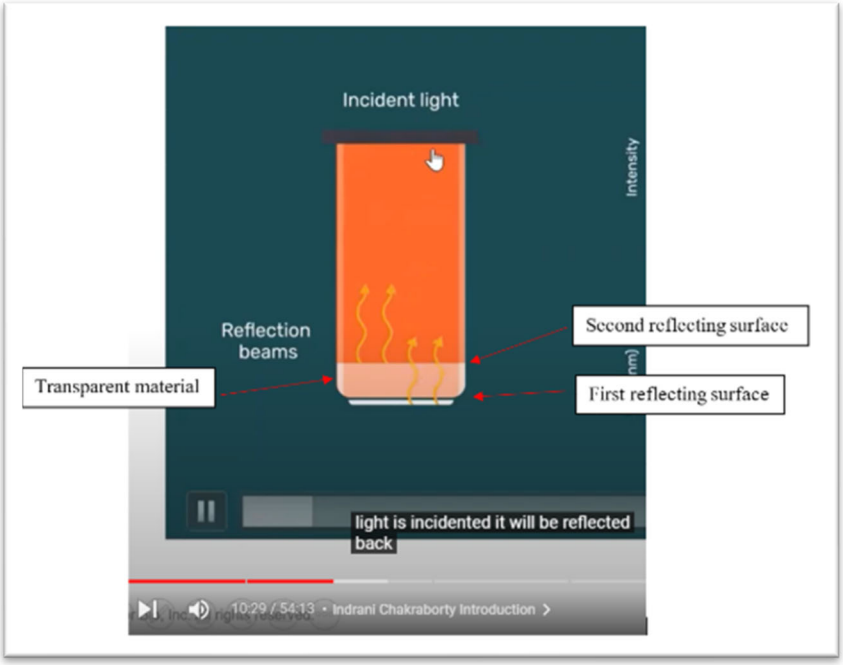
64. Upon information and belief, the GatorPrime second optical element has a first reflecting surface and a second reflecting surface,[9] separated from the first reflecting surface by the transparent material, as shown in Figure 3 below:



(Figure 3).

65. Upon information and belief, GatorPrime's first and second reflecting surfaces are separated by at least 50 nm.

66. Upon information and belief, the GatorPrime's first reflecting surface comprises a layer of analyte binding molecules. Upon further information and belief, the analyte binding molecules are located on the distal end of the first reflecting surface,[10] as shown in Figure 4:

[9] Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 10:27 "When the light is incidented, it will be reflected back from these two surfaces.").

[10] The analyte binding molecules are referred to as ligands. Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 10:53 "Then when you have your ligand bound then you can dip it in another solution where you have analyte of your interest.").

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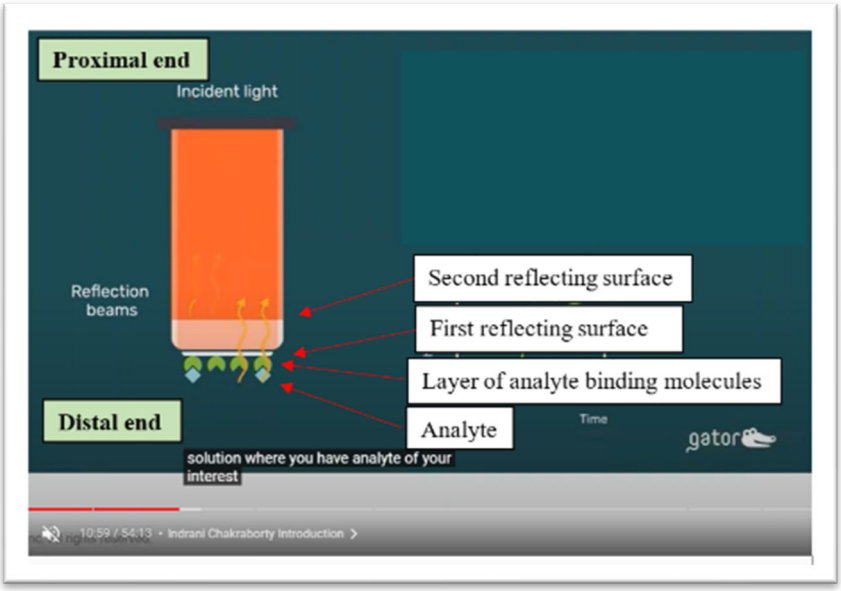

(Figure 4).

67. Upon information and belief, in the GatorPrime, an interference between light reflected into the fiber from said first and second reflecting surfaces varies as analyte in the sample binds to the analyte binding molecules.[11]

68. Defendants have been and are directly infringing, literally and/or under the doctrine of equivalents, one or more claims of the '547 Patent in violation of 35 U.S.C. § 271(a), by making, using, importing, selling, and/or offering for sale in or into the United States Gator Bioanalysis System line of devices.

69. Additionally, Defendants have induced and continue to induce others to directly infringe one or more claims of the '547 Patent under 35 U.S.C. § 271(b) by, among other things, and with specific intent or willful blindness, actively aiding and abetting others to directly infringe, including but not limited to Defendants' partners, clients, customers, and end users, whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '547 Patent.

[11] Technology, Gator Bio, https://www.gatorbio.com/technology ("Biolayer interferometry compares the interference pattern of white light reflected from an internal reference layer within a layer of immobilized biomolecules on the surface chemistry of the biosensor. Shifts in interference due to the accumulation of biomolecules are monitored in real-time to sensitively analyze and calculate rates of association and dissociation among target proteins with high precision.") (last visited Mar. 4, 2022).

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70. Defendants have contributed and continue to contribute to the direct infringement of one or more claims of the '547 Patent under 35 U.S.C. § 271(c) by selling the Gator Biosensor Probes, non-staple articles whose only substantial use infringes the '547 Patent, and thus contributing to the direct infringement of the '547 Patent of others, including but not limited to Defendants' partners, clients, customers, and end users, whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '547 Patent.

71. Defendants have infringed one or more claims of the '547 Patent under 35 U.S.C. § 271(f) by supplying in or from the United States the Gator Biosensor Probes which are especially made for use in the Gator Bioanalysis System line of devices and are non-staple articles whose only substantial use infringes the '547 Patent.

72. Defendants have never been licensed, either expressly or impliedly, under the '547 Patent.

73. Plaintiff has complied with the requirements of 35 U.S.C. § 287 by providing actual or constructive notice to Defendants of their infringement.

74. Plaintiff has been, and continues to be, damaged and irreparably harmed by Defendants' infringement, which will continue unless this Court enjoins these infringements.

75. Plaintiff, under 35 U.S.C. § 284, is entitled to damages adequate to compensate for Defendants' infringement, including lost profits and/or a reasonable royalty.

76. Defendants' infringement of the '547 Patent has been, and continues to be, deliberate, willful, and knowing, thus entitling Plaintiff to treble damages under 35 U.S.C. § 284.

77. This is an exceptional case under 35 U.S.C. § 285, thus entitling Plaintiff to recover its reasonable attorney fees from Defendants.

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SECOND CAUSE OF ACTION

INFRINGEMENT OF U.S. PATENT NO. 7,445,887

78. Plaintiff restates and realleges the foregoing allegations as if fully stated herein.

79. Defendants' Gator Bioanalysis System line of devices, including, but not limited to, the representative GatorPrime device, infringes at least independent claim 1 of the '887 Patent, which recites:

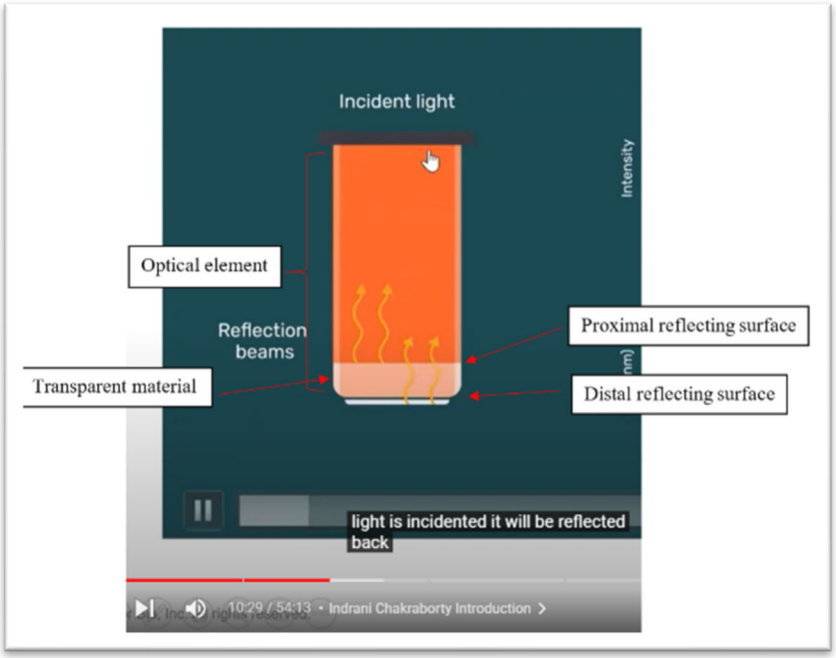
> **1**. A method for assaying enzyme activity, comprising:
> providing an optical element coupled to a light source via a mechanical coupling that engages the optical element with an optical fiber and provides an air gap between the optical element and the fiber, the optical element including (a) a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm, and (b) a layer of enzyme substrate molecules positioned so that interference between a reflected beam from the proximal reflecting surface and a reflected beam from the distal reflecting surface varies as an enzyme reacts with the substrate, and wherein the reflected beams are coupled into the optical fiber;
> exposing the optical element to an enzyme; and
> detecting a change in the interference between the reflected beams, wherein the change is indicative of enzyme activity.

80. Upon information and belief, the GatorPrime practices a method for assaying enzyme activity.[12]

81. Upon information and belief, the GatorPrime practices an optical element coupled to a light source via a mechanical coupling that engages the optical element with an optical fiber and provides an air gap between the optical element and the fiber.

[12] The Gator Bio website provides for small molecules, antibody, and protein (SMAP) Probes. Gator Bio, Gator Bio Small Molecule Sensitivity: The Power of Next Generation BLI, https://www.gatorbio.com/application/files/7716/3337/0223/SMAP.pdf ("This biosensor is sensitive enough to capture small molecules (down to 150 Da), but can also be used for biomolecules and antibodies when sensitivity is a must.") (last visited Mar. 4, 2022).

82. Upon information and belief, the GatorPrime practices an optical element that includes a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm, as shown in Figure 5:



(Figure 5).

83. Upon information and belief, the GatorPrime has a layer of enzyme substrate molecules positioned so that interference between a reflected beam from the proximal reflecting surface and a reflected beam from the distal reflecting surface varies as an enzyme reacts with the substrate.[13]

[13] The Gator Bio website provides for small molecules, antibody, and protein (SMAP) Probes. Gator Bio, Gator Bio Small Molecule Sensitivity: The Power of Next Generation BLI, https://www.gatorbio.com/application/files/7716/3337/0223/SMAP.pdf ("This biosensor is sensitive enough to capture small molecules (down to 150 Da), but can also be used for biomolecules and antibodies when sensitivity is a must.") (last visited Mar. 4, 2022); Gator Bio, Technology, https://www.gatorbio.com/technology ("Biolayer interferometry compares the interference pattern of white light reflected from an internal reference layer within a layer of immobilized biomolecules on the surface chemistry of the biosensor. Shifts in interference due to the accumulation of biomolecules are monitored in real-time to sensitively analyze and calculate rates of association and dissociation among target proteins with high precision.") (last visited Mar. 4, 2022).

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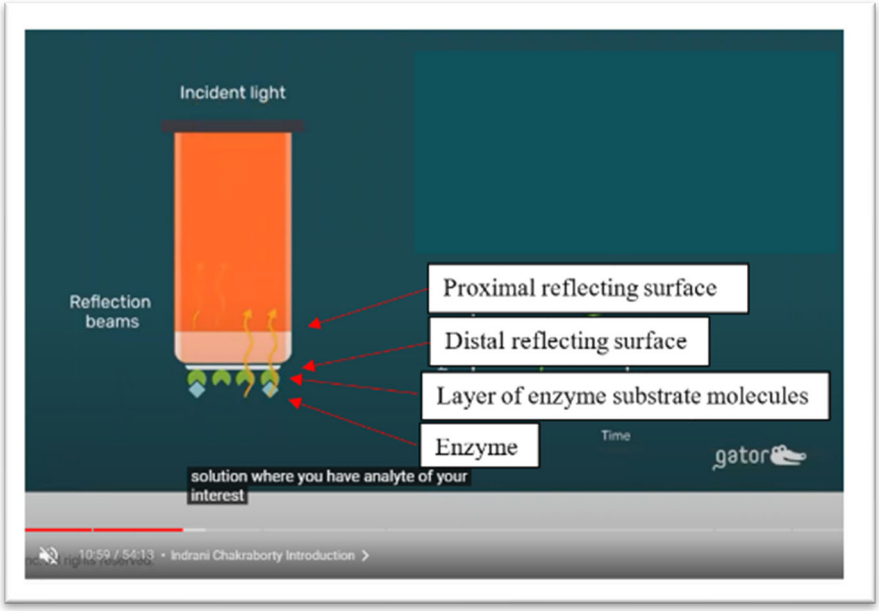
84. Upon information and belief, the GatorPrime reflected beams are coupled into the optical fiber, as shown in Figure 5 above.

85. Upon information and belief, the GatorPrime method includes exposing the optical element to an enzyme, as shown in Figure 6 below:



(Figure 6).

86. Upon information and belief, the GatorPrime method includes detecting a change in the interference between the reflected beams, wherein the change is indicative of enzyme activity.

87. Defendants have been and are directly infringing, literally and/or under the doctrine of equivalents, one or more claims of the '887 Patent in violation of 35 U.S.C. § 271(a), by making, using, importing, selling, and/or offering for sale in or into the United States the Gator Bioanalysis System line of devices.

88. Additionally, Defendants have induced and continue to induce others to directly infringe one or more claims of the '887 Patent under 35 U.S.C. § 271(b) by, among other things, and with specific intent or willful blindness, actively aiding and abetting others to directly infringe, including but not limited to Defendants' partners, clients, customers, and end users,

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whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '887 Patent.

89. Defendants have contributed and continue to contribute to the direct infringement of one or more claims of the '887 Patent under 35 U.S.C. § 271(c) by providing the Gator Biosensor Probes, non-staple articles whose only substantial use infringes the '887 Patent, and thus contributing to the direct infringement of the '887 Patent of others, including but not limited to Defendants' partners, clients, customers, and end users, whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '887 Patent.

90. Defendants have infringed one or more claims of the '887 Patent under 35 U.S.C. § 271(f) by supplying in or from the United States the Gator Biosensor Probes which are especially made for use in the Gator Bioanalysis System line of devices and are non-staple articles whose only substantial use infringes the '887 Patent.

91. Defendants have never been licensed, either expressly or impliedly, under the '887 Patent.

92. Plaintiff has been, and continues to be, damaged and irreparably harmed by Defendants' infringement, which will continue unless this Court enjoins these infringements.

93. Plaintiff, under 35 U.S.C. § 284, is entitled to damages adequate to compensate for Defendants' infringements, including lost profits and/or a reasonable royalty.

94. Defendants' infringement of the '887 Patent has been, and continues to be, deliberate, willful, and knowing, thus entitling Plaintiff to treble damages under 35 U.S.C. § 284.

95. This is an exceptional case under 35 U.S.C. § 285, thus entitling Plaintiff to recover its reasonable attorney fees from Defendants.

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THIRD CAUSE OF ACTION

INFRINGEMENT OF U.S. PATENT NO. 7,728,982

96. Plaintiff restates and realleges the foregoing allegations as if fully stated herein.

97. Defendants' Gator Bioanalysis System line of devices, including, but not limited to, the representative GatorPrime device, infringe at least independent claim 1 of the '982 Patent, which recites:

> **1**. An optical assembly for use in detecting an analyte in a sample based on interference, comprising:
> an optical fiber having a tip; and
> an optical element removably attached to the tip of the optical fiber and configured for receiving a beam of light from the optical fiber, the optical element comprising flexible gripping arms that slide over and grip the optical fiber to removably attach to optical element to the optical fiber,
> said optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface separated from the first reflecting surface by the transparent material, said first and second reflecting surfaces separated by at least 50 nm,
> wherein said first reflecting surface binds a layer of analyte binding molecules positioned so that interference between a beam of light reflected from the first reflecting surface into the optical fiber and a beam of light reflected from the second reflecting surface into the optical fiber varies as analyte binds to the layer of analyte binding molecules.

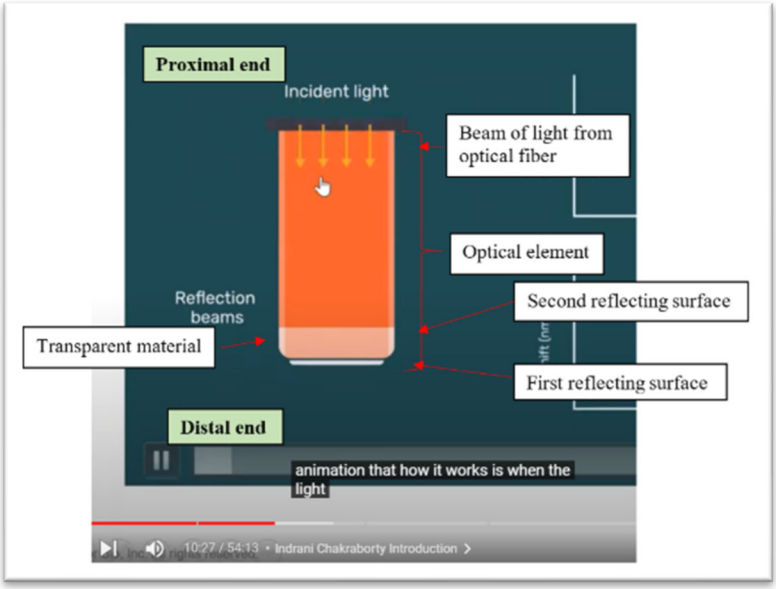
98. Upon information and belief, the GatorPrime has an optical assembly for use in detecting an analyte in a sample based on interference.[14]

99. Upon information and belief, the GatorPrime has an optical fiber that has a tip,[15] as shown in Figure 1 above.

[14] Gator Bio, Technology, https://www.gatorbio.com/technology ("Biolayer interferometry compares the interference pattern of white light reflected from an internal reference layer within a layer of immobilized biomolecules on the surface chemistry of the biosensor. Shifts in interference due to the accumulation of biomolecules are monitored in real-time to sensitively analyze and calculate rates of association and dissociation among target proteins with high precision.") (last visited Mar. 4, 2022).

[15] UF ICBR, Seminar and Demonstration with Gator Bio BLI, YouTube (Oct. 21, 2021), https://www.youtube.com/watch?v=Myc2YUHkiMQ (at 26:29 "The optical fibers are shining through the probes.").

100. Upon information and belief, the GatorPrime has an optical element removably attached to the tip of the optical fiber. Upon further information and belief, the optical element is configured for receiving a beam of light from the optical fiber, as shown in Figure 7 below:



(Figure 7).

101. Upon information and belief, the GatorPrime has a clamp that holds the optical element in place near the tip of the optical fiber.

102. Upon information and belief, the GatorPrime optical element comprises a transparent material, located at the distal end of the optical element, as shown in Figure 7 above.

103. Upon information and belief, the GatorPrime optical element has a first reflecting surface and second reflecting surface,[16] separated from the first reflecting surface by the transparent material, as shown in Figure 7 above.

104. Upon information and belief, the GatorPrime has first and second reflecting surfaces that are separated by at least 50 nm.

[16] Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 10:27 "When the light is incidented, it will be reflected back from these two surfaces.").

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105. Upon information and belief, the GatorPrime has a first reflecting surface that binds a layer of analyte binding molecules positioned so that interference between a beam of light reflected from the first reflecting surface into the optical fiber and a beam of light reflected from the second reflecting surface into the optical fiber varies as analyte binds to the layer of analyte binding molecules.[17]

106. Defendants have been and are directly infringing, literally and/or under the doctrine of equivalents, one or more claims of the '982 Patent in violation of 35 U.S.C. § 271(a), by making, using, importing, selling, and/or offering for sale in or into the United States the Gator Bioanalysis System line of devices.

107. Additionally, Defendants have induced and continue to induce others to directly infringe one or more claims of the '982 Patent under 35 U.S.C. § 271(b) by, among other things, and with specific intent or willful blindness, actively aiding and abetting others to directly infringe, including but not limited to Defendants' partners, clients, customers, and end users, whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '982 patent.

108. Defendants have contributed and continue to contribute to the direct infringement of one or more claims of the '982 Patent under 35 U.S.C. § 271(c) by providing the Gator Biosensor Probes, non-staple articles whose only substantial use infringes the '982 Patent, and thus contributing to the direct infringement of the '982 Patent of others, including but not limited to Defendants' partners, clients, customers, and end users, whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '982 Patent.

109. Defendants have infringed one or more claims of the '982 Patent under 35 U.S.C. § 271(f) by supplying in or from the United States the Gator Biosensor Probes which are

[17] Gator Bio, Technology, https://www.gatorbio.com/technology ("Biolayer interferometry compares the interference pattern of white light reflected from an internal reference layer within a layer of immobilized biomolecules on the surface chemistry of the biosensor. Shifts in interference due to the accumulation of biomolecules are monitored in real-time to sensitively analyze and calculate rates of association and dissociation among target proteins with high precision.") (last visited Mar. 4, 2022).

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especially made for use in the Gator Bioanalysis System line of devices and are non-staple articles whose only substantial use infringes the '982 Patent.

110. Defendants have never been licensed, either expressly or impliedly, under the '982 Patent.

111. Plaintiff has complied with the requirements of 35 U.S.C. § 287 by providing actual or constructive notice to Defendants of their infringement.

112. Plaintiff has been, and continues to be, damaged and irreparably harmed by Defendants' infringements, which will continue unless this Court enjoins these infringements.

113. Plaintiff, under 35 U.S.C. § 284, is entitled to damages adequate to compensate for Defendants' infringement, including lost profits and/or a reasonable royalty.

114. Defendants' infringement of the '982 Patent has been, and continues to be, deliberate, willful, and knowing, thus entitling Plaintiff to treble damages under 35 U.S.C. § 284.

115. This is an exceptional case under 35 U.S.C. § 285, thus entitling Plaintiff to recover its reasonable attorney fees from Defendant.

FOURTH CAUSE OF ACTION

INFRINGEMENT OF U.S. PATENT NO. 8,305,585

116. Plaintiff restates and realleges the foregoing allegations as if fully stated herein.

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117. Defendants' Gator Bioanalysis System line of devices, including, but not limited to, the representative GatorPrime device, infringes at least independent claim 1 of the '585 Patent, which recites:

> **1**. An optical assembly for use in detecting an analyte in a sample based on interference, comprising:
> an optical fiber having a tip; and
> an optical element removably attached to the tip of the optical fiber and configured for receiving a beam of light from the optical fiber,
> said optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface separated from the first reflecting surface by the transparent material, said first and second reflecting surfaces separated by at least 50 nm,
> wherein said first reflecting surface binds a layer of analyte binding molecules positioned so that interference between a beam of light reflected from the first reflecting surface into the optical fiber and a beam of light reflected from the second reflecting surface into the optical fiber varies as analyte binds to the layer of analyte binding molecules.

118. Upon information and belief, the GatorPrime device is an optical assembly for use in detecting an analyte in a sample based on interference.[18]

119. Upon information and belief, the GatorPrime has an optical fiber that has a tip,[19] as shown in Figure 1 above.

120. Upon information and belief, the GatorPrime has an optical element removably attached to the tip of the optical fiber. The optical element is configured for receiving a beam of light from the optical fiber, as shown in Figure 7 above.

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[18] Gator Bio, Technology, https://www.gatorbio.com/technology ("Biolayer interferometry (BLI) is a label free biomolecular detection method created by Gator Bio co-founder, Hong Tan. In biolayer interferometry, biomolecular interactions are detected by measuring the interference pattern of white light reflected from the surface of a biosensor.") (last visited Mar. 4, 2022); Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 8:28 "Bio layer interferometry or BLI is part of the label free systems that has many other biophysical techniques where label free meaning you do not have any dye or fluorophore or any kind of special tags that you need to conjugate to your molecule of interest in order to see binding by using a fluorescence or chemiluminescence or anything all you do in this um technique is we are using uh white light and that's all.").

[19] UF ICBR, Seminar and Demonstration with Gator Bio BLI, YouTube (Oct. 21, 2021), https://www.youtube.com/watch?v=Myc2YUHkiMQ (at 26:29 "The optical fibers are shining through the probes.").

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121. Upon information and belief, the GatorPrime has an optical element that comprises a transparent material.

122. Upon information and belief, the GatorPrime optical element has a first reflecting surface and a second reflecting surface,[20] separated from the first reflecting surface by the transparent material, as shown in Figure 7 above.

123. Upon information and belief, the GatorPrime has first and second reflecting surfaces that are separated by at least 50 nm.

124. Upon information and belief, the GatorPrime has a first reflecting surface that binds a layer of analyte binding molecules. Upon further information and belief, the analyte binding molecules are located on the distal end of the first reflecting surface, as shown in Figure 4 above.

125. Upon information and belief, the GatorPrime has an interference between light reflected into the fiber from said first and second reflecting surfaces that varies as analyte in the sample binds to the analyte binding molecules.[21]

126. Defendants have been and are directly infringing, literally and/or under the doctrine of equivalents, one or more claims of the '585 Patent in violation of 35 U.S.C. § 271(a), by making, using, importing, selling, and/or offering for sale in or into the United States the Gator Bioanalysis System line of devices.

127. Additionally, Defendants have induced and continue to induce others to directly infringe one or more claims of the '585 Patent under 35 U.S.C. § 271(b) by, among other things, and with specific intent or willful blindness, actively aiding and abetting others to directly infringe, including but not limited to Defendants' partners, clients, customers, and end users,

[20] Gator Bio, Strategies for Accurate, Easy and Fast Quantitation of AAV Serotypes, YouTube (Oct. 25, 2021), https://www.youtube.com/watch?v=y-MbZPotvyo (at 10:27 "When the light is incidented, it will be reflected back from these two surfaces.").

[21] Gator Bio, Technology, https://www.gatorbio.com/technology ("Biolayer interferometry compares the interference pattern of white light reflected from an internal reference layer within a layer of immobilized biomolecules on the surface chemistry of the biosensor. Shifts in interference due to the accumulation of biomolecules are monitored in real-time to sensitively analyze and calculate rates of association and dissociation among target proteins with high precision.") (last visited Mar. 4, 2022).

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whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '585 Patent.

128. Defendants have contributed and continue to contribute to the direct infringement of one or more claims of the '585 Patent under 35 U.S.C. § 271(c) by providing the Gator Biosensor Probes, non-staple articles whose only substantial use infringes the '585 Patent, and thus contributing to the direct infringement of the '585 Patent of others, including but not limited to Defendants' partners, clients, customers, and end users, whose use of the Gator Bioanalysis System line of devices constitutes direct infringement of at least one claim of the '585 Patent.

129. Defendants have infringed one or more claims of the '585 Patent under 35 U.S.C. § 271(f) by supplying in or from the United States the Gator Biosensor Probes which are especially made for use in the Gator Bioanalysis System line of devices and are a non-staple articles whose only substantial use infringes the '585 Patent.

130. Defendants have never been licensed, either expressly or impliedly, under the '585 Patent.

131. Plaintiff has complied with the requirements of 35 U.S.C. § 287 by providing actual or constructive notice to Defendants of their infringement.

132. Plaintiff has been, and continues to be, damaged and irreparably harmed by Defendants' infringement, which will continue unless this Court enjoins these infringements.

133. Plaintiff, under 35 U.S.C. § 284, is entitled to damages adequate to compensate for Defendants' infringements, including lost profits and/or a reasonable royalty.

134. Defendants' infringement of the '585 Patent has been, and continues to be, deliberate, willful, and knowing, thus entitling Plaintiff to treble damages under 35 U.S.C. § 284.

135. This is an exceptional case under 35 U.S.C. § 285, thus entitling Plaintiff to recover its reasonable attorney fees from Defendants.

PRAYER FOR RELIEF

Sartorius hereby requests the following relief from the Court:

(a) That judgment be entered for Plaintiff and against Defendants on this Complaint on all claims asserted herein;

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(b) That the Court award Sartorius such damages as may be proven at trial, in accordance with each of the claims asserted in this Complaint;

(c) That the Court find Defendants' infringement of the Asserted Patents to be willful, and that this case be deemed exceptional under 35 U.S.C. § 285, that Plaintiff's damages be trebled, and that Plaintiff be awarded attorney fees and nontaxable costs and expenses;

(d) That the Court award Plaintiff pre-judgment and post-judgment interest;

(e) That the Court immediately enjoin and restrain, preliminarily and permanently, Defendants, their subsidiaries, affiliates, agents, officers, servants, employees, attorneys, successors, and assigns and all other persons in active concert or participation with Defendants, from manufacturing, distributing, selling or offering to sell, in the United States or importing into the United States products infringing the claims of the Asserted Patents;

(f) That the Court award Plaintiff its taxable costs incurred in this action;

(d) That the Court award Plaintiff such further relief as the Court deems just and proper.

Dated: March 4, 2022

By: */s/ Stuart G. Gross*

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DEMAND FOR A JURY TRIAL

Sartorius, under Rule 38 of the Federal Rules of Civil Procedure, requests a trial by jury on all issues so triable.

Dated: March 4, 2022

By: */s/ Stuart G. Gross*

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EXHIBIT A

U 8189137

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

December 13, 2021

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

U.S. PATENT: *7,394,547*
ISSUE DATE: *July 01, 2008*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

SYLVIA HOLLEY
Certifying Officer

US007394547B2

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 7,394,547 B2**
(45) **Date of Patent: *Jul. 1, 2008**

(54) **FIBER-OPTIC ASSAY APPARATUS BASED ON PHASE-SHIFT INTERFEROMETRY**

(75) Inventors: **Hong Tan**, San Jose, CA (US); **Duan Jun Chen**, East Brunswick, NJ (US); **Yushan Tan**, Shanghai (CN); **Krista Leah Witte**, Hayward, CA (US)

(73) Assignee: **ForteBio, Inc.**, Menlo Park, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 197 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/981,901**

(22) Filed: **Nov. 4, 2004**

(65) **Prior Publication Data**

US 2005/0254062 A1 Nov. 17, 2005

Related U.S. Application Data

(60) Provisional application No. 60/558,381, filed on Mar. 31, 2004, provisional application No. 60/518,068, filed on Nov. 6, 2003.

(51) **Int. Cl.**
G01B 9/02 (2006.01)

(52) **U.S. Cl.** **356/480**

(58) **Field of Classification Search** 356/480, 356/519

See application file for complete search history.

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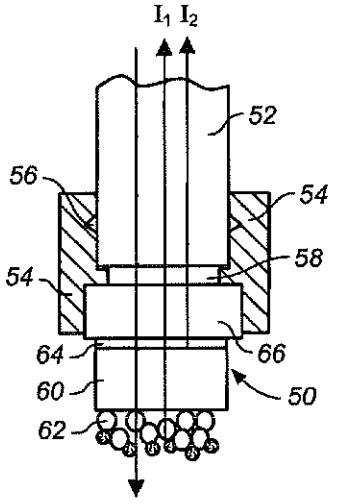
Primary Examiner—Samuel A Turner
(74) *Attorney, Agent, or Firm*—Fenwick & West LLP

(57) **ABSTRACT**

Apparatus and method for detecting the presence or amount or rate of binding of an analyte in a sample solution is disclosed. The apparatus includes an optical assembly having first and second reflecting surfaces separated by a distance "d" greater than 50 nm, where the first surface is formed by a layer of analyte-binding molecules, and a light source for directing a beam of light onto said first and second reflecting surface. A detector in the apparatus operates to detect a change in the thickness of the first reflecting layer resulting from binding of analyte to the analyte-binding molecules, when the assembly is placed in the solution of analyte, by detecting a shift in phase of light waves reflected from the first and second surfaces.

23 Claims, 6 Drawing Sheets



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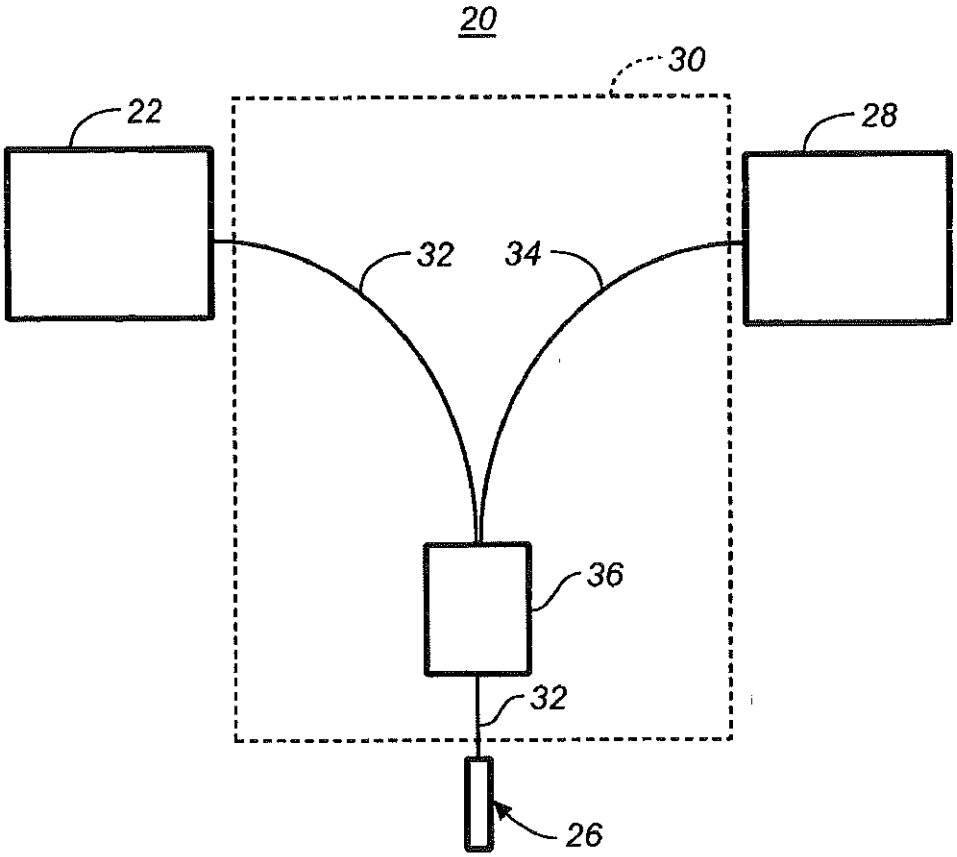

FIG. _1

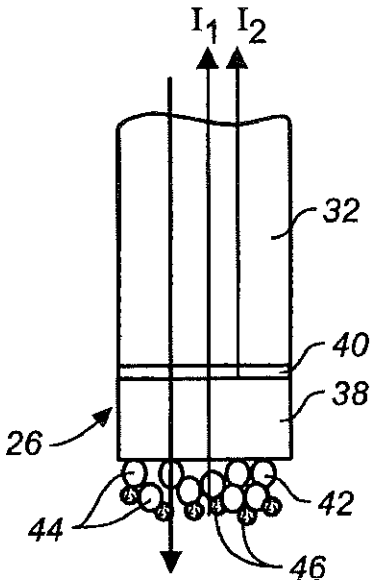

FIG. _2

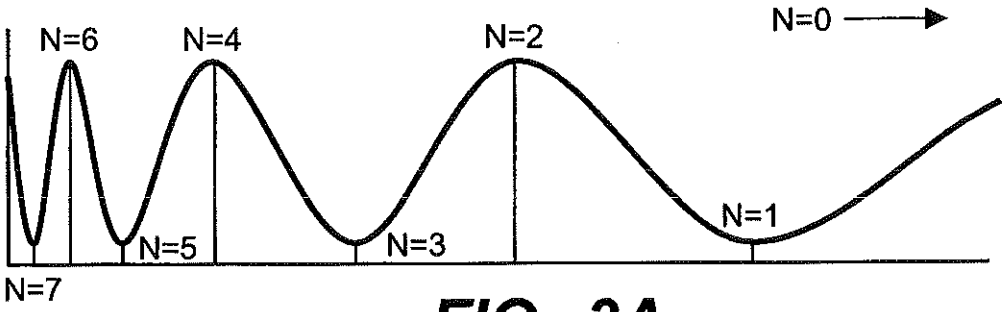

FIG. _3A

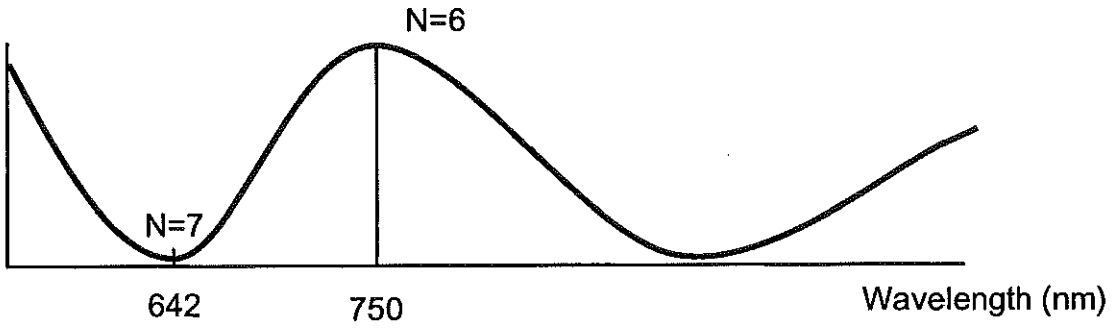

FIG. _3B

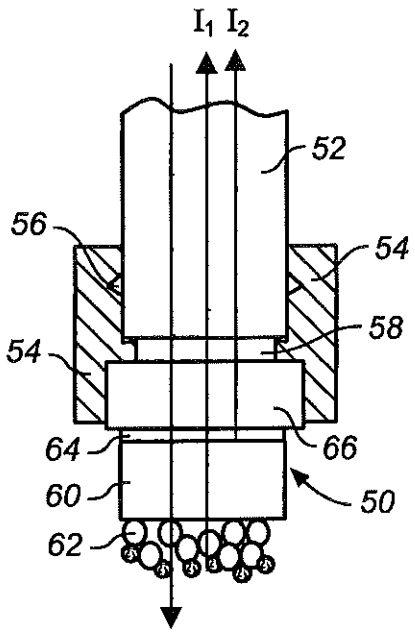

FIG. _4

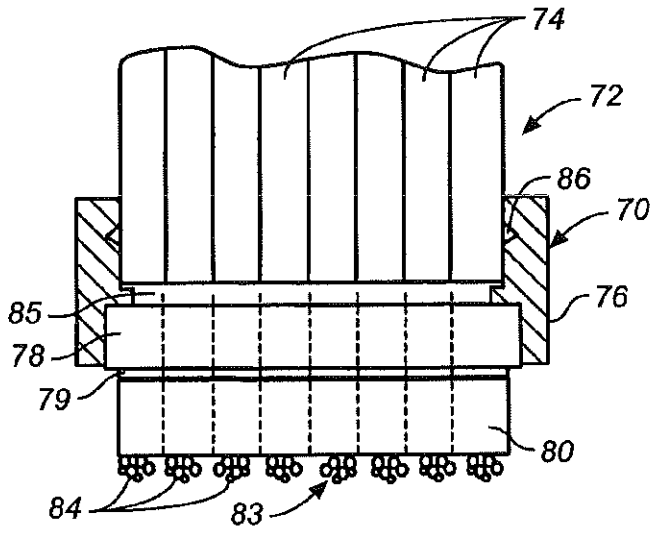

FIG. _5

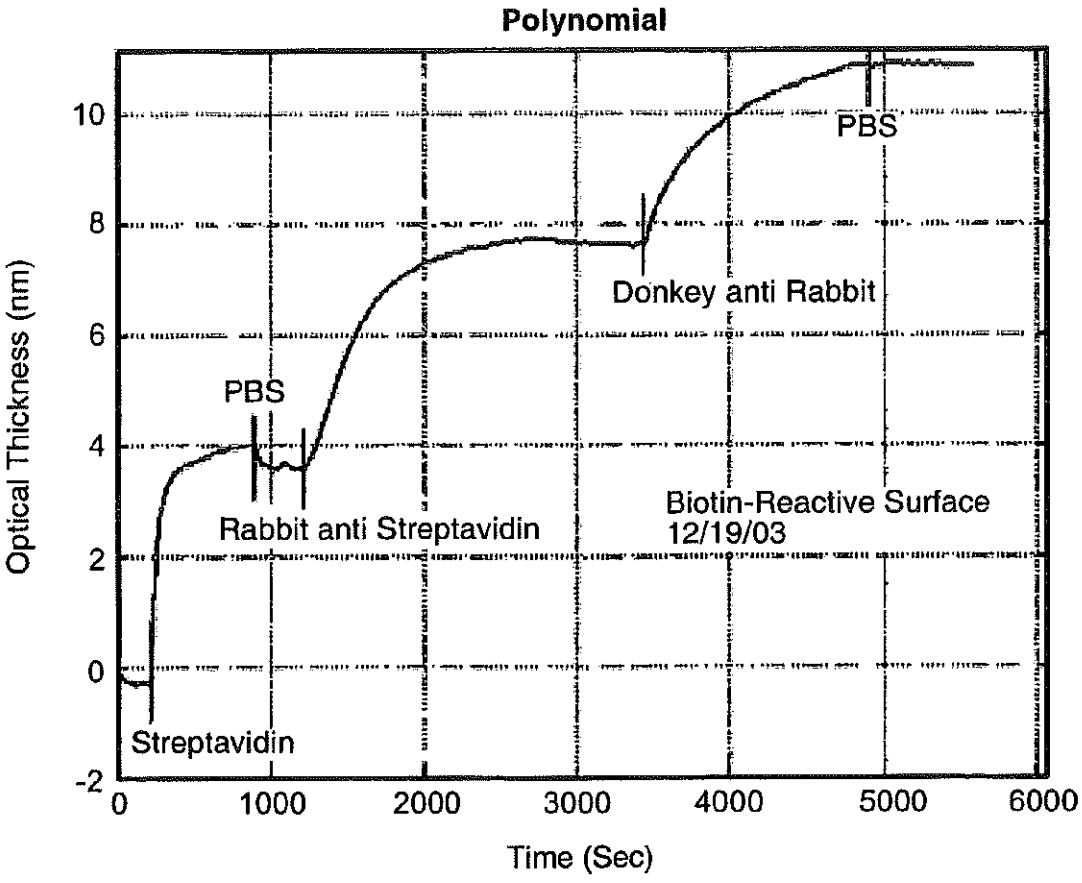

FIG. _6

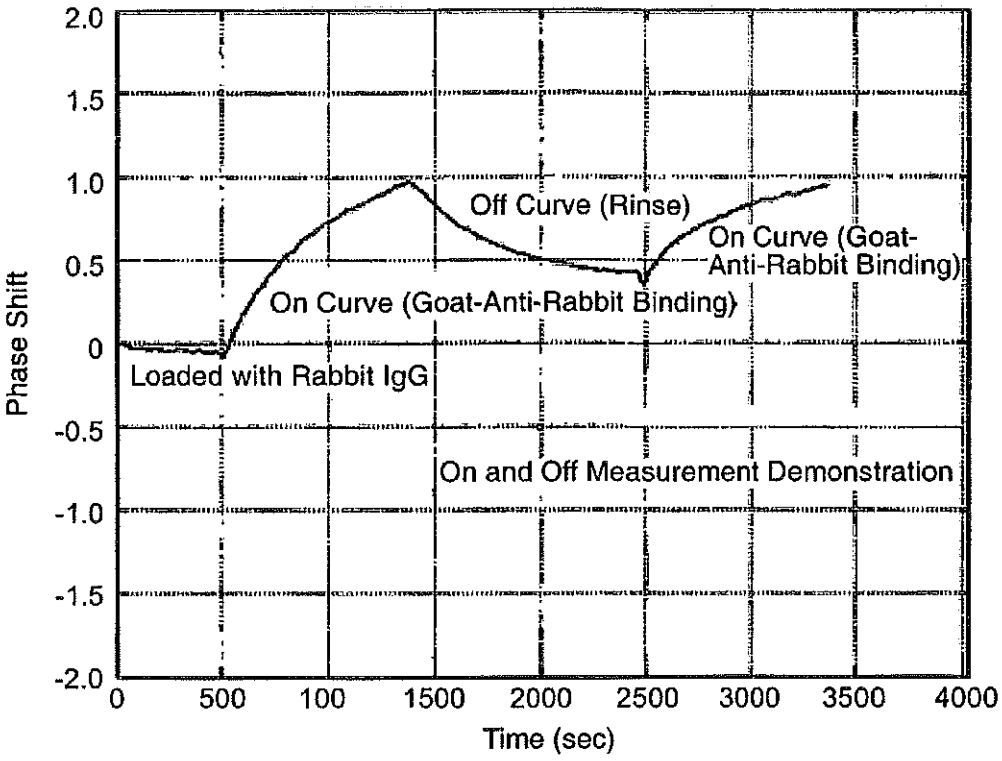

FIG. _7

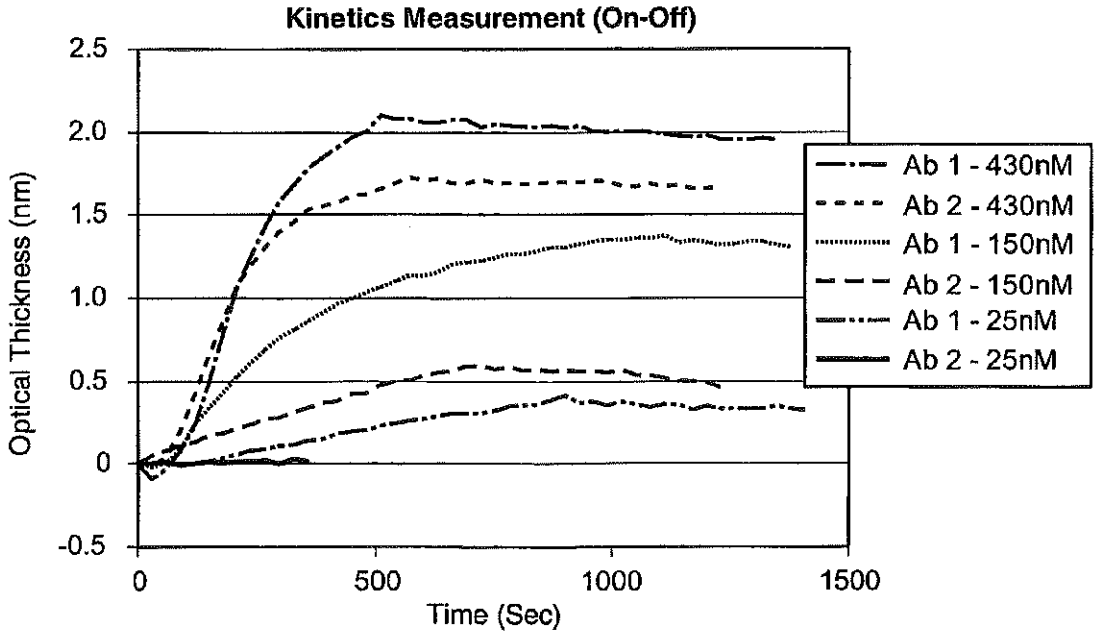

FIG. _8

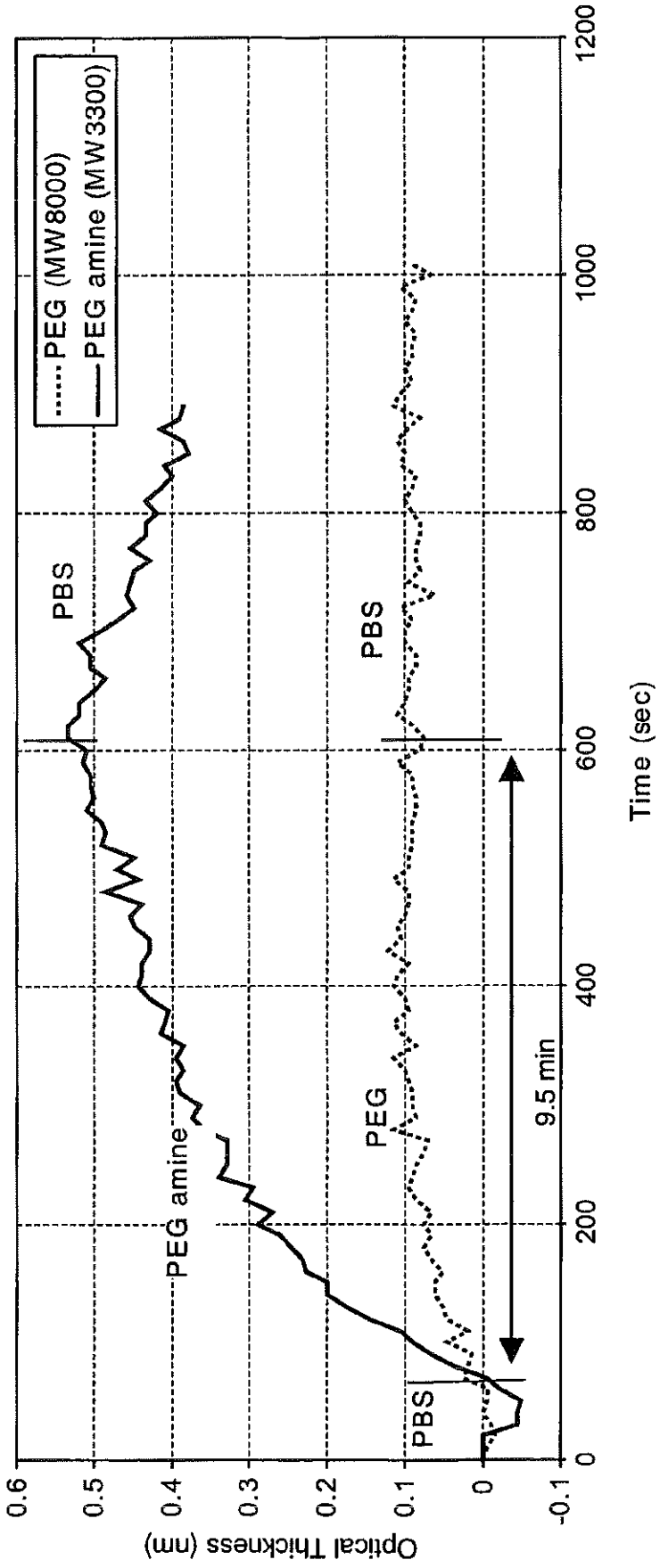

FIG._9

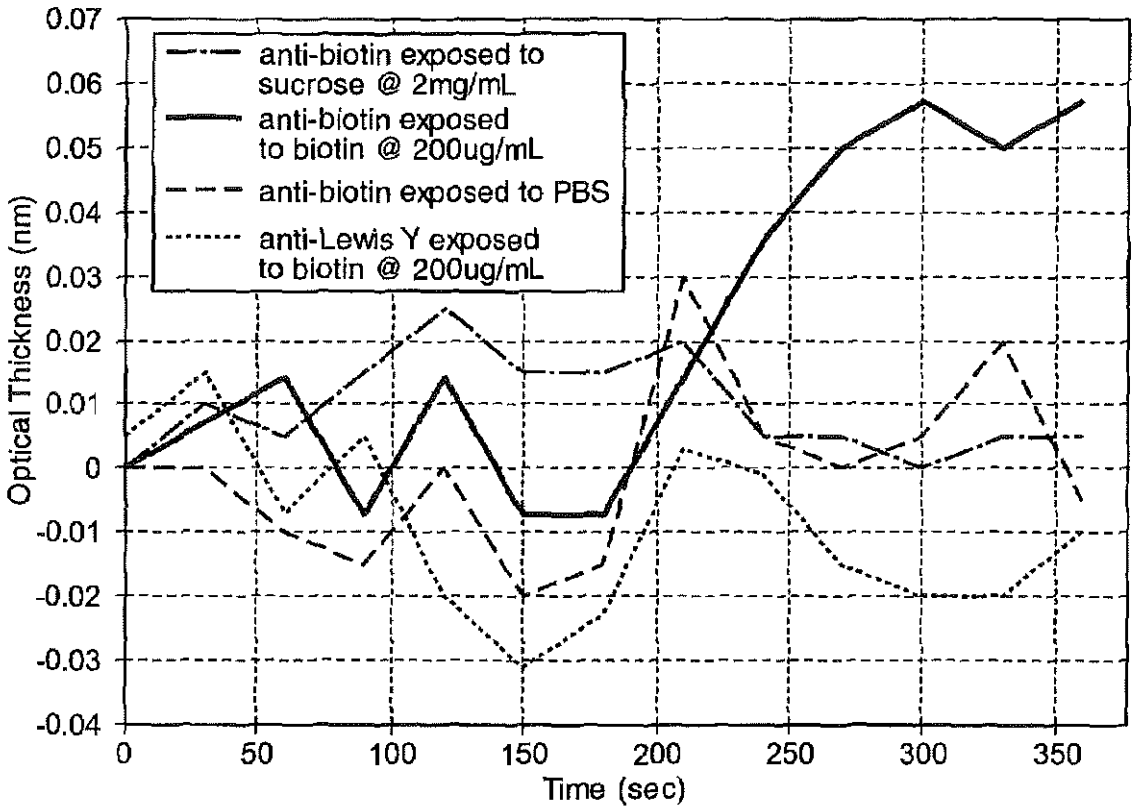

FIG. _10

FIBER-OPTIC ASSAY APPARATUS BASED ON PHASE-SHIFT INTERFEROMETRY

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/518,068, filed Nov. 6, 2003, and U.S. Provisional Application No. 60/558,381, filed Mar. 31, 2004 the entire disclosures of which are hereby incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to an apparatus and method for detecting the presence, amount, or rate of binding of one or more analytes in a sample, and in particular, to apparatus and method based on fiber optic interferometry.

2. Description of the Related Art

Diagnostic tests based on a binding event between members of an analyte-anti-analyte binding pair are widely used in medical, veterinary, agricultural and research applications. Typically, such methods are employed to detect the presence or amount or an analyte in a sample, and/or the rate of binding of the analyte to the anti-analyte. Typical analyte-anti-analyte pairs include complementary strands of nucleic acids, antigen-antibody pairs, and receptor-receptor binding agent, where the analyte can be either member of the pair, and the anti-analyte molecule, the opposite member.

Diagnostics methods of this type often employ a solid surface having immobilized anti-analyte molecules to which sample analyte molecules will bind specifically and with high affinity at a defined detection zone. In this type of assay, known as a solid-phase assay, the solid surface is exposed to the sample under conditions that promote analyte binding to immobilized anti-analyte molecules. The binding event can be detected directly, e.g., by a change in the mass, reflectivity, thickness, color or other characteristic indicative of a binding event. Where the analyte is pre-labeled, e.g., with a chromophore, or fluorescent or radiolabel, the binding event is detectable by the presence and/or amount of detectable label at the detection zone. Alternatively, the analyte can be labeled after it is bound at the detection zone, e.g., with a secondary, fluorescent-labeled anti-analyte antibody.

Co-owned U.S. Pat. No. 5,804,453, (the '453 patent) which is incorporated herein by reference, discloses a fiber-optic interferometer assay device designed to detect analyte binding to a fiber-optic end surface. Analyte detection is based on a change in the thickness at the end surface of the optical fiber resulting from the binding of analyte molecules to the surface, with greater amount of analyte producing a greater thickness-related change in the interference signal. The change in interference signal is due to a phase shift between light reflected from the end of the fiber and from the binding layer carried on the fiber end, as illustrated particularly in FIGS. 7*a* and 7*b* of the '453 patent. The device is simple to operate and provides a rapid assay method for analyte detection.

Ideally, an interferometer assay device will yield readily observable changes in spectral peak and valley (extrema) positions within the range of a conventional visible-light spectrometer, that is, in the visible light range between about

450-700 nm, such that relatively small optical thickness changes at the fiber end can be detected as significant changes in the spectral positions of interference wavelength peaks and valleys. One limitation which has been observed with the device described in the '453 patent is the absence of readily identified wavelength spectral extrema over this spectral range.

The present invention is designed to overcome this limitation, preserving the advantages of speed and simplicity of the earlier-disclosed device, but significantly enhancing sensitivity and accuracy. The present invention also provides a more convenient disposable-head format, as well as a multi-analyte array format, e.g., for gene-chip and protein-chip applications.

SUMMARY OF THE INVENTION

The invention includes, in one aspect, an apparatus for detecting an analyte in a sample, including detecting the presence of analyte, the amount of analyte or the rate of association and/or dissociation of analyte to analyte-binding molecules. The apparatus includes an optical element with a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm. A beam of light from an optical fiber is directed to and reflected from the two reflecting surfaces. The reflected beams are coupled back into the optical fiber and interfere. The optical element also includes a layer of analyte binding molecules that is positioned so that the interference between the reflected beams varies as analyte binds to the layer of analyte binding molecules.

The change in interference can be caused by different physical phenomenon. For example, analyte binding can cause a change in the optical path length or in the physical distance between the two reflecting surfaces. Alternately, analyte binding can cause a change in the index or in the optical absorption of material located between the reflecting surfaces. Analyte binding can also cause the layer of analyte binding molecules to swell, resulting in a change in the interference.

In one particular design, the distal reflecting surface includes the layer of analyte binding molecules. As analyte binds to the layer of analyte binding molecules, the optical path length or the physical distance between the two reflecting surfaces may increase, for example. In another aspect of the invention, a transparent solid material is located between the reflecting surfaces and, optionally, the proximal reflecting surface includes a material with an index greater than that of the transparent solid material. Alternately, an air gap may be located between the reflecting surfaces. In yet another design, the distal reflecting surface is positioned between the proximately reflecting surface and the layer of analyte binding molecules. For example, analyte binding may cause the layer of analyte binding molecules to swell, moving the distal reflecting surface closer to the proximal reflecting surface. In yet another design, the layer of analyte binding molecules is positioned between the two reflecting surfaces. Analyte binding may cause the layer to swell or to change its index, thus changing the interference between the two reflected beams.

In another aspect, the apparatus includes an optical assembly having first and second reflecting surfaces separated by a distance "d" greater than 50 nm. The optical assembly is composed of a transparent optical element that can have a thickness defined between proximal and distal faces of the element of at least 50 nm, preferably between 400-1,000 nm. The first reflecting surface is carried on the distal face of optical element, and is formed of a layer of analyte-binding molecules. The second reflecting surface is formed by a coat-

ing of transparent material having an index of refraction greater than that of the optical element. This coating can be formed of a Ta_2O_5 layer having a preferred thickness of between 5 and 50 nm. The optical element can be SiO_2, and has a thickness of between about 100-5,000 nm, preferably 400-1,000 nm.

Also included are a light source for directing a beam of light onto the first and second reflecting surfaces, and a detector unit that operates to detect a change in the optical thickness of the first reflecting layer resulting from binding of analyte to the analyte-binding molecules, when the assembly is placed in the solution of analyte. The optical thickness change at the first reflecting layer is related to a shift in a phase characteristic of the interference wave formed by the two light waves reflected from said first and second surfaces. This phase characteristic can be a shift in the spectral position(s) of one or more peaks and valleys of the interference wave, or by a change in the period of a full cycle of the wave.

The light source can include an optical fiber having a distal end adapted to be placed adjacent the second reflecting surface in the assembly, and the apparatus further includes an optical coupling for directing reflected light waves reflected from the assembly to the detector.

In a first embodiment, the optical assembly is fixedly mounted on the optical fiber, with the distal end of the optical fiber in contact with the second reflecting surface. In a second embodiment, the optical assembly further includes a second transparent optical element having an index of refraction less than that of the second coating and a thickness greater than about 100 nm, where the coating of high index of refraction material is sandwiched between the two transparent optical elements. In this latter embodiment, the assembly is removably attached to the distal end region of the fiber with a spacing of less than 100 nm or greater than 2 μm between the distal end of the fiber and the confronting face of the second transparent optical element in the assembly.

For detecting multiple analytes, such as multiple nucleic acid species, the layer of analyte-binding molecules can be composed of an array of discrete analyte-binding regions, such as single strands of nucleic acid. The regions are effective to bind different analytes. The optical fiber includes a plurality of individual fibers, each aligned with one of the regions, the detector includes a plurality of detection zones, and the optical coupling functions to couple each of the plurality of fibers with one of the zones.

The analyte-binding molecules in the assembly can be, for example, (i) an anti-species antibody molecules, for use in screening hybridoma libraries for the presence of secreted antibody, (ii) antigen molecules, for use in detecting the presence of antibodies specific against that antigen; (iii) protein molecules, for use in detecting the presence of a binding partner for that protein; (iv) protein molecules, for use in detecting the presence of multiple binding species capable of forming a multi-protein complex with the protein; or (v) single stranded nucleic acid molecules, for detecting the presence of nucleic acid binding molecules.

The detector can be a spectrometer for measuring reflected light intensity over a selected range of wavelengths. Alternatively, or in addition, the light source can include a plurality of light-emitting diodes, each with a characteristic spectral frequency, and the detector functions to record light intensity of reflected light at each of the different LED frequencies. In still another embodiment, the light source includes a white-light source and the detector is designed to record light intensity of reflected light at each of a plurality of different wavelengths.

In another aspect, the invention includes a method for detecting the presence or amount of an analyte in a sample solution. The method involves reacting the sample solution with a first reflecting surface formed by a layer of analyte-binding molecules carried on the distal surface of a transparent optical element having a thickness of at least 50 nm, thereby to increase the thickness of the first reflecting layer by the binding of analyte to the analyte-binding molecules in the layer. The change in thickness of the first reflecting layer is measured by detecting a shift in a phase characteristic of the interference wave formed by the two light waves reflected from the first layer and from a second reflecting layer that is formed on the opposite, proximal surface of the optical element and which has an index of refraction greater than that of the optical element.

The detecting step can include directing light from an optical fiber onto the two reflecting surfaces, and directing reflected light from the two surfaces onto a detector through an optical coupling. The detector can be a spectrometer, where the detecting includes measuring a shift in the spectral position of one or more of the interference extrema produced by the two reflecting lightwaves.

Where the method is used for measuring the rate of association of analyte to the second layer, the reacting step can be carried out until a near-maximum increase in thickness of the first reflecting layer is observed. Where the method is used for measuring the rate of dissociation of analyte to the second layer, the reacting steps can include immersing the second layer in a dissociation buffer for a period of time until a decrease in thickness of the first reflecting layer is observed. Where the method is used for measuring the amount of analyte present in the sample, the detecting is carried out over a period sufficient to measure the thickness of the first reflecting layer at a plurality of different time points.

Where the method is used measuring one or more of a plurality of analytes in a sample, the first reflecting layer is composed of an array of discrete analyte-binding regions, the different regions being effective to bind different analytes, and the detecting is effective to detect a change in the thickness of each of the regions resulting from binding of analyte to the analyte-binding molecules.

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

FIG. **1** shows the basic system setup for the bioprobe and its apparatus;

FIG. **2** shows an optical assembly formed accordance to one embodiment of the invention;

FIGS. **3**A and **3**B show a portion of an interference wave over 7 peak and valley orders (**3**A), and over in a visible portion of the spectrum (**3**B);

FIG. **4** shows an optical assembly constructed according to another embodiment of the invention;

FIG. **5** shows a disposable multi-analyte optical assembly having an analyte-binding array and constructed according to another embodiment of the invention;

FIG. **6** shows a sequential binding of three molecules;

FIG. **7** shows on and off curves generated from the association and dissociation of antibodies;

FIG. **8** shows the curves of two antibodies binding to their antigen at different concentrations;

FIG. **9** shows immobilization of bis amino PEG (MW 3300) specifically through an amide bond formation. The PEG (MW 8000) is used as a negative control to monitor non-specific binding of the PEG polymer; and

FIG. **10** shows a small molecule binding to a large molecule, negative controls and the base line measurement.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Terms used in the claims and specification are to be construed in accordance with their usual meaning as understood by one skilled in the art except and as defined as set forth below. Numeric ranges recited in the claims and specification are to be construed as including the limits bounding the recited ranges.

The term "in vivo" refers to processes that occur in a living organism.

An "analyte-binding" molecule refers to any molecule capable of participating in a specific binding reaction with an analyte molecule. Examples include but are not limited to, e.g., antibody-antigen binding reactions, and nucleic acid hybridization reactions.

A "specific binding reaction" refers to a binding reaction that is saturable, usually reversible, and that can be competed with an excess of one of the reactants. Specific binding reactions are characterized by complementarity of shape, charge, and other binding determinants as between the participants in the specific binding reaction.

An "antibody" refers to an immunoglobulin molecule having two heavy chains and two light chains prepared by any method known in the art or later developed and includes polyclonal antibodies such as those produced by inoculating a mammal such as a goat, mouse, rabbit, etc. with an immunogen, as well as monoclonal antibodies produced using the well-known Kohler Milstein hybridoma fusion technique. The term includes antibodies produced using genetic engineering methods such as those employing, e.g., SCID mice reconstituted with human immunoglobulin genes, as well as antibodies that have been humanized using art-known resurfacing techniques.

An "antibody fragment" refers to a fragment of an antibody molecule produced by chemical cleavage or genetic engineering techniques, as well as to single chain variable fragments (SCFvs) such as those produced using combinatorial genetic libraries and phage display technologies. Antibody fragments used in accordance with the present invention usually retain the ability to bind their cognate antigen and so include variable sequences and antigen combining sites.

A "small molecule" refers to an organic compound having a molecular weight less than about 500 daltons. Small molecules are useful starting materials for screening to identify drug lead compounds that then can be optimized through traditional medicinal chemistry, structure activity relationship studies to create new drugs. Small molecule drug compounds have the benefit of usually being orally bioavailable. Examples of small molecules include compounds listed in the following databases: MDL/ACD (http://www.mdli.com/), MDL/MDDR (http://www.mdli.com/), SPECS (http://www.specs.net/), the China Natural Product Database (CNPD) (http://www.neotrident.com/), and the compound sample database of the National Center for Drug Screening (http://www.screen.org.cn/).

Abbreviations used in this application include the following: "ss" refers to single-stranded; "SNP" refers to single nucleotide polymorphism; "PBS" refers to phosphate buffered saline (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4); "NHS" refers to N-hydroxysuccinimide; "MW" refers to molecular weight; "Sulfo-SMCC" refers to sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Advantages and Utility

The advantages and utility of the invention are illustrated by reference to the Figures and Examples as described in greater detail below. These include the ability to monitor in real time analyte binding reactions without the use of labels, diminishing cost and potential toxicity. A further advantage includes the ability to practice the method using visible wavelength light sources. Yet other advantages are provided by the fiber optic nature of the detector tip that allows binding reactions to be monitored in very small sample volumes, including in "in vitro" spaces, and to bundle fibers to carry out highly multiplexed analyses of binding reactions.

FIG. **1** shows, in schematic view, an interferometer apparatus **20** constructed in accordance with the invention. In its most basic elements, the apparatus includes a light source **22**, an optical assembly **26** that functions as a sensing element or detector tip and that will be detailed further with respect to FIGS. **2**, **4** and **5** below, and a detector unit **28** for detecting interference signals produced by interfering light waves reflected from the optical assembly **26**.

Light from source **22** is directed onto the optical assembly **26**, and reflected back to the detector through an optical coupling assembly indicated by dashed lines at **30**. In a preferred embodiment, the coupling assembly includes a first optical waveguide or fiber **32** extending from the light source to the optical assembly, a second optical waveguide or fiber **34** which carries reflected light from the optical assembly to the detector, and an optical coupler **36** which optically couples fibers **32**, **34**. Suitable optical fiber and coupling components are detailed in the above-cited '453 patent. One exemplary coupler is commercially available from many vendors including Ocean Optics (Dunedin, Fla.).

Alternatively, the coupling assembling can include a lens system constructed to focus a light beam onto the upper surface of the optical assembly and to direct reflected interfering light from the optical assembly to the detector. The latter system would not require optical fibers, but would impose relatively stringent requirements on the positioning of the lens elements used for the optical coupling.

The light source in the apparatus can be a white light source, such as a light emitting diode (LED) which produces light over a broad spectrum, e.g., 400 nm or less to 700 nm or greater, typically over a spectral range of at least 100 nm. Alternatively, the light source can be a plurality of sources each having a different characteristic wavelength, such as LEDs designed for light emission at different selected wavelengths in the visible light range. The same function can be achieved by a single light source, e.g., white light source, with suitable filters for directing light with different selected wavelengths onto the optical assembly.

The detector is preferably a spectrometer, such as charge-coupled device (CCD), capable of recording the spectrum of the reflected interfering light from the optical assembly. Alternatively, where the light source operates to direct different selected wavelengths onto the optical assembly, the detector can be a simple photodetector for recording light intensity at each of the different irradiating wavelengths. In still

another embodiment, the detector can include a plurality of filters which allows detection of light intensity, e.g., from a white-light source, at each of a plurality of selected wavelengths of the interference reflectance wave. Exemplary light source and detector configurations are described in the above-cited '453 patent, particularly with respect to FIGS. **8** and **10** of that patent, and it will be understood that these configurations are suitable for use in the present invention.

FIG. **2** shows an optical assembly **26** constructed in accordance with one embodiment of the invention, and an adjoining portion of the distal end region of an optical fiber **32** to which the optical assembly is fixedly attached. As seen, the assembly **26** includes a transparent optical element **38** having first and second reflecting surfaces **42**, **40** formed on its lower (distal) and upper (proximal) end faces, respectively. According to an important feature of the invention, the thickness "d" of the optical element between its distal and proximal surfaces, i.e., between the two reflecting surfaces, is at least 50 nm, and preferably at least 100 nm. An exemplary thickness is between about 100-5,000 nm, preferably 400-1,000 nm. The first reflecting surface **42** is formed of a layer of analyte-binding molecules, such as molecules **44**, which are effective to bind analyte molecules **46** specifically and with high affinity. That is, the analyte and anti-analyte molecules are opposite members of a binding pair of the type described above, which can include, without limitations, antigen-antibody pairs, complementary nucleic acids, and receptor-binding agent pairs.

The index of refraction of the optical element is preferably similar to that of the first reflecting surface, so that reflection from the lower distal end of the end optical assembly occurs predominantly from the layer formed by the analyte-binding molecules, rather than from the interface between the optical element and the analyte-binding molecules. Similarly, as analyte molecules bind to the lower layer of the optical assembly, light reflection form the lower end of the assembly occurs predominantly from the layer formed by the analyte-binding molecules and bound analyte, rather than from the interface region. One exemplary material forming the optical element is SiO_2, e.g., a high-quality quality glass having an index of refraction of about 1.4-1.5. The optical element can also be formed of a transparent polymer, such as polystyrene or polyethylene, having an index of refraction preferably in the 1.3-1.8 range.

The second reflecting surface in the optical assembly formed as a layer of transparent material having an index of refraction that is substantially higher than that of the optical element, such that this layer functions to reflect a portion of the light directed onto the optical assembly. Preferably, the second layer has a refractive index greater than 1.8. One exemplary material for the second layer is Ta_2O_5 with refractive index equal to 2.1. The layer is typically formed on the optical element by a conventional vapor deposition coating or layering process, to a layer thickness of less than 50 nm, typically between 5 and 30 nm.

The thickness of the first (analyte-binding) layer is designed to optimize the overall sensitivity based on specific hardware and optical components. Conventional immobilization chemistries are used in chemically, e.g., covalently, attaching a layer of analyte-binding molecules to the lower surface of the optical element. For example, a variety of bifunctional reagents containing a siloxane group for chemical attachment to SiO_2, and an hydroxyl, amine, carboxyl or other reaction group for attachment of biological molecules, such as proteins (e.g., antigens, antibodies), or nucleic acids. It is also well known to etch or otherwise treat glass a glass surface to increase the density of hydroxyl groups by which analyte-binding molecules can be bound. Where the optical element is formed of a polymer, such as polystyrene, a variety of methods are available for exposing available chemically-active surface groups, such as amine, hydroxyl, and carboxyl groups.

The analyte-binding layer is preferably formed under conditions in which the distal surface of the optical element is densely coated, so that binding of analyte molecules to the layer forces a change in the thickness of the layer, rather than filling in the layer. The analyte-binding layer can be either a monolayer or a multi-layer matrix.

The measurement of the presence, concentration, and/or binding rate of analyte to the optical assembly is enabled by the interference of reflected light beams from the two reflecting surfaces in the optical assembly. Specifically, as analyte molecules attach to or detach from the surface, the average thickness of the first reflecting layer changes accordingly. Because the thickness of all other layers remains the same, the interference wave formed by the light waves reflected from the two surfaces is phase shifted in accordance with this thickness change.

Assume that there are two reflected beams: The first beam is reflected from the first surface, which is the distal end interface between analyte-binding molecules and bound analyte and the surrounding medium; and the second beam is reflected from the second surface, which is the proximal interface between the optical element (the first layer) and the high-index of refraction layer (the second layer). The overall wavelength-dependent intensity of the interference wave is:

$$I = I_1 + I_2 + 2\sqrt{I_1 I_2}\cos\left(\frac{2\pi\Delta}{\lambda}\right)$$

where I is the intensity, I_1 and I_2 are the intensity of two interference beams, Δ is the optical path difference, and λ is the wavelength.

When $(2\pi\Delta/\lambda)=N\pi$, the curve is at its peak or valley if N is an integer 0, 1, 2, ...

The thickness of the first layer $d=\Delta/2n$. Therefore, $\lambda=4nd/N$ at peaks or valleys (extrema).

For the first several values of N, i.e., 0, 1, 2, . . . 7, and assuming a d of 770 nm, the equation gives:

N=0: $\lambda=\infty$ (peak)
N=1: $\lambda=4nd$=4,496.80 nm (Valley)
N=2: $\lambda=2nd$=2,248.40 nm (Peak)
N=3: $\lambda=4nd/3$=1,498.9 nm (Valley)
N=4: $\lambda=nd$=1,124.20 nm (Peak)
N=5: $\lambda=4nd/5$=899.36 nm (Valley)
N=6: $\lambda=2nd/3$=749.47 nm (Peak)
N=7: $\lambda=4nd/7$=642 nm (Valley)
N=8: $\lambda=nd/2$=562 nm (Peak)
N=9: $\lambda=4nd/9$=499.64 nm (Valley)
N=10: $\lambda=4nd/10$=449.6 nm (Peak)

As can be seen, and illustrated further in FIGS. **3**A and **3**B, at least three peaks/valleys (N=7-9) occur in the visible spectral range.

If the 7^{th} order valley is used to calculate the change in molecular layer thickness, when the molecular layer attached to the first layer increases from 0 nm to 10 nm, the 7^{th} order valley will shift to 650.74 nm. Therefore, the ratio between the actual the phase shift of the 7^{th} order valley and thickness change equals (650.74-642.40)/10=0.834.

By contrast, if the initial spacing between the two reflecting layers is made up entirely of the analyte-binding molecules on the end of the fiber, assuming a thickness of this layer of 25

nm, then the first order peak will occur at 146 nm, clearly out of the range of the visible spectrum, so that the device will only see a portion of the region between the O-order valley and the first order peak, but will not see any peaks, making a shift in the spectral characteristics of the interference wave difficult to measure accurately.

Not until the total thickness of the reflecting layer approaches about 100 nm will the first-order peak appear in the visible spectrum. Assuming a total thickness change of up to 50 nm, the thickness of the optical element can then be as small as 50 nm, but is preferably on the order of several hundred nm, so that the phase shift or change in periodicity of the interference wave can be measured readily by a shift in the spectral positions of higher-order peaks or valleys, e.g., where N=3-10.

The ratio between the actual thickness and the measured phase shift is considered as a key factor of measurement sensitivity. It can be appreciated how one can adjust the thickness of the optical element and its refractive index to improve and optimize the sensitivity to accommodate the electronics and optical designs.

FIG. **4** shows an optical assembly **50** that is removably carried on the distal end of an optical fiber **52** in the assay apparatus. The optical element includes a plurality of flexible gripping arms, such as arms **54**, that are designed to slide over the end of the fiber and grip the fiber by engagement of an annular rim or detente **56** on the fiber with complementary-shaped recesses formed in the arms, as shown. This attachment serves to position the optical assembly on the fiber to provide an air gap **58** between the distal end of the fiber and the confronting (upper) face of the assembly, of less than 100 nm or greater than 2 μm. With an air gap of greater than about 100 nm, but less that 2 μm, internal reflection from the upper surface of the optical assembly can contribute significantly to undesirable fringes that can adversely impact the detection accuracy.

With continued reference to FIG. **4**, the optical assembly includes a first optical element **60** similar to optical element **38** described above, and having first and second reflective layers **62**, **64**, respectively, corresponding to above-described reflective layers **40**, **42**, respectively. The assembly further includes a second optical element **66** whose thickness is preferably greater than 100 nm, typically at least 200 nm, and whose index of refraction is similar to that of first optical element **60**. Preferably, the two optical elements are constructed of the same glass or a polymeric material having an index of refraction of between about 1.4 and 1.6. Layer **64**, which is formed of a high index of refraction material, and has a thickness preferably less than about 30 nm, is sandwiched between the 2 optical elements as shown.

In operation, the optical assembly is placed over the distal fiber end and snapped into place on the fiber. The lower surface of the assembly is then exposed to a sample of analyte, under conditions that favor binding of sample analyte to the analyte-binding molecules forming reflective layer **62**. As analyte molecules bind to this layer, the thickness of the layer increases, increasing the distance "d" between reflective surfaces **62** and **64**. This produces a shift in the extrema of the interference wave produced by reflection from the two layers, as described above with reference to FIGS. **3**A and **3**B. This shift in extrema or wavelength, or wavelength period, in turn, is used to determine the change in thickness at the lower (distal-most) reflecting layer. After use, the optical assembly can be removed and discarded, and replaced with fresh element for a new assay, for assaying the same or a different analyte.

FIG. **5** illustrates an optical assembly and fiber bundle in an embodiment of the invention designed for detecting one or more of a plurality of analytes, e.g., different-sequence nucleic acid analytes, in a sample. A fiber bundle **72** is composed of an array, e.g., circular array, for individual optical fibers, such as fibers **74**. The optical assembly, indicated generally at **70**, is composed of the basic optical elements described above with reference to FIG. **4**, but in an array format. Specifically, a first optical element **80** in the element provides at its lower distal surface, an array of analyte-reaction regions, such as regions **84**, each containing a layer of analyte-binding molecules effective to bind to one of the different analytes in the sample. Each region forms a first reflective layer in the optical assembly. One preferred sensing provides an array of different-sequence nucleic acids, e.g., cDNAs or oligonucleotides, designed to hybridize specifically with different-sequence nucleic acid analyte species in a sample. That is, the array surface forms a "gene chip" for detecting each of a plurality of different gene sequences.

Also included in the optical assembly are a second optical element **78** and a layer **79** of high index of refraction material sandwiched between the two optical elements, and which provides the second reflecting surface in the optical assembly. The assembly is carried on the fiber bundle **72** by engagement between a pair of flexible support arm, such as arm **76** and an annular rim or detente **86** on the bundle. With the assembly placed on the fiber bundle, the lower distal ends of the fibers are spaced from the confronting surface of optical element **78** by an air gap **85** whose spacing is preferably less than 100 nm or greater than 2 μm. Further, each of the fibers is aligned with a corresponding assay region of the optical assembly, so that each fiber is directing light on, and receiving reflected light from, its aligned detection region. Similarly, the optical coupler in the apparatus, which serves to couple multiple fibers to the detector, preserves the alignment between the array regions and corresponding positions on an optical detector, e.g., two-dimensional CCD. The materials and thickness dimensions of the various optical-assembly components are similar to those described above with respect to FIG. **4**.

The apparatus described in this invention can be used more specifically for the following applications:

(i) with an anti-species antibody carried on the tip, for screening hybridoma expression lines for cell lines with high antibody expression;

(ii) with an antigen carried on the tip, to characterize high affinity antibodies against that antigen;

(iii) with a protein carried on the tip, for identifying and characterizing binding partners (DNA, RNA, proteins, carbohydrates, organic molecules) for that protein;

(iv) with a carbohydrate or glycosyl moiety carried on the tip, for identifying and characterizing binding partners (such as, e.g., DNA, RNA, proteins, carbohydrates, organic molecules) for that carbohydrate;

(v) with a protein thought to participate in a multi-protein complex carried on the tip, for characterizing the binding components and/or kinetics of complex formation;

(vi) with a small protein-binding molecule carried on the tip, for identifying and characterizing protein binders for that molecule;

(vii) with an antibody carried on the tip, for constructing a calibration curve for the analyte using a set of analytes standards. Using this calibration curve, one can then determine the concentration of the analyte in unknown solutions (cell culture supernatants, biological samples, process mixtures, etc).

(viii) with a single-stranded nucleic acid, e.g., ssDNA or RNA carried on the tip, for identifying and molecules that bind specifically to the nucleic acid.

Using a temperature control block, the apparatus and method can also be used to monitor the binding and characterize the binding of an immobilized ssDNA to an oligonucleotide in solution to perform SNP analysis.

The following examples illustrate various methods and applications of the invention, but are in no way intended to limit its scope.

EXAMPLES

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, *Proteins: Structures and Molecular Properties* (W. H. Freeman and Company, 1993); A. L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences,* 18th Edition (Easton, Pa.: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry* 3rd *Ed.* (Plenum Press) Vols A and B (1992).

Example 1

Small Molecule-protein Binding Reaction

This example demonstrates the capability to detect the binding of protein to small molecule immobilized on a sensor tip and subsequent bindings of multiple antibodies. The two-layer configuration on the tip of an optic fiber is used for this test. The thickness of the first Ta_2O_5 layer is 25 nm and the thickness of the second SiO_2 layer is 770 nm. The fiber was purchased from Ocean Optics (Dunedin, Fla.). It was manually cut into segments that are 40 mm long. Both ends of these segments were polished to standard mirror surface quality. The polishing method used here was exactly the same as those for optical lenses and mirrors. One surface of these fiber segments was outsourced to an optical coating house for Ta_2O_5 layer and SiO_2 layer. This vendor employed an ion-beam assisted physical vapor deposition (IAPVD) coater made by Leybold. IAPVD is a commonly used coating technique for anti-reflection and optical filters. The experimental steps included the following (all steps are performed at room temperature unless otherwise noted):

The fiber tip was coated with a polymer monolayer derivatized with biotin. The polymer monolayer was prepared using a biotinylated lipid (custom). This lipid was using to form a lipid monolayer on the surface of water solution. The monolayer was cross linked using UV light for 15 minutes. Clean, dry fibers were then brought in contact with the floating thin film and the biotin polymer was adsorbed onto the fiber tip. The fibers were then dryed at 60° C. for 1 hour. The fiber were then stored under ambient conditions

The biosensor tip was immersed in 50 μg/ml streptavidin streptavidin (Pierce Biotechnology, Rockford Ill., cat # 21122) in PBS (Invitrogen, Carlsbad, Calif.; cat # 14190078) for 9 minutes and then rinsed briefly with PBS.

The same tip was dipped into 10 μg/ml rabbit-anti-streptavidin solution (AbCam, Cambridge, Mass.; cat # ab6676-1000) in PBS for 36 minutes and then washed with PBS briefly.

Finally, the tip was immersed in 50 μg/mL donkey-anti-rabbit antibody solution antibody (Jackson ImmunoResearch, West Grove, Pa.; cat# 711-005-152) in PBS for 25 minutes. A final 10 minute rinse was performed in PBS solution.

FIG. **6** shows the real-time response curve for this sequential binding test. The vertical axis is the 7th order valley phase shift in nanometers. It clearly shows the binding of streptavidin to the biotin already immobilized on the tip, and subsequent bindings of anti-streptavidin antibody to streptavidin and a second antibody to this first antibody. The dissociation of the streptavidin layer from the tip was visible (a small reduction in the optical thickness) at 900 seconds.

Example 2

Biomolecular Interaction Analysis of Kinetics and Affinity of Biomolecular Interactions

This example illustrates use of the invention to carry out a biomolecular interaction analysis (BIA) measuring kinetics and affinity of biomolecular interactions. The same tip configuration as described in Example 1 was used. The experimental steps included the following (all steps are performed at room temperature unless otherwise noted):

Mercaptosilane coated tips were prepared using the following procedure. Clean, dry fibers were incubated in a mixture of Toluene: hexanoic acid: mercaptopropyltrioxysilane (10:2:1 volumetric ratio) at room temperature for 24 hours. The fibers were rinsed 2× with 10 mL toluene for 5 minutes each. The fibers were then rinsed 1× with 10 mL of ethanol and dried under a stream of argon and stored at ambient conditions.

The biosensor tip was first derivatized by immersion in a with 10 μg/ml solution of rabbit-IgG (Jackson ImmunoResearch, West Grove, Pa.; cat# 309-005-003) in PBS for 1 hour.

The coated tip was dipped into 10 μg/ml goat-anti-rabbit antibody solution (Jackson ImmunoResearch, West Grove, Pa.; cat# 111-005-003) in PBS and remained in it for 15 minutes.

The tip was removed and washed in PBS. To facilitate the dissociation of the second antibody from the first antibody, the PBS was agitated manually for 20 minutes.

The tip was then dipped into the same goat-anti-rabbit solution again to show the reproducible association of goat-anti-rabbit to rabbit-IgG.

FIG. **7** shows the on and off curves generated from the association and dissociation of rabbit-IgG and goat-anti-rabbit. The vertical axis is again the 7th order valley phase shift. The phase shift is directly related to the average thickness with a ratio of 0.834. The ability to detect the on and off curves reliably is essential for measuring interaction kinetics and affinity.

Example 3

Calculating Affinity Constants from Antibody-antigen Binding and Release Curves

This experiment demonstrates the calculation of affinity constants from measuring on and off curves for two antibodies and their antigen. The proprietary antibodies were labeled as Ab-1 and Ab-2. The molecular weight of the antigen was about 30 kilodaltons. The same tip configuration as described in Example 1 was used. The same mercaptosilane fiber preparation as described in Example 2 was used. The experimental steps included (all steps are performed at room temperature unless otherwise noted):

The fiber tip was activated for covalent attachment of the antigen. Mercaptosilane coated fibers were activated by immersing the sensor tips in 50 μL of a 50 mg/mL solution of sulfo-SMCC (Pierce Biotechnology, Rockford Ill.; cat # 22322) in DMF (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat # 494488) at for 2 hours. The sensor tips were rinsed briefly in DMF and dried;

The antigen was covalently bound to the activated fiber tip by immersing the activated tip in a 20 μg/ml solution of antigen in PBS for 20 minutes. The tip was rinsed with PBS for 2 minutes. Following the PBS rinse, the tip was quenched with an aqueous solution of 100 μM ethanolamine pH 8.5 (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat # E9508) for 5 minutes and then was rinsed again in PBS for 2 minutes.

The same tip was immersed in antibody for an association test and the real-time binding data were recorded for 9-15 minutes (depending on the antibody identity and concentration). Once those data were recorded, the tip was again immersed in PBS and agitated to measure the off curve (i.e., dissociation between the immobilized antigen and bound antibody) for 9-15 minutes. The binding (on curve) and dissociation (off curve) measurements were repeated using different concentrations of antibody (25 nM, 150 nM, and 430 nM) and with two different antibodies identified as Ab-1 and Ab-2.

FIG. **8** shows the association and dissociation curves at different concentrations. The test of 25 nM Ab-2 was not completed because the association was extremely slow at this concentration. These illustrated curves are plots of the raw data.

K_{on}, K_{off} and K_D were derived from these curves by fitting the raw data with a first order exponential function. By averaging two sets of data, kinetic and affinity coefficients were obtained as follows:

Ab-1	Ab-2
$K_{on} = 1.35 \times 10^5$ ($M^{-1}S^{-1}$)	$K_{on} = 2.01 \times 10^5$ ($M^{-1}S^{-1}$)
$K_{off} = 5.55 \times 10^{-5}$ (S^{-1})	$K_{off} = 8.15 \times 10^{-5}$ (S^{-1})
$K_D = K_{off}/K_{on} = 3.99 \times 10^{-9}$ (M)	$K_D = K_{off}/K_{on} = 4.45 \times 10^{-9}$ (M)

Example 4

NHS-ester Activated Tips

The same tip configuration as described in Example 1 was used. The same mercaptosilane fiber preparation as described in Example 2 was used. Mercaptosilane coated fibers were activated by immersing the sensor tips in 50 μL of a 50 mg/mL solution of sulfo-SMCC (Pierce Biotechnology, Rockford Ill.; cat # 22322) in DMF (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat # 494488) at for 2 hours. The sensor tips were rinsed briefly in DMF and dried.

Amine containing molecules can be covalently bound to this surface through formation of a stable amide linkage. Molecules that do not contain free amines are not immobilized through the NHS moiety, but these molecules can still bind to the surface through non-specific binding. This non-specific binding can be multi-layered whereas the covalent immobilization through the NHS esters will be in a single layer controlled by the availability and accessibility of the NHS ester.

In this set of experiments, a bis amino PEG (MW 3300) (Shearwater Polymers, San Carlos, Calif.) was used as a test compound to covalently bind to the activated surface. A PEG (MW 8000) (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat # 04162) that contained no free amino groups was used as a negative control. This negative control was used to look for any non-specific or multi-layered binding that might be inherent to PEG polymers on this surface.

FIG. **9** shows the time course of the treatment of the activated mercaptosilane tip with the test molecules. The activated tip showed a distinct increase in optical thickness upon exposure to the 0.1 mg/mL bis amino PEG (MW 3300) in PBS. This increase is stopped when the bis amino PEG solution is replaced by the PBS buffer. The activated tip exposed to 0.1 mg/mL PEG (MW 8000) in PBS, which contains no amines, shows a small initial increase in optical thickness but the trace quickly becomes flat. From this it can be concluded that the PEG polymer does not have intrinsic non-specific binding and that the binding seen for the bis amino PEG is attributed to the specific covalent immobilization of the amine group.

Example 5

Antibody Derivatized Tips Using NHS-ester Chemistry

This example illustrates the binding of a low molecular weight molecule binding to an immobilized high molecular weight molecule. Using the same NHS ester terminated surface described in Example 4 and the same tip configuration as described in Example 1, an anti-biotin antibody was immobilized to 3 fibers. Immobilization of the antibody was accomplished by immersing the activated fiber in a 20 μg/mL solution of mouse anti-biotin antibody (Biodesign, Saco Minn.; cat # H61504M) in PBS for 1 hour at room temperature. The tip was rinsed with PBS for 2 minutes. Following the PBS rinse, the tip was quenched with an aqueous solution of 100 μM ethanolamine pH 8.5 (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat # E9508) for 5 minutes and then was rinsed again in PBS for 2 minutes.

The first fiber was exposed to a solution of 200 μg/mL biotin (Pierce Biotechnology, Rockford Ill.; cat # 29129) in PBS. Controls using a solution of sucrose (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat # S8501) (2 mg/mL) and PBS were carried out on the second and the third fibers to determine baseline noise. Data from these tests are shown in FIG. **10**. Biotin binding is seen as an increase in optical thickness, whereas exposure to sucrose shows no detectable increase over baseline (PBS).

Another negative control was carried out using an irrelevant antibody (anti-Lewis Y antibody from Calbiochem, San Diego Calif.; cat# 434636) immobilized in an identical fashion to the anti-biotin antibody above. This immobilized antibody was exposed to a solution of 200 μg/mL biotin. The lack of biotin binding to this antibody indicates that the biotin binding to the anti-biotin antibody is a result of specific interactions and not due to non-specific binding.

While the invention has been particularly shown and described with reference to a preferred embodiment and vari-

ous alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the invention.

All references, issued patents and patent applications cited within the body of the instant specification are hereby incorporated by reference in their entirety, for all purposes.

What is claimed is:

1. An assembly for use in detecting an analyte in a sample based on interference, comprising:

an optical fiber having a tip;

a first optical element adapted for coupling to a light source through a mechanical coupling that engages the first optical element with the fiber and provides an air gap between the first optical element and the fiber; and

second optical element attached to the first optical element, the second optical element commensurate in size with the fiber tip and adapted for coupling to the first optical element,

said second optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface separated from the first reflecting surface by the transparent material, said first and second reflecting surfaces separated by at least 50 nm, wherein said first reflecting surface comprises a layer of analyte binding molecules, and an interference between light reflected into the fiber from said first and second reflecting surfaces varies as analyte in the sample binds to the analyte binding molecules.

2. The assembly of claim **1**, wherein said second reflecting surface comprises a layer of material having refractive index greater than the refractive index of said first optical element transparent material.

3. The assembly of claim **1**, wherein the separation between said first and second reflecting surfaces is between 100 nm and 5,000 nm.

4. The assembly of claim **3**, wherein the separation between said first and second reflecting surfaces is between 400 nm and 1,000 nm.

5. The assembly of claim **1**, wherein the refractive index of said first optical element transparent material is less than 1.8.

6. The assembly of claim **5**, wherein said first optical element transparent material is a material selected from the group consisting of SiO_2 and a transparent polymer.

7. The assembly of claim **6**, wherein said transparent polymer comprises polystyrene or polyethylene.

8. The assembly of claim **1**, wherein the second reflecting surface comprises a layer of material having a refractive index greater than 1.8.

9. The assembly of claim **8**, wherein said second reflecting surface layer comprises Ta_2O_5.

10. The assembly of claim **9**, wherein the thickness of said second reflecting surface layer is between 5 nm and 50 nm.

11. The assembly of claim **1**, wherein said layer of analyte binding molecules comprises a molecule selected from the group consisting of a protein, a small molecule, a nucleic acid and a carbohydrate.

12. The assembly of claim **11**, wherein said protein is selected from the group consisting of an avidin, a streptavidin, an antibody, and an antibody fragment.

13. The assembly of claim **1**, wherein the thickness of said first optical element is greater than 100 nm.

14. The assembly of claim **13**, wherein the thickness of said first optical element is greater than 200 nm.

15. The assembly of claim **1**, wherein said air gap is less than 100 nm.

16. The assembly of claim **1**, wherein said air gap is greater than 2 μm.

17. A two dimensional array of the assemblies of claim **1**.

18. An apparatus for detecting an analyte, comprising:

the two dimensional array of the assemblies of claim **17**;

a light source for directing light onto said first and said second reflecting surfaces; and

a detector that receives light from said first and said second reflecting surfaces and detects a change in optical thickness of said first reflecting surface upon exposure of said first reflecting surface to said analyte.

19. A method for detecting analyte in a sample, comprising:

providing the apparatus of claim **18** and a sample;

exposing said first reflecting surface to said sample, and

determining whether said exposure results in a change in optical thickness of said first reflecting surface.

20. An apparatus for detecting an analyte, comprising:

the assembly of claim **1**;

a light source for directing light onto said first and said second reflecting surfaces; and

a detector that receives light from said first and said second reflecting surfaces and detects a change in optical thickness of said first reflecting surface upon exposure of said first reflecting surface to said analyte.

21. A method for detecting analyte in a sample, comprising:

providing the apparatus of claim **20** and a sample;

exposing said first reflecting surface to said sample, and

determining whether said exposure results in a change in optical thickness of said first reflecting surface.

22. A kit, comprising:

an assembly comprising an optical fiber having a tip; first optical element adapted for coupling to a light source through a mechanical coupling that engages the first optical element with the fiber and provides an air gap between the first optical element and the fiber; a second optical element attached to the first optical element, the second optical element commensurate in size with the fiber tip and adapted for coupling to the first optical element, said second optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface separated from the first reflecting surface by the transparent material, said first reflecting surface and said second reflecting surface separated by at least 50 nm,

wherein said first reflecting surface binds a layer of analyte binding molecules and an interference between light reflected into the fiber from said first and second reflecting surfaces varies as analyte binds to the analyte binding molecules, and said second reflecting surface comprises a layer of material having an index of refraction greater than the refractive index of said optical element transparent material; and

instructions for binding said layer of analyte binding molecules to said first reflecting surface.

23. The kit of claim **22**, further comprising a reagent for chemically modifying said first reflecting surface and instructions for using said reagent.

* * * * *

EXHIBIT B

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THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

U.S. PATENT: *7,445,887*
ISSUE DATE: *November 04, 2008*

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Under Secretary of Commerce for Intellectual Property
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SYLVIA HOLLEY
Certifying Officer


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(12) **United States Patent**
Zuk et al.

(10) **Patent No.: US 7,445,887 B2**
(45) **Date of Patent: Nov. 4, 2008**

(54) **ENZYME ACTIVITY MEASUREMENTS USING BIO-LAYER INTERFEROMETRY**

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(58) **Field of Classification Search** 435/4
See application file for complete search history.

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(57) **ABSTRACT**

Disclosed are enzyme assays using biolayer interferometry. Assays may be carried out using immobilized substrate or with a substrate capture format. In certain embodiments, the assays are carried out using unlabeled substrates. The methods are broadly applicable to enzyme assay measurements, can be carried out in vivo or in vitro, and are easily multiplexed.

18 Claims, 13 Drawing Sheets

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Fig. 1

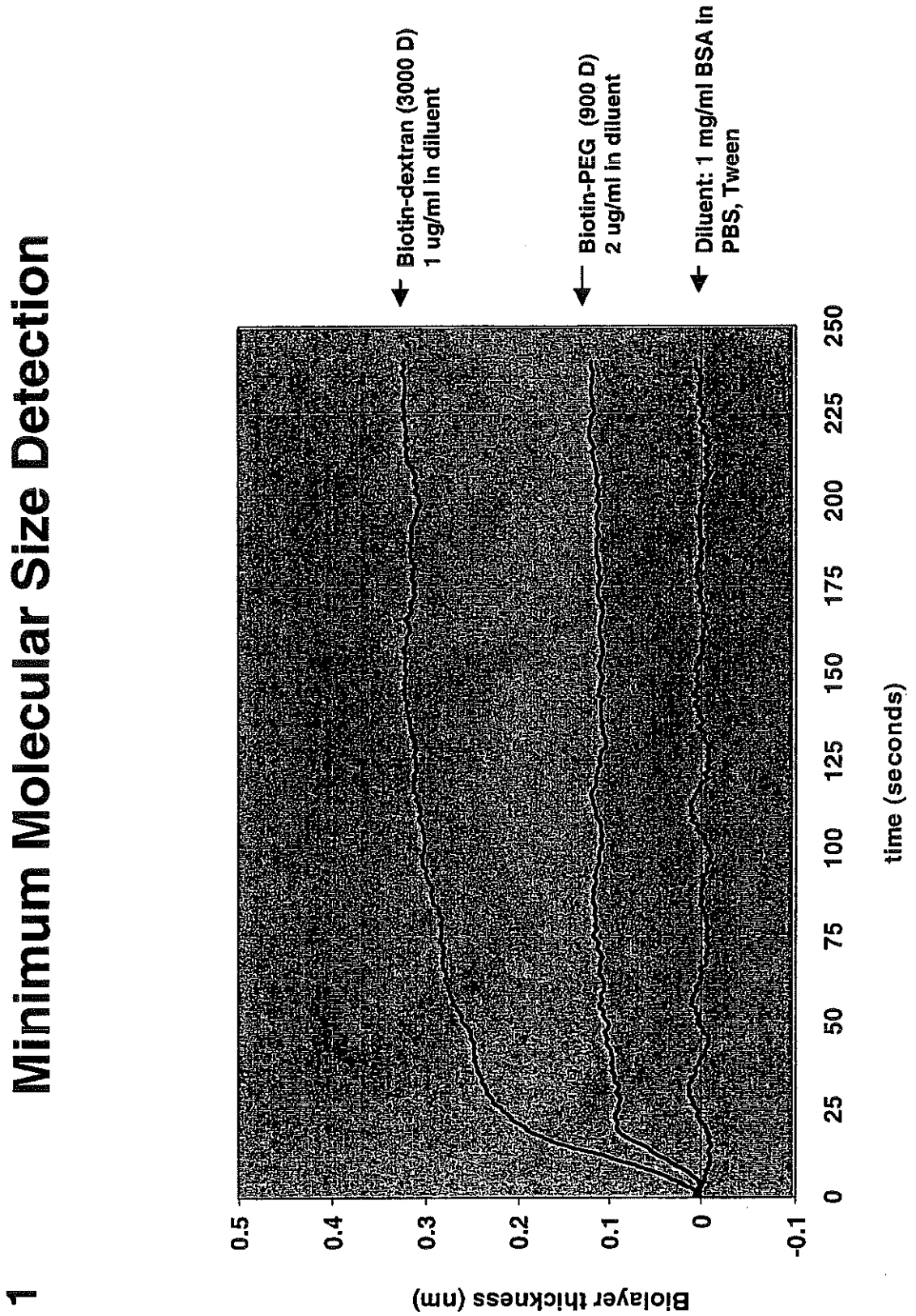
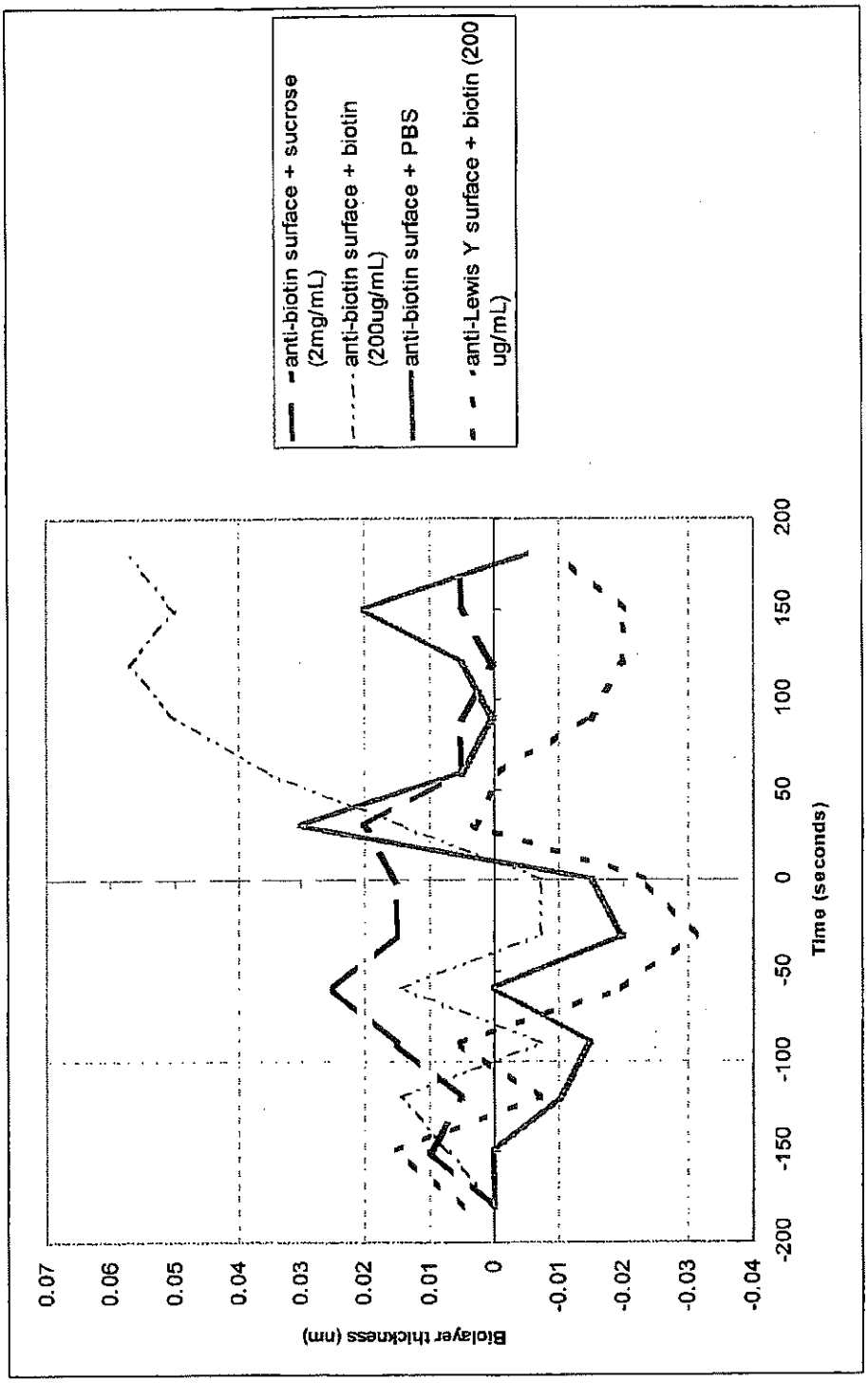


Fig. 2 Minimum Molecular Size Detection



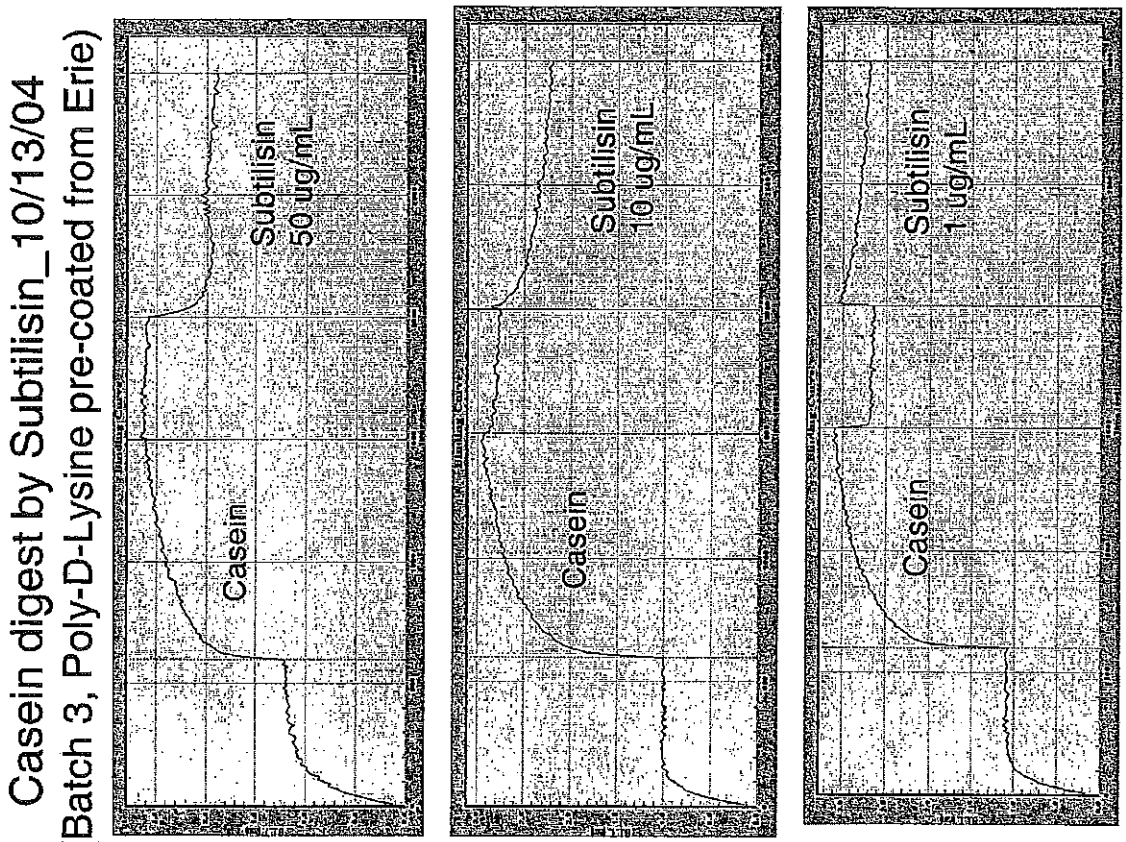


Fig. 3

Protease Assay
(Casein / Subtilisin Pair)

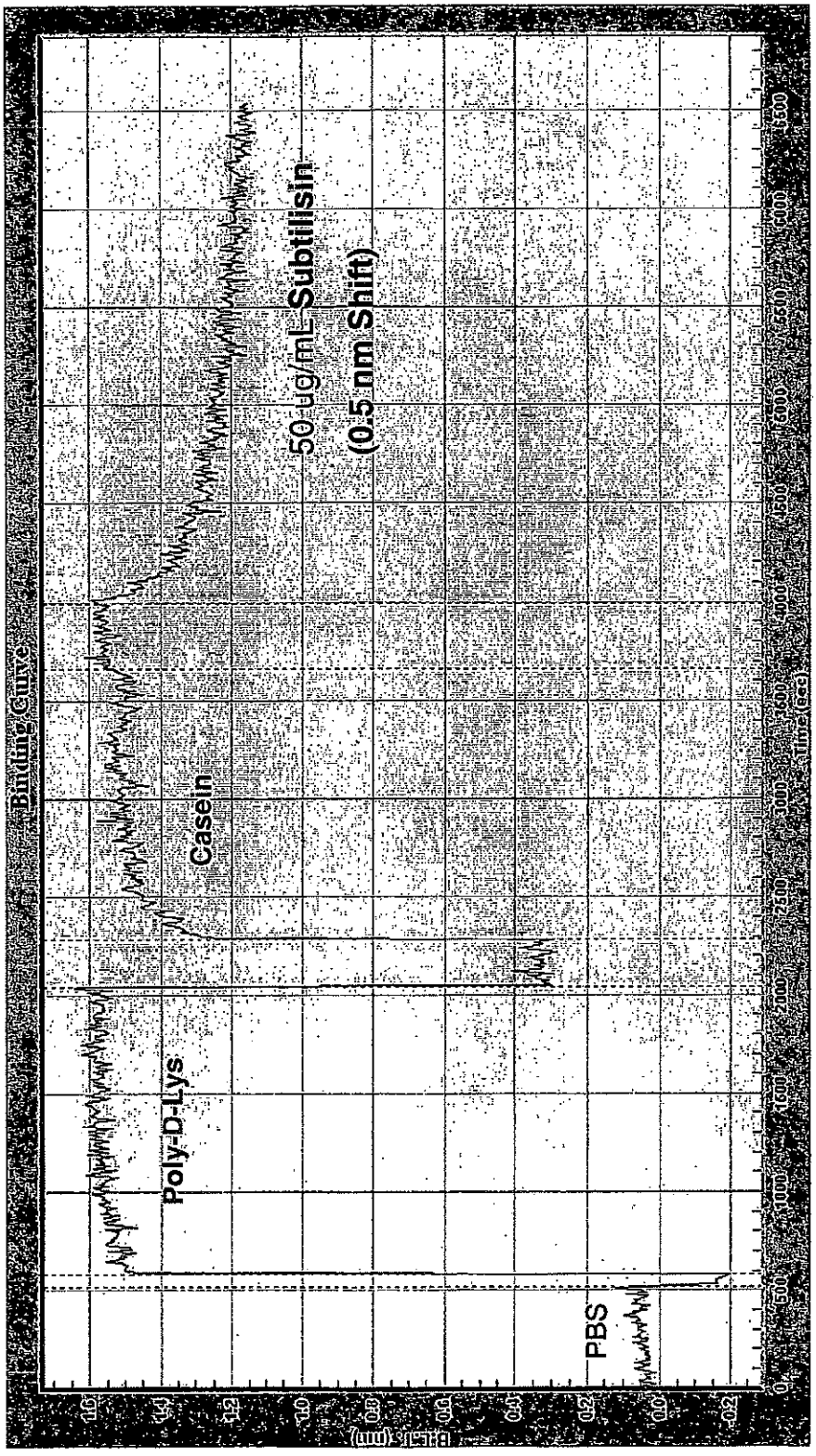


Fig. 4

Fig. 5 **The Effect of Protease Inhibitor**
(Subtilisin / PMSF Pair)

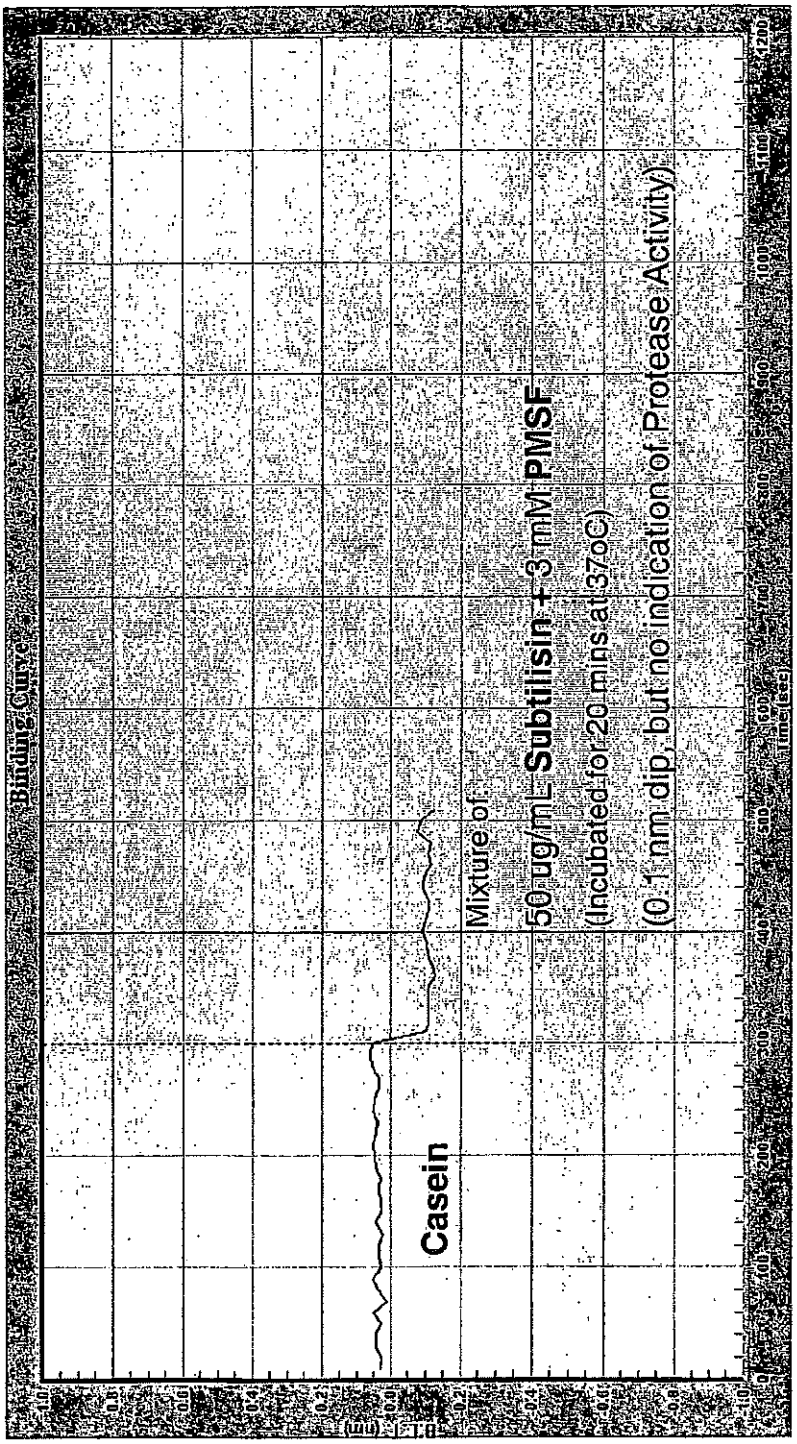


Fig. 6 **The Effect of Protease Inhibitor**
(Subtilisin / PMSF Pair)

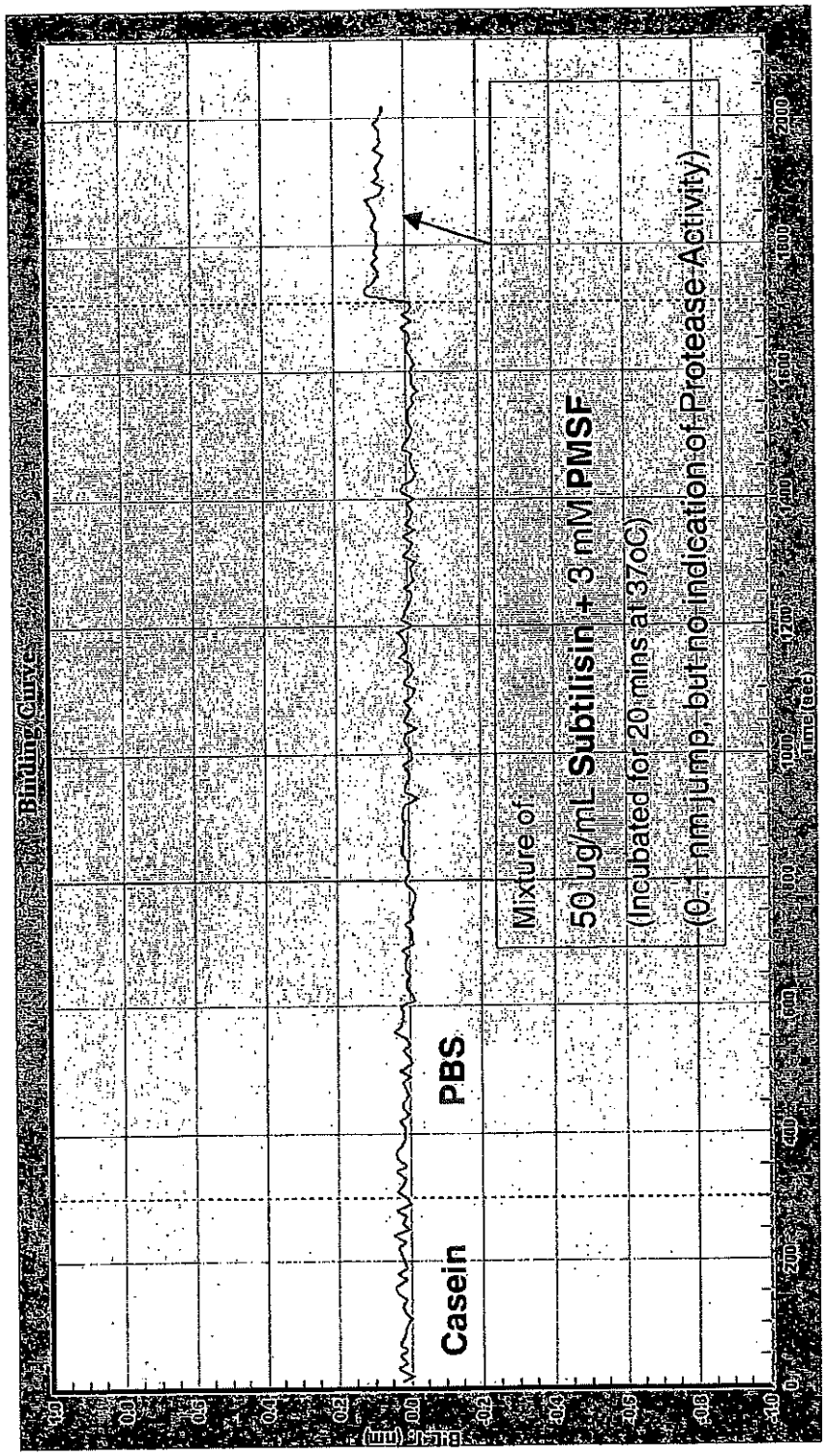
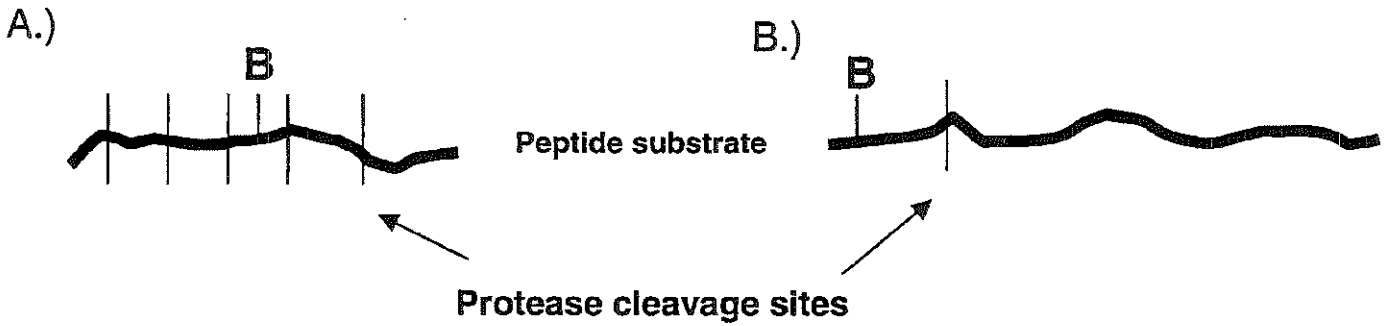


Fig. 7

Substrate Capture Format

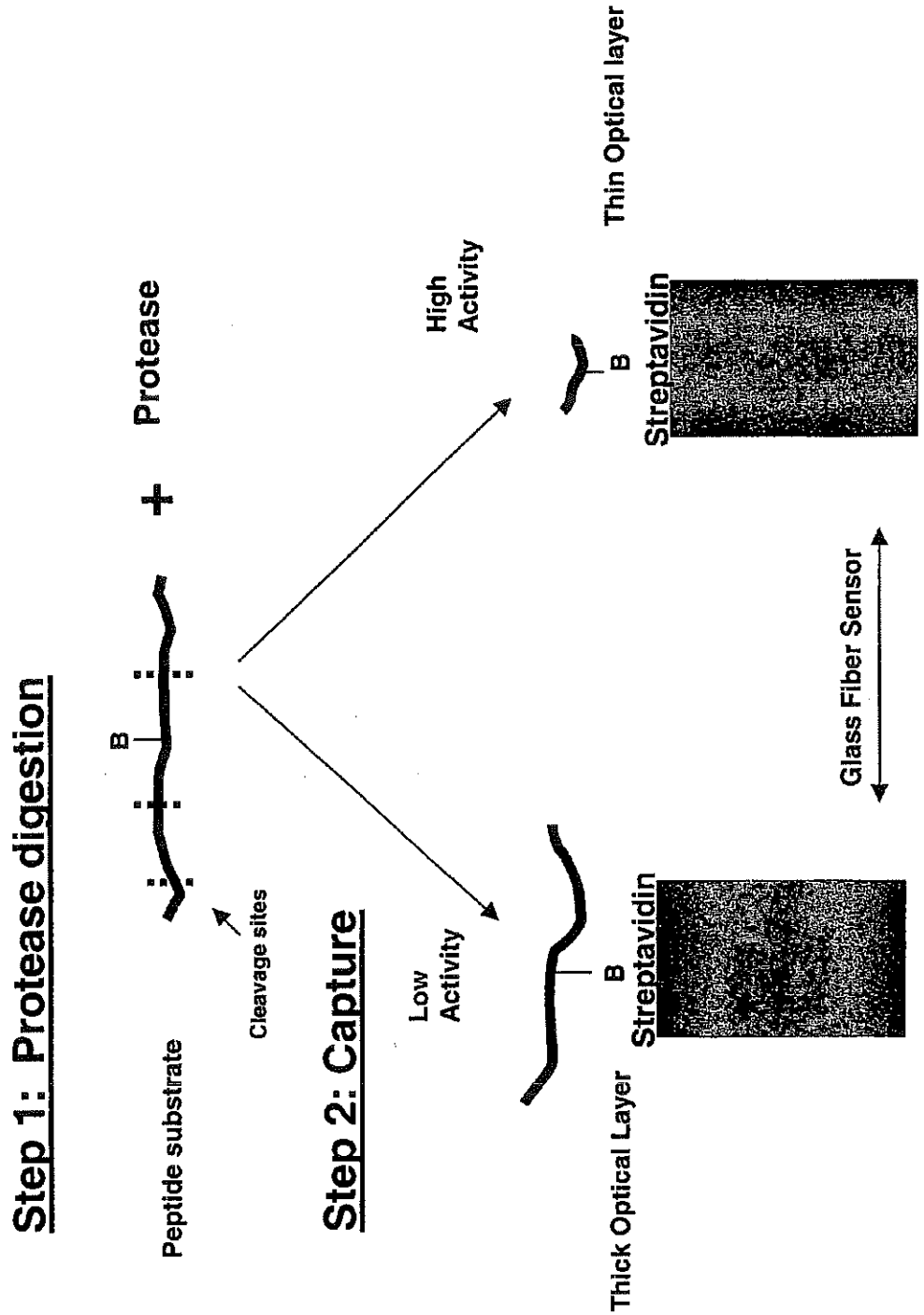
Peptide substrate: biotin substitution positions



A.) Protease has multiple cleavage sites, Biotin substitution does not have to be site specific for significant molecular size change

B.) Protease has single cleavage site, Biotin substitution is adjacent to cleavage site to produce smallest fragment

Fig. 8



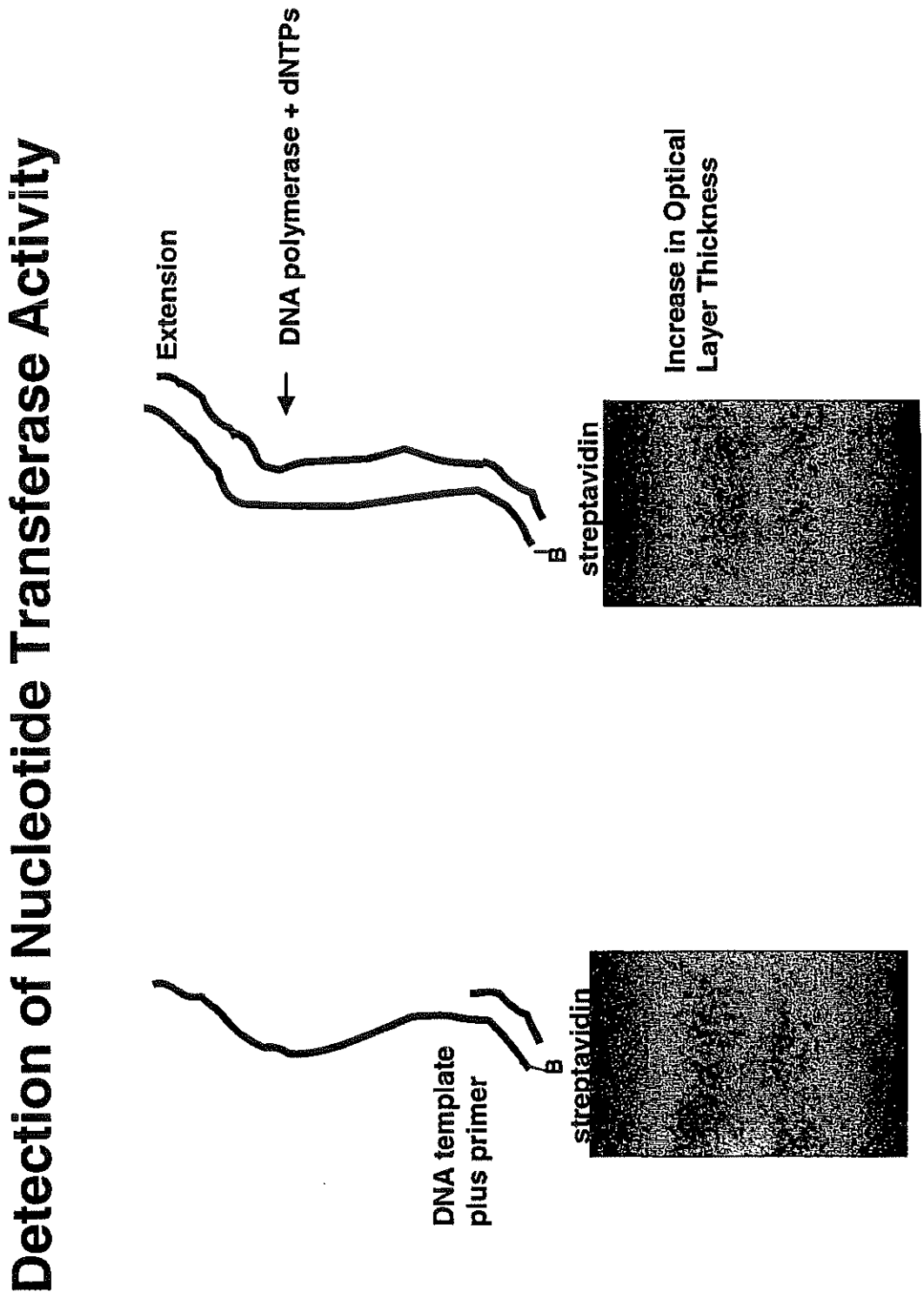


Fig. 9

Fig. 10

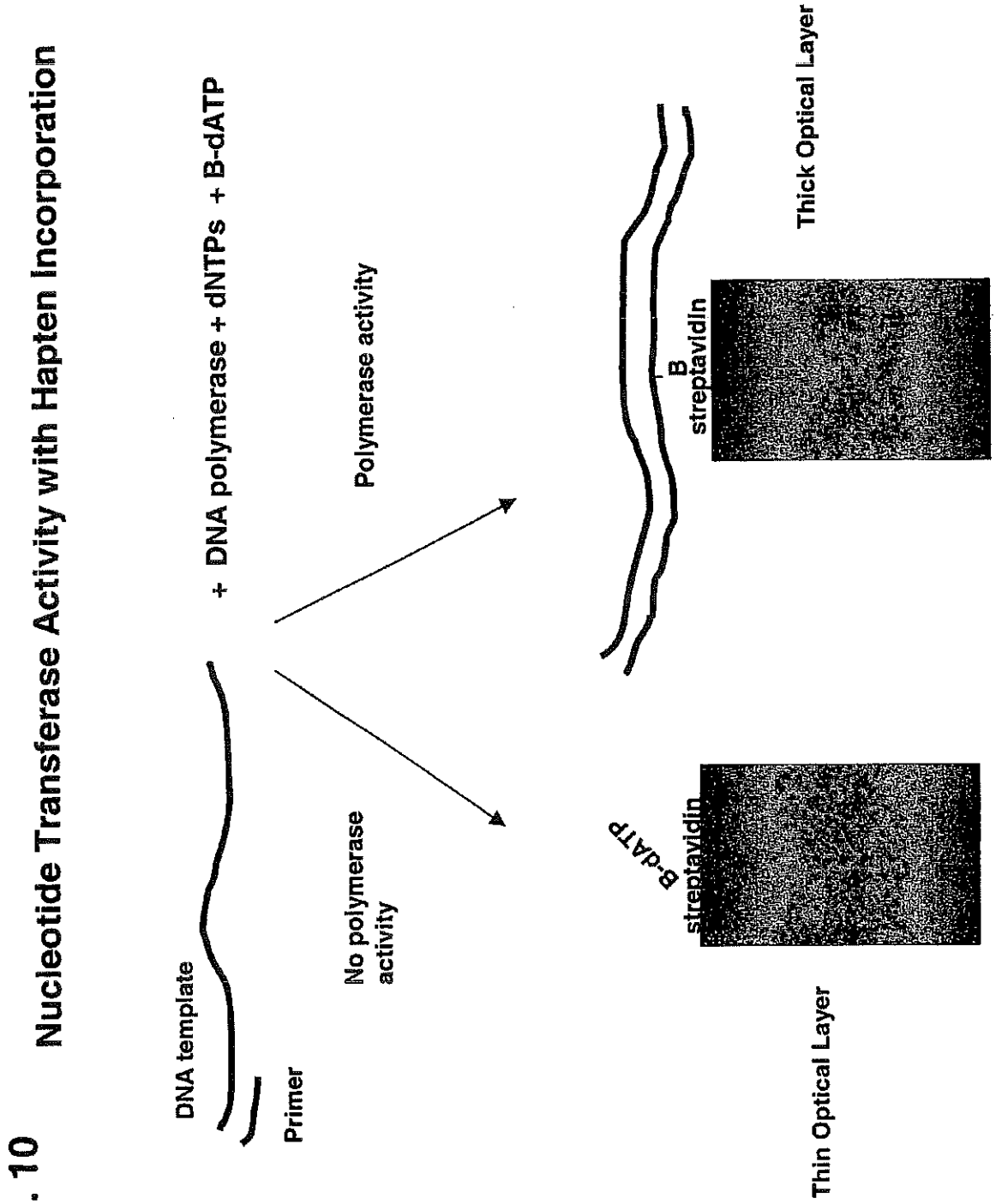
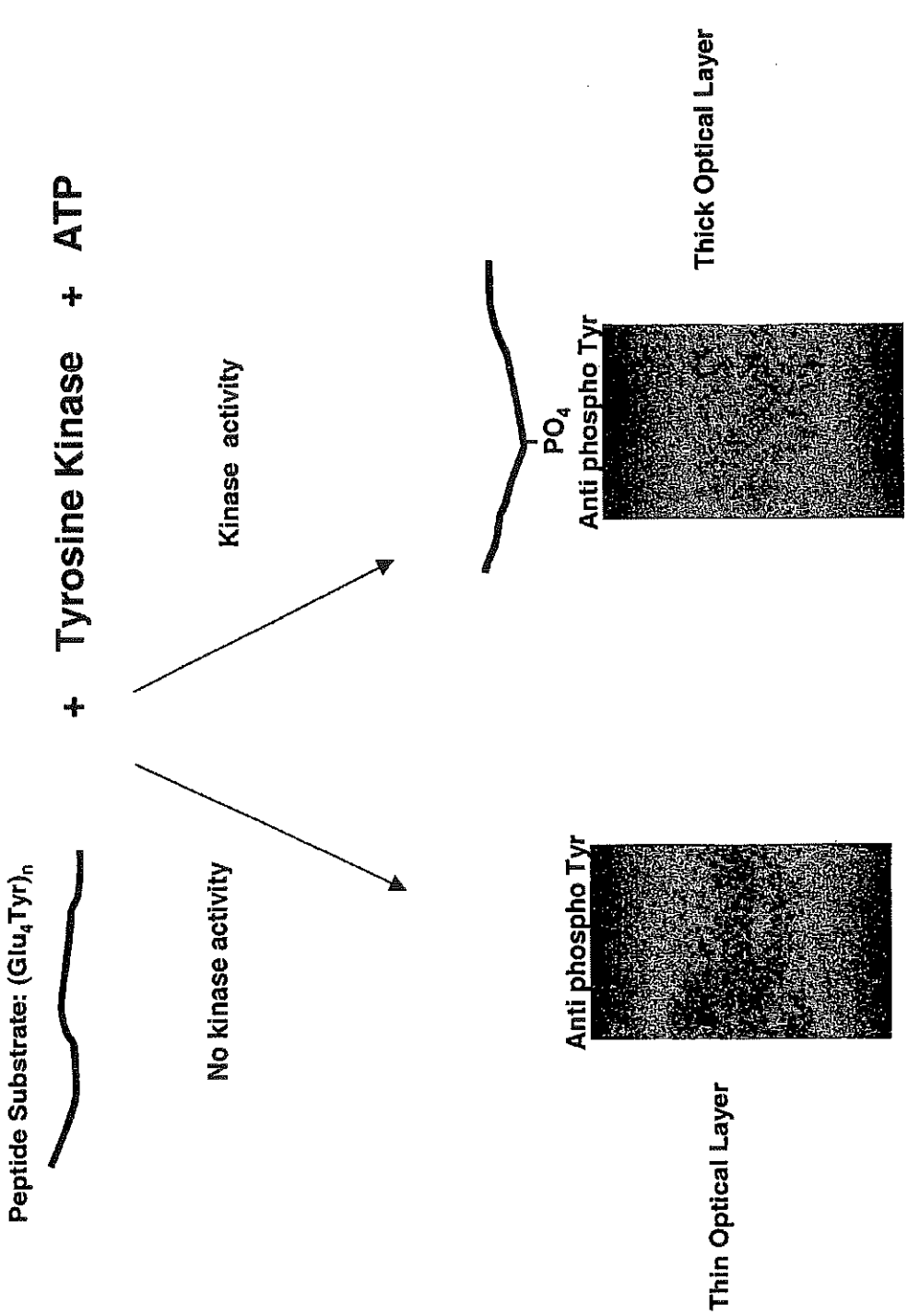


Fig. 11



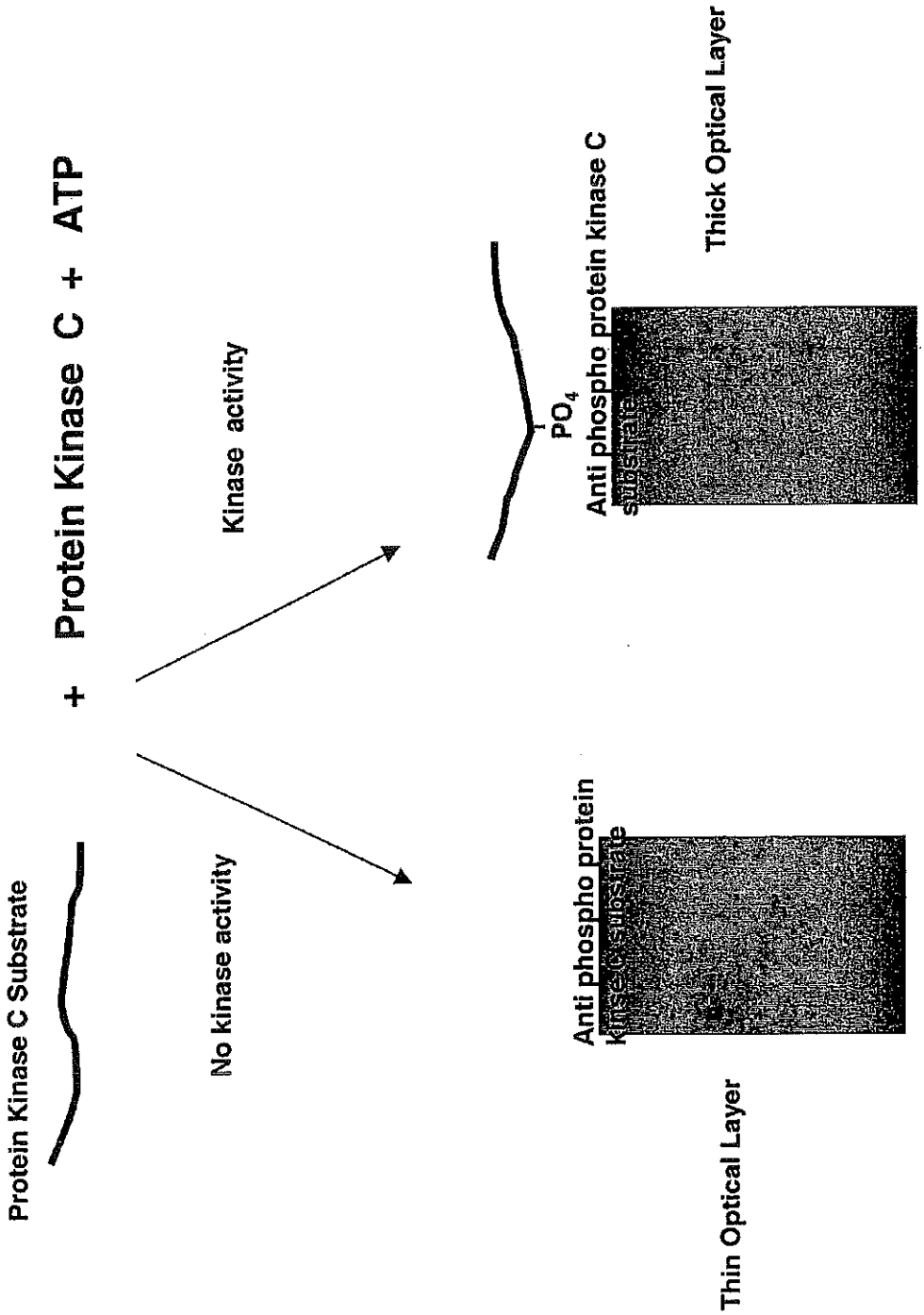
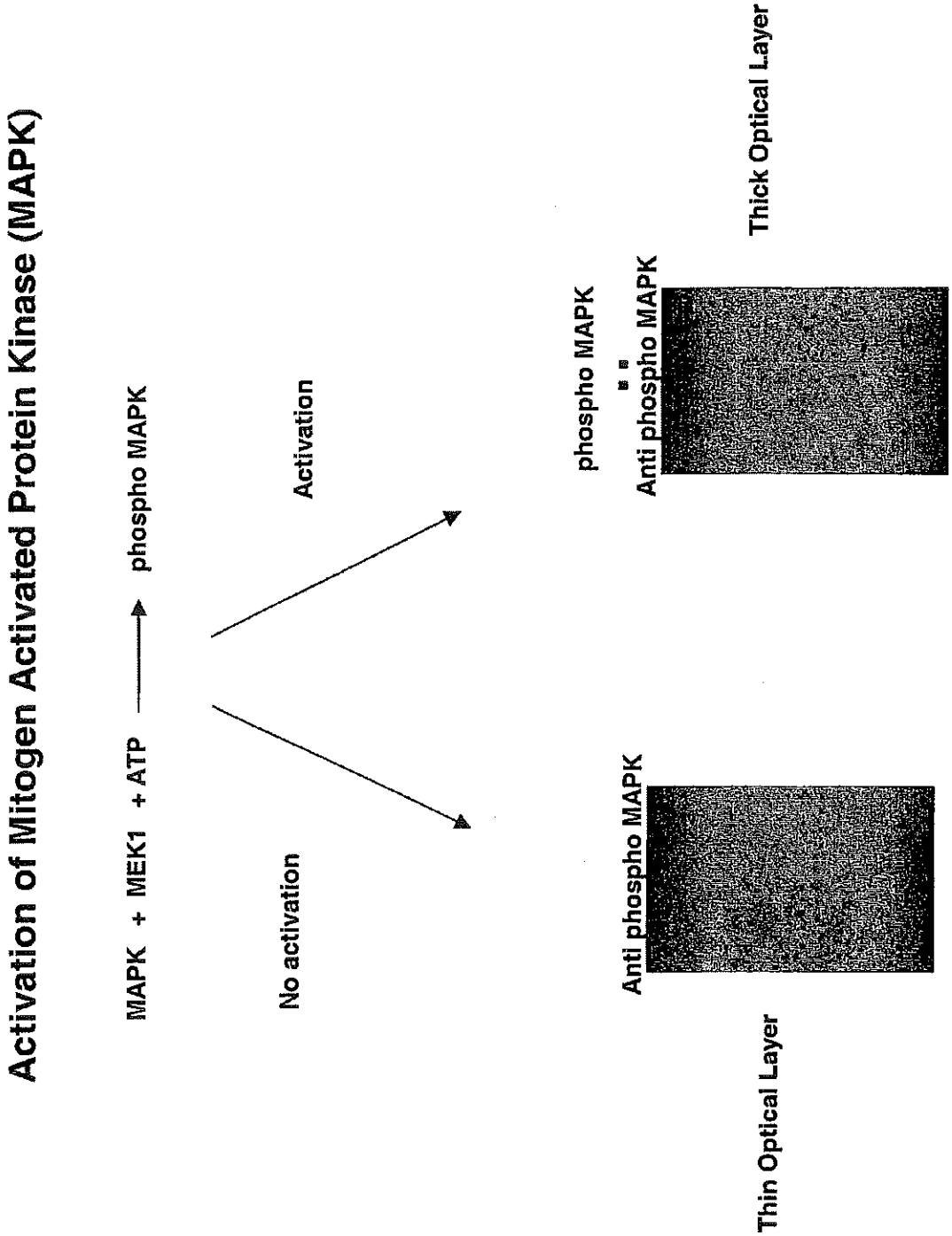


Fig. 12

Fig. 13



ENZYME ACTIVITY MEASUREMENTS USING BIO-LAYER INTERFEROMETRY

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application Ser. No. 60/645,153, filed Jan. 19, 2005 and U.S. Provisional Application Ser. No. 60/642,454, filed Jan. 7, 2005, both of which are incorporated herein by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to interferometry-based methods and compositions useful for measuring enzyme activity.

2. Description of the Related Art

Enzymes represent a broad class of proteins that catalyze biochemical reactions and have many therapeutic and industrial applications. Often in the course of development and manufacture of enzyme products it is necessary to measure the activity of the enzyme. A simple enzyme activity method, preferably label free thereby avoiding perturbation of the enzyme/substrate interaction, would find wide application. In the development and manufacture of enzyme or enzyme inhibitor based products for therapeutic or industrial applications, it is critical to monitor the activity of the enzyme throughout the process. Enzyme assays typically require labeling the substrate in such a way that the enzyme acting on the substrate produces a detectable change in signal. Labeled enzyme substrates are often not commercially available, in which case their synthesis can be complex. For companies developing a multitude of enzyme products, the implementation of activity methods that are simple and easy to perform in research & development and manufacturing environments becomes a major task. The need for labeled substrates adds to the time, expense and inconvenience of enzyme activity measurements. Specific activity measurements also require quantifying the amount of enzyme present in a sample. Quantitation, as by, e.g., enzyme-linked immunosorbant (ELISA)-based assays are also adds to the time and expense of specific activity measurements and requires additional sample. The present invention addresses these and other shortcomings of the prior art by providing simple, fiber based, real-time enzyme activity assays, capable of providing specific activity measurements, suitable for low-volume samples, that are highly multiplexable and in some embodiments can be carried out using unlabeled substrates.

SUMMARY OF THE INVENTION

The present invention is defined by the following claims, and nothing in this section should be taken as a limitation on those claims. Disclosed herein are assemblies, kits, and methods for assaying enzyme activity using fiber-based interferometry. In one embodiment, the assay comprises providing an optical element coupled to a light source via an optical fiber and the element includes proximal and distal reflecting surfaces separated by at least 50 nm. A layer of enzyme substrate molecules is positioned so that interference between beams reflected from the proximal and distal reflecting surfaces varies as an enzyme reacts with the substrate. The reflected beams are coupled into the optical fiber. The element is exposed to an enzyme and a change is detected in the interference between the reflected beams. The detected change is indicative of enzyme activity.

In still another embodiment, a layer of analyte binding molecules substituted in the optical element for the layer of enzyme substrate molecules. Interference between beams reflected from the proximal and distal reflecting surfaces varies as an enzyme reacts with the substrate and the acted-upon substrate or portion thereof binds to the analyte binding molecules. In preferred embodiments, the analyte binding molecules comprise an antibody, an antibody fragment, a single chain Fv molecule ("scFv"), an avidin, a streptavidin, or a biotin.

In another embodiment, a semi-permeable membrane is placed between the optical element and the assay solution. In another embodiment, the substrate is coupled to a support such as a microtitre well or a bead.

In yet another embodiment, a similar, second element is provided that includes a layer of molecules that specifically binds to the enzyme. The second element is exposed to an enzyme (either at the same time or at a different time as the first element is exposed) and a change is detected in the interference between the reflected beams. The change is indicative of enzyme concentration or amount. This is useful for carrying out specific activity measurements. In preferred embodiments, the enzyme-binding molecules comprise an anti-enzyme antibody, an antibody fragment or an scFv molecule.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

FIG. **1** is a graph illustrating minimum molecular size detection;

FIG. **2** is another graph illustrating minimum molecular size detection;

FIG. **3** is a graph illustrating subtilisin activity measurement at three enzyme concentrations;

FIG. **4** is a graph illustrating subtilisin activity measurement at 50 μg/ml;

FIG. **5** is a graph illustrating effect of protease inhibitor on subtilisin activity;

FIG. **6** is a second graph illustrating effect of protease inhibitor on subtilisin activity.

FIG. **7** is a diagram illustrating substrate preparation for substrate capture format; FIG. **7**A illustrates substrate having multiple protease cleavage sites; FIG. **7**B illustrates substrate having single (or few) cleavage sites;

FIG. **8** is a schematic illustrating principle of substrate capture format assays;

FIG. **9** is a schematic illustrating method to detect nucleotide transferase activity;

FIG. **10** is a schematic illustrating method to detect nucleotide transferase activity with hapten incorporation;

FIG. **11** is a schematic illustrating method to detect phosphotransferase activity using antibody to phosphorylated substrate;

FIG. **12** is a schematic illustrating method to detect protein kinase C activity;

FIG. **13** is a schematic illustrating method to detect activation of mitogen activated protein kinase (MAPK).

DETAILED DESCRIPTION OF THE INVENTION

Advantages and Utility

Briefly, and as described in more detail below, described herein are assemblies, kits and methods for assaying enzyme activity using fiber-based interferometry.

Several features of the current approach should be noted. Measurements can be carried out using extremely small sample volumes (e.g., nL). Measurements can be carried out in vivo or in vitro. In some embodiments, measurements can be carried out on unlabeled substrates while in other embodiments, substrates include a moiety to allow capture by the assembly. In preferred embodiments, the moiety is one member of a binding pair such as, e.g., avidin, streptavidin, biotin, a hapten, an antibody, antibody fragment, an scFv, or a lectin, and the optical element comprises the complementary member of the pair. In these embodiments, the same type of optical element (i.e., carrying one member of the binding pair) can be used in a wide variety of enzyme assays provided the substrate includes the other member of the pair.

Advantages of this approach are numerous. Because the invention provides for fiber-based interferometry measurements, it is sensitive, capable of being highly multiplexed, and easily adapted for specific activity measurements by including a module for measuring enzyme amount.

The invention is useful for measuring enzyme activity in any context for which such activity measurements are useful including, e.g., for discovery, modification, optimization, production, etc. of enzymes or enzyme inhibitors. The invention may be practiced using any type of enzyme such as, e.g., hydrolases, glycosylases, esterases, and transferases, or inhibitors of such enzymes.

Definitions

Terms used in the claims and specification are defined as set forth below unless otherwise specified.

The term "in vivo" refers to processes that occur in a living organism.

Abbreviations used in this application include the following: dsDNA—double-stranded DNA; dNTPs—deoxynucleotide triphosphates; B-ATP—biotinylated-ATP; PEG—polyethylene glycol, PMSF—phenylmethylsulfonyl fluoride.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Assemblies of the Invention

Assemblies of the invention include biosensor tips adapted for coupling to a bio-layer interferometer and carrying a layer of substrate or an analyte binding molecule. The analyte binding molecule may be by way of example and not limitation, a member of a binding pair such as, e.g., avidin, streptavidin, biotin, a hapten, an antibody, an antibody fragment, an scFv, or a lectin.

Kits of the Invention

Kits of the invention include a glass fiber adapted for coupling to a bio-layer interferometer, reagents and instructions for derivatizing the glass fiber with a substrate layer or an analyte binding molecular layer, optionally reagents and instructions for optically activating an end of the glass fiber and packaging.

Methods of the Invention

In general, methods of the invention are practiced using assemblies and apparatus, including a bio-layer interferometry (BLI) sensor, such as those described in co-owned U.S. Non-provisional application Ser. No. 10/981,901, filed Nov. 4, 2004, for Hong Tan, et al., entitled "Fiber-Optic Assay Apparatus Based on Phase-Shift Intepferometry," the contents of which are herein incorporated by reference in their entirety.

In brief, the sensor is prepared by optically activating one end of a glass fiber. The activation steps include buffing the fiber surface, coating the surface with Ta_2O_5 followed by coating with an SiO_2 layer, and cleaning, and immobilization of enzyme substrate or one member of a binding pair by passive adsorption and/or covalent attachment.

Included within the scope of the invention are two broad and general formats for assaying enzyme activity. In the first format, substrate is immobilized on a surface of a bio-layer interferometry (BLI) sensor. In the second format, the BLI sensor includes a surface having a molecule capable of binding substrate. In the second format, a semi-permeable membrane optionally is included between the BLI sensor and the assay solution, or the substrate is bound to a support such as a microtitre well or a bead. These embodiments are particularly useful with hydrolases to prevent or slow binding of full-length substrate to the BLI sensor. In the second format, information about enzyme activity can be derived both from kinetic and steady state components of the interference signal.

A Bio-Layer Interferometry (BLI) sensor is capable of measuring sub nanometer changes in the thickness of its optical layer detection surface. Analysis of biological samples is possible by designing assay formats where biomolecules bind at the sensor surface and change the optical layer thickness. The magnitude of the optical layer thickness change is proportional to the mass or molecular weight of the binding molecule. The Bio-Layer Interferometer can be configured to have substrate immobilized to the sensor surface to measure enzymes whose activity creates a change in the substrate molecular weight, either increasing or diminishing the molecular weight, to produce a corresponding change in the optical layer thickness.

The invention is broadly applicable to enzyme activity measurements, including by way of example but not limitation, measurements of hydrolases (including proteases), glycosylases, esterases, transferases (including nucleotide transferases and phosphotransferases). These are considered in greater detail below.

EXAMPLES

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for. Unless otherwise specified, procedures are carried out at room temperature (typically 20-23 degrees Celsius).

The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T. E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Free-

man and Company, 1993); A. L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences,* 18th Edition (Easton, Pa.: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry* 3rd Ed. (Plenum Press) Vols A and B(1992).

Example 1

BLI Molecular Weight Detection Characterization

The minimum molecular weight of a binding molecule that BLI can detect is illustrated in FIGS. **1** & **2**. In FIG. **1** data of a biotin-PEG conjugate with a molecular weight of 900 Daltons binding to a streptavidin coated BLI sensor is depicted. The BLI sensor and methods for coating the sensor are described in detail in co-owned U.S. Non-provisional application Ser. No. 10/981,901, filed Nov. 4, 2004, for Hong Tan, et al., entitled "Fiber-Optic Assay Apparatus Based on Phase-Shift Interferometry," FIG. **2** shows the binding of biotin (M.W. ~230 D) to a streptavidin coated BLI sensor. The data indicate that the described interferometry methods readily detect binding of molecules around 250 Dalton molecular weight, and that molecules in the 500 to 1000 Dalton molecular weight range generate a substantial change in optical layer thickness. Based on the minimum molecular size detection of BLI, one can configure the BLI sensor with immobilized substrates to monitor the activity of enzymes producing molecular size changes in the substrate as small as 250 to 1000 Daltons.

The small minimum molecular size detection limit of BLI makes it possible to apply BLI to a large number of enzymes. The following are by way of example, but not limitation, enzyme classes whose activities can be measured in accordance with the present invention, and specific examples of such measurements.

Example 2

Hydrolase Activity Measurements

Hydrolyases are enzymes that catalyze cleavage of C—O, C—N, C—C or phosphoric anhydride bonds.

Subgroup 1: Proteases (Enzymes Acting on Peptide Bonds) Immobilized Substrate Format

This format features the protease substrate immobilized to the surface of the BLI glass fiber sensor using methods described in co-owned U.S. Non-provisional application Ser. No. 10/981,901, filed Nov. 4, 2004, for Hong Tan, et al., entitled "Fiber-Optic Assay Apparatus Based on Phase-Shift Interferometry," (incorporated herein by reference) and below. The fiber is immersed in an enzyme-containing sample, and monitored for changes in optical layer thickness.

The basic assay protocol is to incubate a Bio-Layer Interferometer (BLI) sensor on which has been immobilized an enzyme substrate in an enzyme-containing solution enzyme. The amount of the substrate depletion is quantified by, e.g., a change in optical phase shift using the Bio-Layer Interferometry (BLI) technique fully described in co-owned and pending U.S. Non-provisional application Ser. No. 10/981,901, filed Nov. 4, 2004, for Hong Tan, et al., entitled "Fiber-Optic Assay Apparatus Based on Phase-Shift Interferometry," incorporated herein by reference. The change in, e.g., optical phase shift is proportional to the amount of enzyme activity in the solution because hydrolytic activity is estimated by measuring the depletion of substrate from the BLI sensor resulting from the enzyme activity.

Subtilisin Activity Measurements on Immobilized Casein Substrate

Methods

An optical signal baseline was established by immersing the optically-activated end of a fiber sensor tip in PBS and monitoring the optical signal using interferometry methods and instrumentation described in co-owned U.S. Non-provisional application Ser. No. 10/981,901, filed Nov. 4, 2004, for Hong Tan, et al, entitled "Fiber-Optic Assay Apparatus Based on Phase-Shift Interferometry," (incorporated herein by reference in its entirety). Next the fiber was coated with Poly-D-Lysine by incubating the tip in a 0.5 mg/mL Poly-D-Lysine solution (in PBS, pH 7.4) for 15 minutes. Unbound Poly-D-Lysine was rinsed by incubating the tips in PBS for 10 minutes.

The fiber was coated with a layer of casein [Sigma Chemical Company, St Louis, Mo.] by incubating the tip in a 50 μg/mL casein solution (50 mM Na Phosphate, 150 mM NaCl, pH 7) for 15 minutes. Unbound casein was rinsed by incubating the tip in PBS for 10 minutes.

Fibers with immobilized casein were incubated in various concentrations (1, 10, 50 μg/mL) of subtilisin [Sigma Chemical Company, St Louis, Mo.] solution (in 50 mM Na Phosphate, 150 mM NaCl, pH 7). Each of the procedures described above was carried out while monitoring the optical signal.

Results and Discussion

FIG. **3** illustrates the result of this example. The optical traces show calculated illustrate biolayer thickness as a function of time. Apparent are increases in biolayer thickness during casein loading and subsequent decreases following subtilisin incubation. The traces show a clear dose-response effect with more rapid and greater changes occurring with higher subtilisin concentration over the tested range.

Effect of Protease Inhibitor on Enzymatic Activity

Methods

Fiber sensor tips were prepared and coated with casein as described above. One fiber was incubated in 50 μg/mL of subtilisin solution (in 50 mM Na Phosphate, 150 mM NaCl, pH 7).

Other fibers were incubated in a pre-mixed and pre-incubated solution of 50 μg/mL of subtilisin and 3 mM PMSF [Sigma Chemical Company, St Louis, Mo.] (the mixture was incubated at 37° C. for 20 minutes) solution (50 mM Na Phosphate, 150 mM NaCl, pH 7). Each of the procedures described above was carried out while monitoring the optical signal.

Result and Discussion

Traces obtained from a fiber incubated in subtilisin solution show the expected depletion of casein from the tip surface (FIG. **4**). This shows up as smooth, time-dependent change in the trace following incubation in the enzyme solution. Fibers incubated in the subtilisin/PMSF mixture of subtilisin and (protease inhibitor) PMSF did not show the any time-dependent changes in the optical signal (FIGS. **5** and **6**), illustrating inhibition of subtilisin by 3 mM PMSF.

Substrate Capture Format

The substrate capture format entails digestion of the substrate by a protease in liquid phase followed by binding of the substrate to the surface of the BLI sensor. The binding of the substrate is designed so that proteolytic cleavage of the substrate produces a detectable to change in the optical layer thickness. In one preferred embodiment, a streptavidin/biotin

binding pair is used to effect substrate capture. FIG. **7** shows two approaches to tag the peptide substrate with biotin. In the case where the protease has multiple cleavage sites, the biotin does not have to be substituted at a specific site since the resulting digestion will produce a peptide with a sufficiently small molecular size. The substrate can also be designed so that there is a single cleavage site and the location of the biotin substitution is such that it remains on the smallest peptide fragment upon proteolysis. Methods for derivatizing substrate with biotin or another member of a binding pair are well known to ordinarily-skilled practitioners and include biochemical modification of existing substrate molecules, or, synthesis using derivatized sub-units. Such methods are described in, e.g., [*Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, 1988); *Bioconjugation Protocols: Strategies and Methods* (Methods in Molecular Biology (Clifton, N.J.), V. 283, 2004)] incorporated by reference, and exemplified below.

Example 3

Trypsin Activity Measurements Using Substrate Capture

Trypsin activity measurements are made using the substrate capture format and cytochrome C as a substrate. Cytochrome C is about 12 kDaltons in molecular weight and includes has 8 trypsin cleavage sites. A standard biotin-NHS derivative [Pierce Biotechnology, Rockford Ill.] is used to tag the cytochrome C. Coupling conditions employ standard phosphate buffered saline (PBS) pH 7 buffer. Biotin-NHS is mixed with cytochrome C at a molar coupling ratio of 5 to 1 (biotin to cytochrome C) typically resulting in one biotin substitution per cytochrome C molecule. Streptavidin-coated BLI sensors are prepared from 0.6 mm diameter glass fibers with a tantalum oxide layer of about 20 nm and a silicon dioxide layer of about 700 nm are dipped in a PBS solution containing 0.5 mg/ml of poly-D-lysine as described above. After 15 minutes at room temperature, the fibers are washed in PBS then immersed in a 1 mg/ml solution of bovine serum albumin (BSA) labeled with N-succinimidiyl 3-(2-pyridyldithio) propionate (SPDP) [Pierce Biotechnology, Rockford Ill.] and incubated for 20 minutes followed by a wash step with PBS. The fibers are then immersed in a solution of 50 mM dithiolthreitol for 30 minutes at room temperature. After a wash in PBS, the fibers are placed in the solution containing 20 μg/ml of streptavidin labeled with SMCC and incubated for 60 minutes, followed a by PBS wash. The fibers are stored in PBS until use.

The basic assay for protease activity using the substrate capture format entails adding to a biotinylated cytochrome C solution, in the range of about 1 μg/ml to 1 mg/ml, a trypsin sample, typically at a 1% wt./wt. ratio. After a digestion time period, a streptavidin coated sensor is placed in the enzyme/substrate mixture for substrate capture. To avoid trypsin acting upon the streptavidin, the enzyme can be inactivated either by snap boiling the substrate mixture or adding a protease inhibitor, such as aprotinin, just prior to adding the glass fiber. The BLI sensor can assess the change in substrate size by a measuring the optical layer thickness upon binding the biotinylated peptide. FIG. **8** illustrates the assay format where protease activity produces smaller peptides with a thinner optical layer.

Subgroup 2: Glycosylases (Enzymes that Hydrolyse O or S or N Glycosyl Bonds) Immobilized Substrate Format

The following example is described for dextranase, but the same methods can be applied to other polysaccharide processing enzymes such as amylase and cellulase. Dextran is linked to a protein coated BLI sensor by initially reacting the dextran with sodium periodate to introduce reactive aldehyde groups on the dextran polymer. A protein coated BLI sensor (such as prepared according to the methods described in the example above) is then immersed in the dextran solution. The aldehyde groups on the dextran form bonds with free amino groups on the protein resulting in a BLI sensor with a dextran as the final layer. Measurement of dextranase activity is possible when the dextran coated sensor is immersed in a dextranase containing sample. Hydrolytic activity of the dextranase reduces the molecular size of the immobilized dextran, which is detected as a thinner optical layer.

Substrate Capture Format

Measurement of glycosylases in the substrate capture format is accomplished by biotinylating the polysaccharide substrate. In the dextranase example, dextran is reacted with sodium periodate to generate aldehyde groups followed by the addition of large molar excess of a bis-amine, such as ethylenediamine. One amino group of the bis-amine couples to the aldehyde on the dextran leaving the second amine free for coupling to biotin-NHS in a subsequent reaction. The molar coupling ratio of the biotin-NHS reaction is selected to yield about one biotin substitution per dextran. The assay for dextranase activity in the substrate capture format is similar to the protease second example where the biotinylated dextran substrate is incubated with the dextranase sample followed by binding of the biotinylated dextran to a streptavidin coated BLI sensor. Dextranase activity creates smaller dextran fragments which are measured as a thinner optical layer.

Example 4

Esterase Activity Measurements

Subgroup 3: Esterases (Enzymes that Act on Ester Bonds)

Some examples of esterases include nucleases (RNase, DNase, etc.), alkaline phosphatase, acid phosphatase, and serine/threonine phosphatase. DNase I is used as an example of measuring esterase activity. Since DNase I cleaves at all 4 bases in oligonucleotides as small as three bases in length, dsDNA is prepared by a commercial vendor using standard DNA synthesis techniques having a length of about 30-40 base pairs with a biotin group at one of the terminal ends. The biotinylated dsDNA is bound to a streptavidin coated BLI sensor. The sensor is then immersed in sample containing DNase I in the buffer: 10 mM Tris pH 7.5, 2.5 $MgCl_2$, 0.5 nM $CaCl_2$ and the decrease in optical layer thickness is monitored. The alternative substrate capture format is performed by mixing the biotin labeled dsDNA with the DNase I in liquid phase before the binding of the substrate to the streptavidin coated sensor.

Example 5

Transferase Activity Measurements

Transferases are enzymes that catalyze the transfer of methyl, glycosyl, or phospho groups to other compounds. In contrast to the hydrolytic enzymes, the transferases increase the molecular size of the substrate and their activity is detected by the BLI sensor as an increase in optical layer thickness.

Subgroup 1: Nucleotide Transferases

Nucleotides transferases catalyze the incorporation of nucleotides into DNA or RNA polymers. FIG. **9** shows the

format for measuring DNA polymerase I activity as an example. A DNA template from 10 to 30 nucleotides is obtained from a commercial vendor with a biotin incorporation at either of the terminal nucleotides. The DNA template is hybridized with an oligonucleotide primer enabling the DNA polymerase I to incorporate nucleotides in the 5' to 3' direction. The biotin tagged DNA template can be bound to a streptavidin coated sensor either before or after the DNA polymerase step. Conditions for the polymerase step such as enzyme loading, dNTPs, buffer formulation, etc. follow established protocols. Since the dNTPs have molecular weights around 400 D, incorporation of as little as 1-2 nucleotides can be detected as a change in optical thickness.

An alternative approach to measure activity of nucleotide transferases is based on hapten incorporation as shown in FIG. **10**. A DNA template with a hybridized primer as mixed with DNA polymerase I and a mixture of dNTPs including biotinylated ATP. B-ATP is obtained from commercial sources since it is commonly used in nick translation. After the polymerase step, a streptavidin coated BLI sensor is placed in the sample mixture. Unincorporated B-ATP having a molecular weight around 600 D produces a relatively small increase in the optical layer upon binding to streptavidin, whereas B-ATP incorporated in the DNA template produces a greater increase in the optical layer depending on the molecular weight of the DNA.

Subgroup 2: Phosphotransferases

Phosphotransferases catalyze the transfer of phosphate groups to hydroxyl containing compounds, typically peptides with tyrosine, serine, or threonine residues. There are over 100 commercially monoclonal antibodies that bind to a phosphorylated amino acid or specific sequences encompassing a phosphorlyated amino acid. Many assays of kinase activity have been reported and are commercially available utilizing antibodies to phosphorylated peptide substrates. FIG. **11** describes an assay using an anti phosphotyrosine antibody. The antibody is initially biotinylated by standard methods then bound to a streptavidin coated sensor. The substrate $(Glu_4Tyr)_n$ is incubated with tyrosine kinase plus ATP, after which the sensor coated with anti phosphotyrosine is placed in the sample. The binding of the phosphorylated peptide produces an increase in the optical layer thickness.

FIG. **12** illustrates an assay using an antibody to a phosphorylated amino acid sequence for a specific kinase. In this case, the kinase is protein kinase C and commercial antibody to phosphorylated protein kinase C substrate. FIG. **13** shows an exemplary format for a kinase activation assay. In this example, mitogen activated protein kinase (MAPK) is activated by phosphorylation by another enzyme, MEK1. A commercial anti phospho MAPK bound to a BLI sensor detects the activation of MAPK.

While the invention has been particularly shown and described with reference to a preferred embodiment and various alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the invention.

All references, issued patents and patent applications cited within the body of the instant specification are hereby incorporated by reference in their entirety, for all purposes.

What is claimed is:

1. A method for assaying enzyme activity, comprising:

providing an optical element coupled to a light source via a mechanical coupling that engages the optical element with an optical fiber and provides an air gap between the optical element and the fiber, the optical element including (a) a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm, and (b) a layer of enzyme substrate molecules positioned so that interference between a reflected beam from the proximal reflecting surface and a reflected beam from the distal reflecting surface varies as an enzyme reacts with the substrate, and wherein the reflected beams are coupled into the optical fiber;

exposing the optical element to an enzyme; and

detecting a change in the interference between the reflected beams, wherein the change is indicative of enzyme activity.

2. The method of claim **1**, wherein the enzyme is a hydrolase or a transferase.

3. The method of claim **2**, wherein the enzyme is a protease, a phosphatase, a glycosylase, or an esterase.

4. The method of claim **2**, wherein the enzyme is a nucleotide transferase, a glycosyl transferase or a phosphotransferase.

5. The method of claim **1**, further comprising providing a second optical element coupled to the light source via a second fiber, the second optical element including (c) a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm, and (d) a layer of enzyme binding molecules positioned so that interference between a second reflected beam from the second optical element proximal reflecting surface and a reflected beam from the second optical element distal reflecting surface varies as the enzyme binds to the layer of enzyme binding molecules, and wherein the second reflected beams are coupled into the fiber; and detecting a change in the interference between the second reflected beams, wherein the change is indicative of enzyme activity.

6. The method of claim **5**, wherein the layer of enzyme binding molecules comprises an anti-enzyme antibody, a fragment of an anti-enzyme antibody, or an anti-enzyme scFv molecule.

7. The method of claim **1**, wherein the optical element is a first optical element, and wherein the method further comprises providing a second optical element positioned between the proximal reflecting surface of the first optical element and the fiber, the second optical element being coupled to the light source via the mechanical coupling that engages the second optical element with the fiber and provides an air gap between the second optical element and the fiber, and wherein the first optical element is coupled to the second optical element at the proximal reflective surface.

8. A method for assaying enzyme activity, comprising:

providing an optical element coupled to a light source via a mechanical coupling that engages the first optical element with a fiber and provides an air gap between the first optical element and the fiber, the optical element including (a) a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm, and (b) a layer of analyte binding molecules;

exposing the optical element to an enzyme substrate reacted or reacting with an enzyme, whereby the enzyme substrate or a portion of the enzyme substrate binds to the layer of analyte binding molecules, and wherein interference between a reflected beam from the proximal reflecting surface and a reflected beam from the distal reflecting surface varies as the enzyme substrate or portion thereof binds to the layer of enzyme binding molecules, the reflected beams coupled into the fiber; and

detecting a change in optical thickness of the first reflecting surface, wherein the change is indicative of enzyme activity.

9. The method of claim 8, wherein the enzyme is a hydrolase or a transferase.

10. The method of claim 9, wherein the enzyme is a protease, a phosphatase, a glycosylase, or an esterase.

11. The method of claim 9, wherein the enzyme is a nucleotide transferase, a glycosyl transferase or a phosphotransferase.

12. The method of claim 8, further comprising interposing a semi-permeable membrane between said optical element and said substrate.

13. The method of claim 8, wherein said substrate is bound to a support.

14. The method of claim 8, further comprising providing a second optical element coupled to the light source via a second fiber, the second optical element including (c) a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm, and (d) a layer of enzyme binding molecules positioned so that interference between a second reflected beam from the second optical element proximal reflecting surface and a reflected beam from the second optical element distal reflecting surface varies as the enzyme binds to the layer of enzyme binding molecules, and wherein the second reflected beams are coupled into the fiber; and detecting a change in the interference between the second reflected beams, wherein the change is indicative of enzyme activity.

15. The method of claim 14, wherein the layer of enzyme binding molecules comprises an anti-enzyme antibody, a fragment of an anti-enzyme antibody, or an anti-enzyme scFv molecule.

16. The method of claim 8, wherein the layer of analyte binding molecules comprises avidin, streptavidin, biotin, an antibody, an antibody fragment, an scFv, or a lectin.

17. The method of claim 8, further comprising the step of inactivating the enzyme before the exposing step.

18. The method of claim 8, wherein the optical element is a first optical element, and wherein the method further comprises providing a second optical element positioned between the proximal reflecting surface of the first optical element and the fiber, the second optical element being coupled to the light source via the mechanical coupling that engages the second optical element with the fiber and provides an air gap between the second optical element and the fiber, and wherein the first optical element is coupled to the second optical element at the proximal reflective surface.

* * * * *

EXHIBIT C

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THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

U.S. PATENT: *7,728,982*
ISSUE DATE: *June 01, 2010*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

SYLVIA HOLLEY
Certifying Officer

US007728982B2

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 7,728,982 B2**
(45) **Date of Patent: *Jun. 1, 2010**

(54) **FIBER-OPTIC ASSAY APPARATUS BASED ON PHASE-SHIFT INTERFEROMETRY**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 39 days.

This patent is subject to a terminal disclaimer.

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(51) **Int. Cl.**
G01B 9/02 (2006.01)

(52) **U.S. Cl.** **356/478**; 356/480

(58) **Field of Classification Search** 356/478, 356/480, 519

See application file for complete search history.

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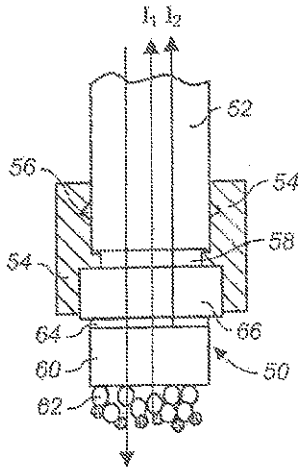
(57) **ABSTRACT**

Apparatus and method for detecting the presence or amount or rate of binding of an analyte in a sample solution is disclosed. The apparatus includes an optical assembly having first and second reflecting surfaces separated by a distance "d" greater than 50 nm, where the first surface is formed by a layer of analyte-binding molecules, and a light source for directing a beam of light onto said first and second reflecting surface. A detector in the apparatus operates to detect a change in the thickness of the first reflecting layer resulting from binding of analyte to the analyte-binding molecules, when the assembly is placed in the solution of analyte, by detecting a shift in phase of light waves reflected from the first and second surfaces.

35 Claims, 6 Drawing Sheets



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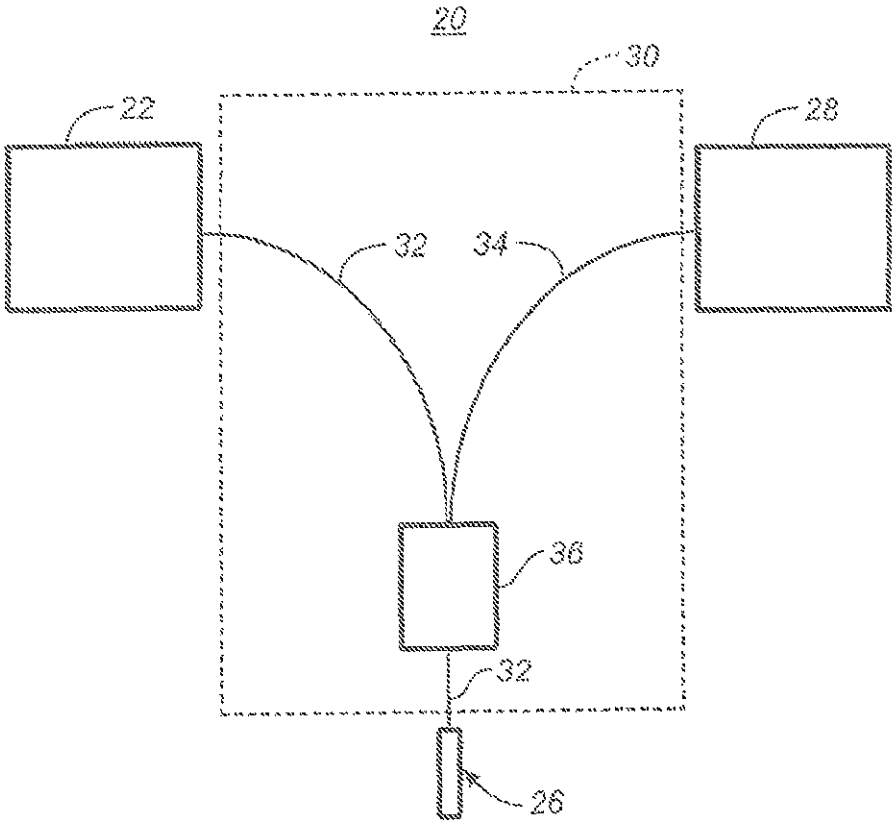

FIG. 1

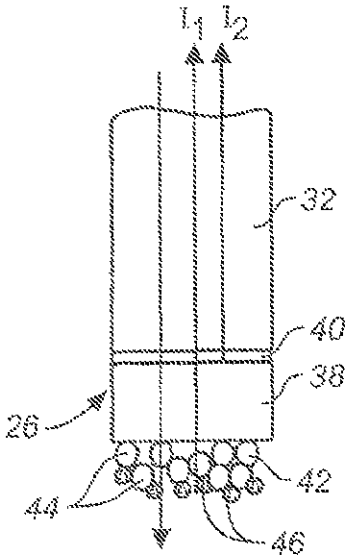

FIG. 2

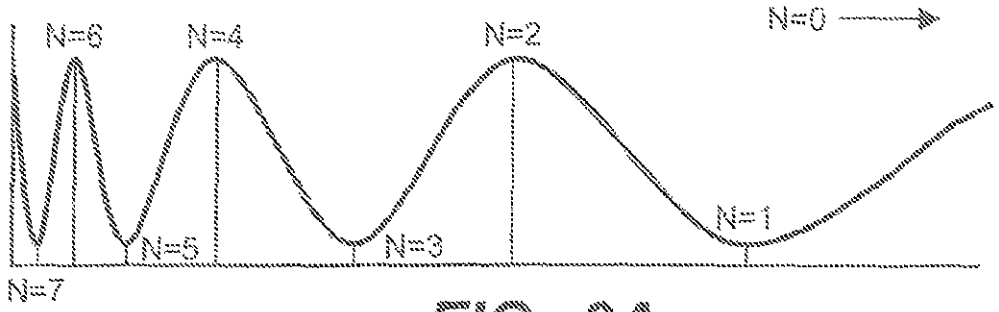

FIG. 3A

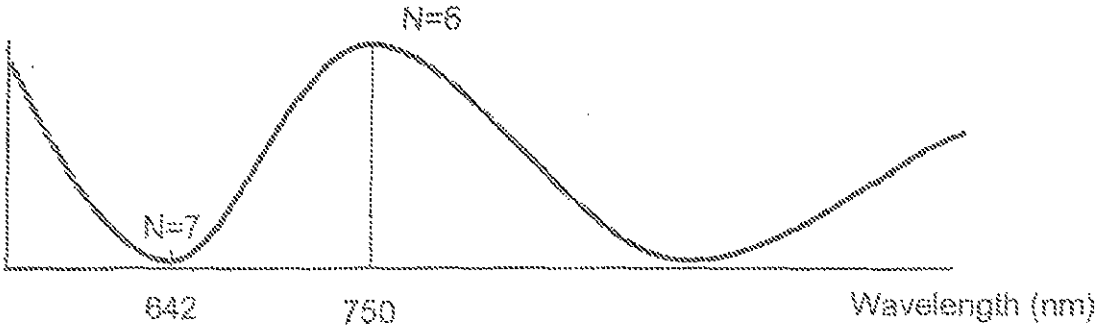

FIG. 3B

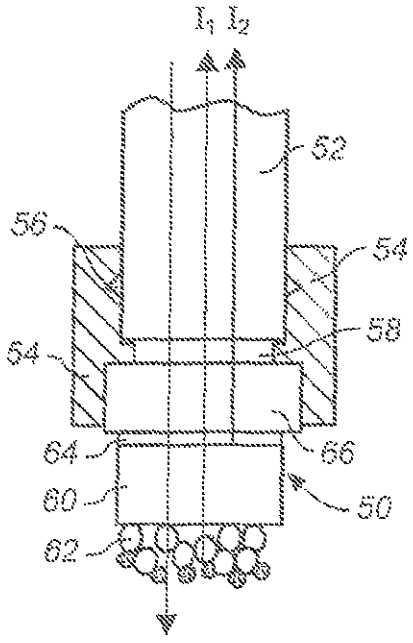

FIG. 4

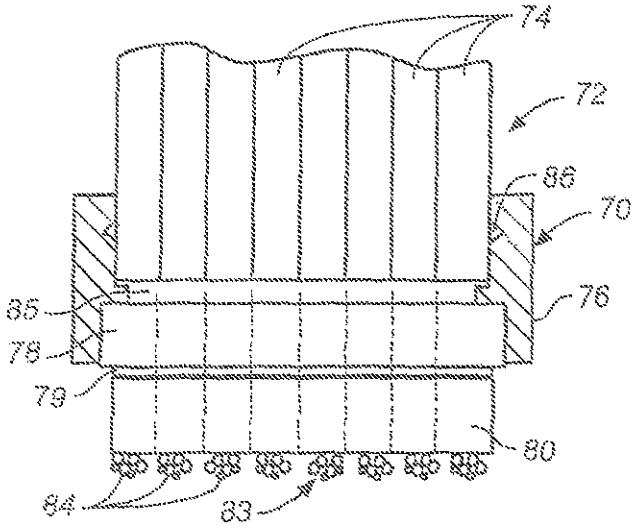

FIG. _5

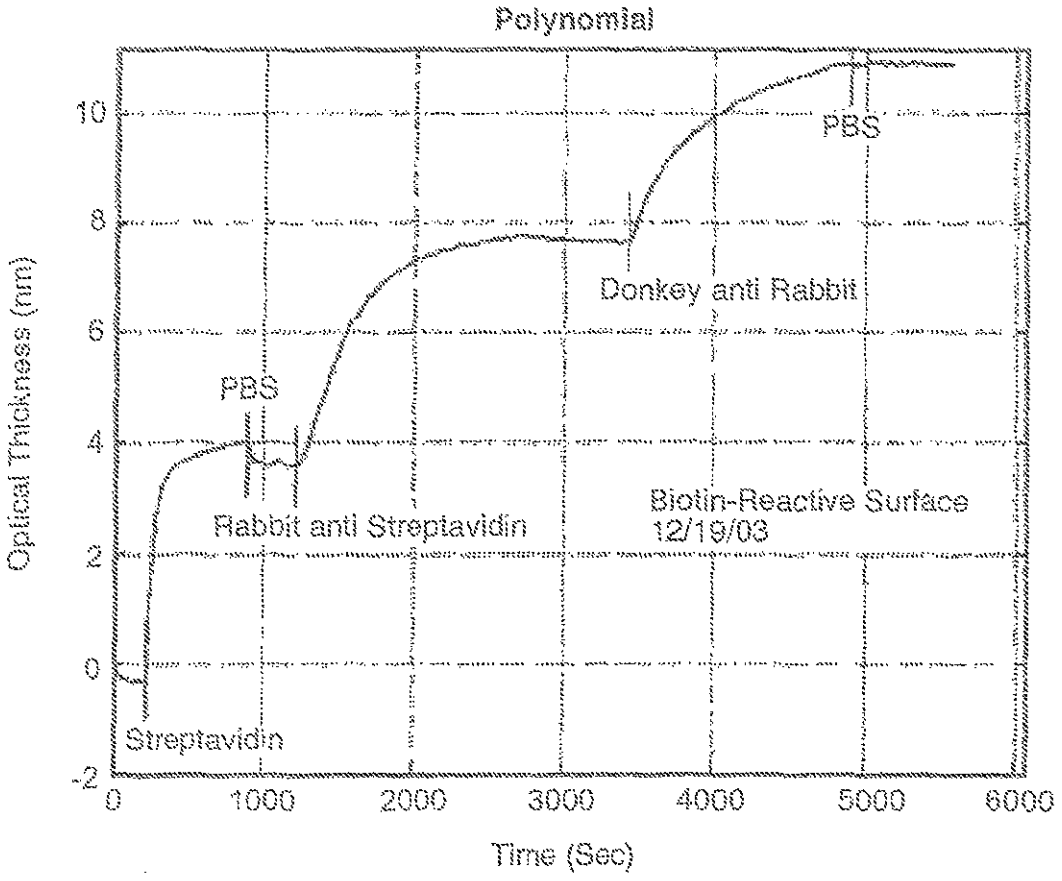

FIG. _6

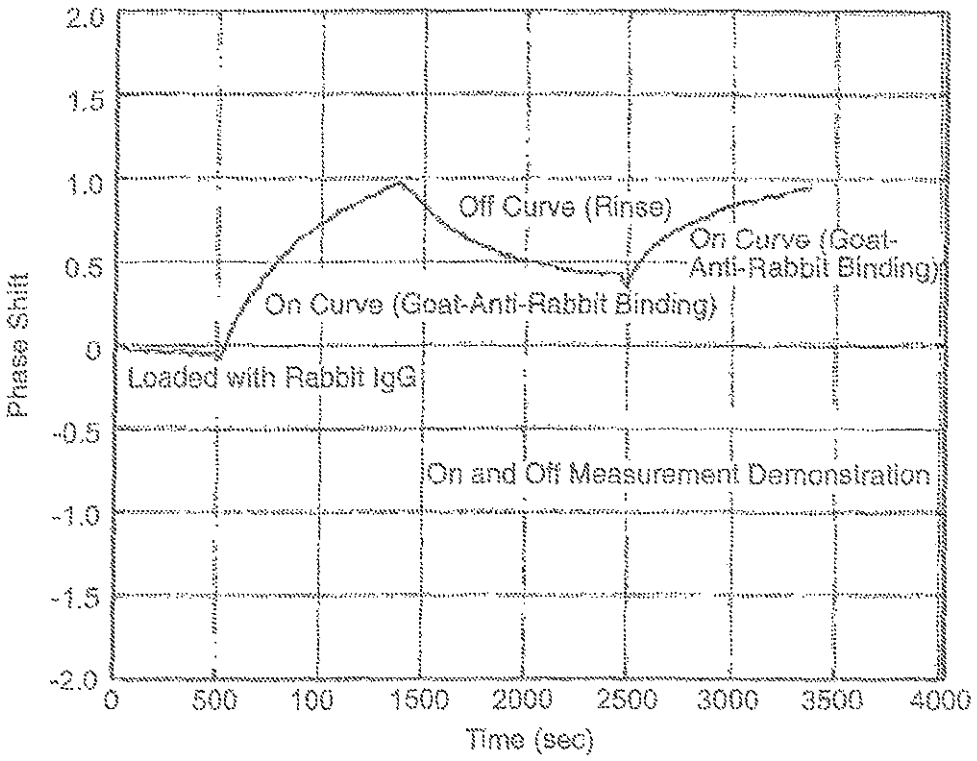

FIG. 7

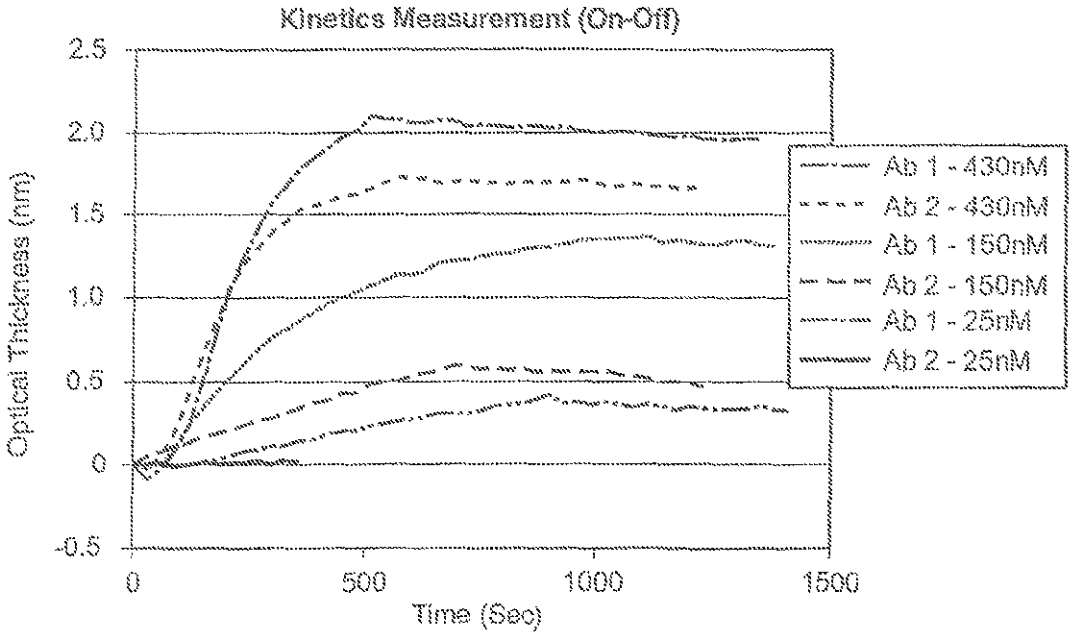

FIG. 8

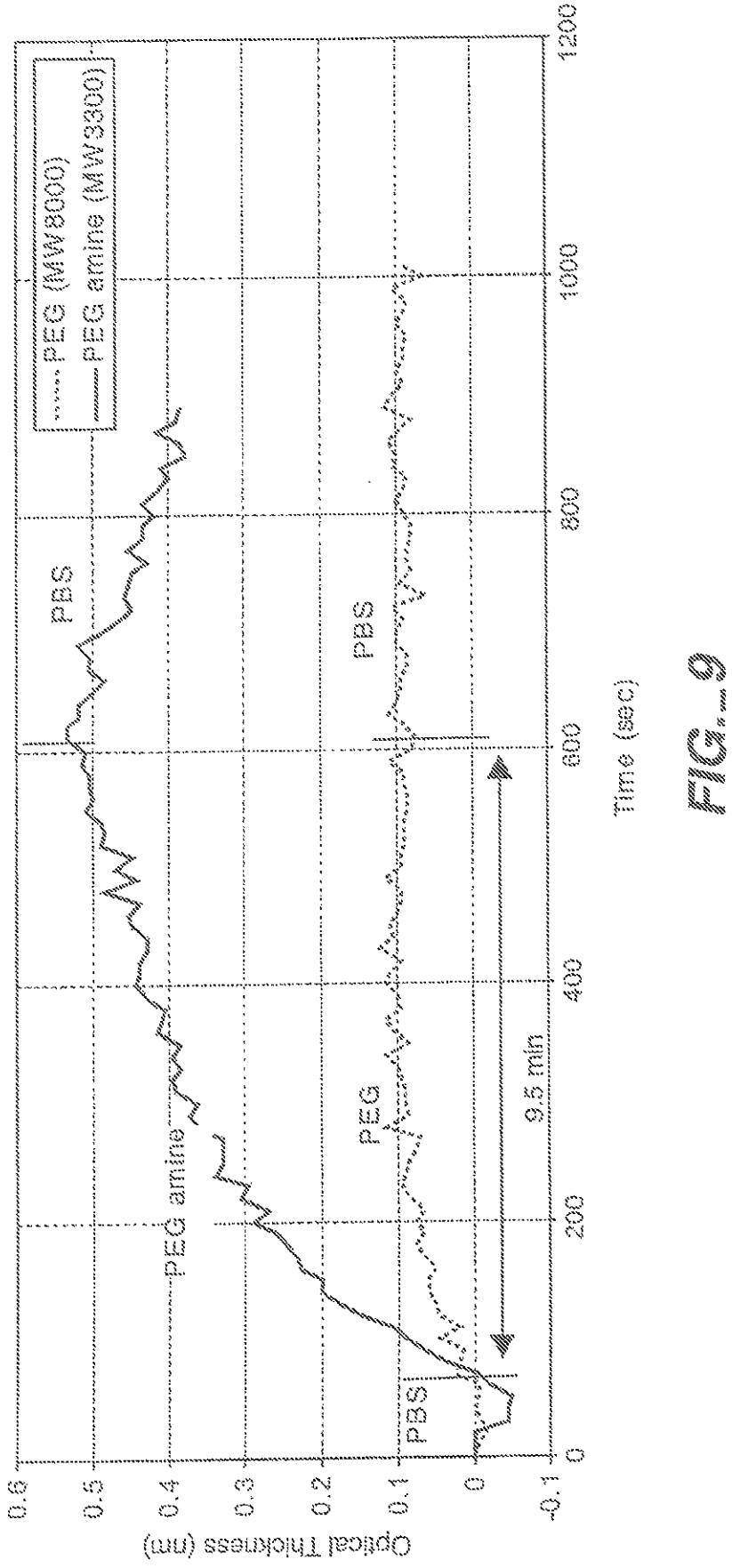

FIG. 9

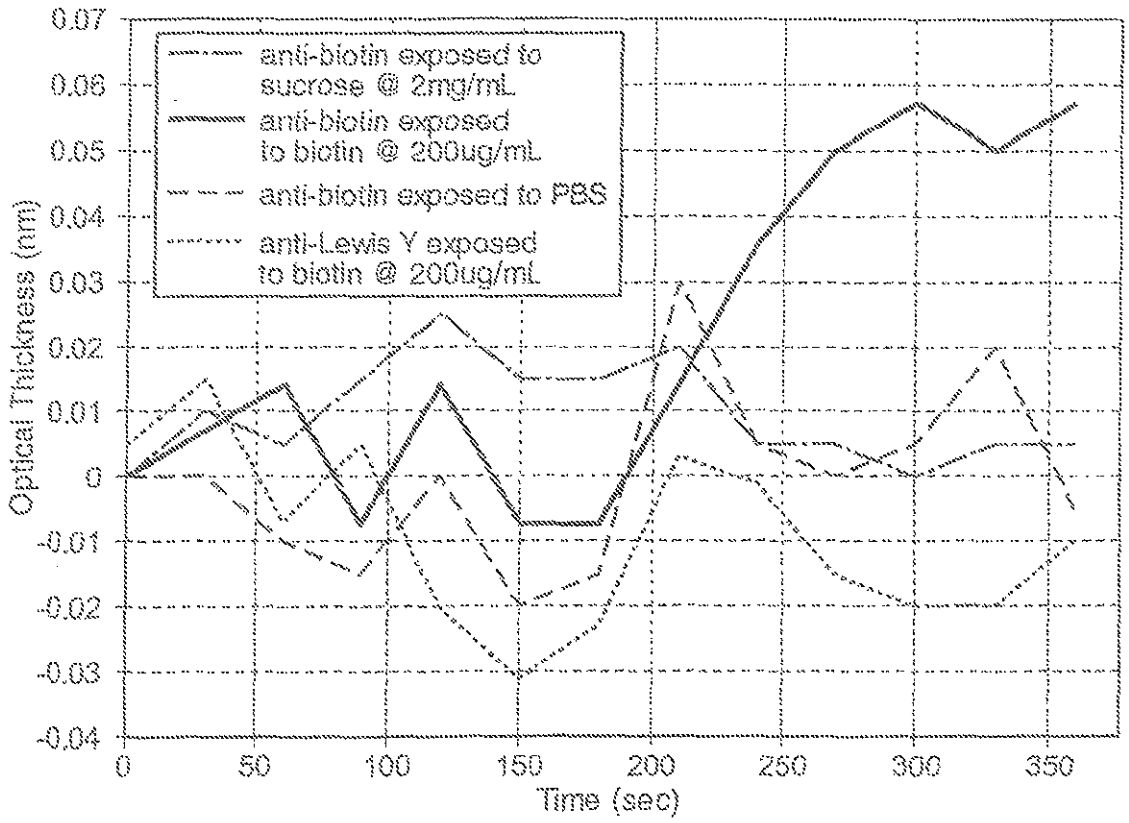

FIG. 10

FIBER-OPTIC ASSAY APPARATUS BASED ON PHASE-SHIFT INTERFEROMETRY

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 10/981,901, filed Nov. 4, 2004, entitled "Fiber-Optic Assay Apparatus Based on Phase-Shift Interferometry," which claims the benefit of (1) U.S. Provisional Application No. 60/518,068, filed Nov. 6, 2003, and (2) U.S. Provisional Application No. 60/558,381, filed Mar. 31, 2004, the entire disclosures of which are hereby incorporated by reference in their entireties, including any appendices or attachments thereof, for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to an apparatus and method for detecting the presence, amount, or rate of binding of one or more analytes in a sample, and in particular, to apparatus and method based on fiber optic interferometry.

2. Description of the Related Art

Diagnostic tests based on a binding event between members of an analyte-anti-analyte binding pair are widely used in medical, veterinary, agricultural and research applications. Typically, such methods are employed to detect the presence or amount or an analyte in a sample, and/or the rate of binding of the analyte to the anti-analyte. Typical analyte-anti-analyte pairs include complementary strands of nucleic acids, antigen-antibody pairs, and receptor-receptor binding agent, where the analyte can be either member of the pair, and the anti-analyte molecule, the opposite member.

Diagnostics methods of this type often employ a solid surface having immobilized anti-analyte molecules to which sample analyte molecules will bind specifically and with high affinity at a defined detection zone. In this type of assay, known as a solid-phase assay, the solid surface is exposed to the sample under conditions that promote analyte binding to immobilized anti-analyte molecules. The binding event can be detected directly, e.g. by a change in the mass, reflectivity, thickness, color or other characteristic indicative of a binding event. Where the analyte is pre-labeled, e.g., with a chromophore, or fluorescent or radiolabel, the binding event is detectable by the presence and/or amount of detectable label at the detection zone. Alternatively, the analyte can be labeled after it is bound at the detection zone, e.g., with a secondary, fluorescent-labeled anti-analyte antibody.

Co-owned U.S. Pat. No. 5,804,453, (the '453 patent) which is incorporated herein by reference, discloses a fiber-optic interferometer assay device designed to detect analyte binding to a fiber-optic end surface. Analyte detection is based on a change in the thickness at the end surface of the optical fiber resulting from the binding of analyte molecules to the surface, with greater amount of analyte producing a greater thickness-related change in the interference signal. The change in interference signal is due to a phase shift between light reflected from the end of the fiber and from the binding layer carried on the fiber end, as illustrated particularly in FIGS. 7a and 7b of the '453 patent. The device is simple to operate and provides a rapid assay method for analyte detection.

Ideally, an interferometer assay device will yield readily observable changes in spectral peak and valley (extrema) positions within the range of a conventional visible-light spectrometer, that is, in the visible light range between about 450-700 nm, such that relatively small optical thickness changes at the fiber end can be detected as significant changes in the spectral positions of interference wavelength peaks and valleys. One limitation which has been observed with the device described in the '453 patent is the absence of readily identified wavelength spectral extrema over this spectral range.

The present invention is designed to overcome this limitation, preserving the advantages of speed and simplicity of the earlier-disclosed device, but significantly enhancing sensitivity and accuracy. The present invention also provides a more convenient disposable-head format, as well as a multi-analyte array format, e.g., for gene-chip and protein-chip applications.

SUMMARY OF THE INVENTION

The invention includes, in one aspect, an apparatus for detecting an analyte in a sample, including detecting the presence of analyte, the amount of analyte or the rate of association and/or dissociation of analyte to analyte-binding molecules. The apparatus includes an optical element with a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm. A beam of light from an optical fiber is directed to and reflected from the two reflecting surfaces. The reflected beams are coupled back into the optical fiber and interfere. The optical element also includes a layer of analyte binding molecules that is positioned so that the interference between the reflected beams varies as analyte binds to the layer of analyte binding molecules.

The change in interference can be caused by different physical phenomenon. For example, analyte binding can cause a change in the optical path length or in the physical distance between the two reflecting surfaces. Alternately, analyte binding can cause a change in the index or in the optical absorption of material located between the reflecting surfaces. Analyte binding can also cause the layer of analyte binding molecules to swell, resulting in a change in the interference.

In one particular design, the distal reflecting surface includes the layer of analyte binding molecules. As analyte binds to the layer of analyte binding molecules, the optical path length or the physical distance between the two reflecting surfaces may increase, for example. In another aspect of the invention, a transparent solid material is located between the reflecting surfaces and, optionally, the proximal reflecting surface includes a material with an index greater than that of the transparent solid material. Alternately, an air gap may be located between the reflecting surfaces. In yet another design, the distal reflecting surface is positioned between the proximately reflecting surface and the layer of analyte binding molecules. For example, analyte binding may cause the layer of analyte binding molecules to swell, moving the distal reflecting surface closer to the proximal reflecting surface. In yet another design, the layer of analyte binding molecules is positioned between the two reflecting surfaces. Analyte binding may cause the layer to swell or to change its index, thus changing the interference between the two reflected beams.

In another aspect, the apparatus includes an optical assembly having first and second reflecting surfaces separated by a distance "d" greater than 50 nm. The optical assembly is composed of a transparent optical element that can have a thickness defined between proximal and distal faces of the

element of at least 50 nm, preferably between 400-1,000 nm. The first reflecting surface is carried on the distal face of optical element, and is formed of a layer of analyte-binding molecules. The second reflecting surface is formed by a coating of transparent material having an index of refraction greater than that of the optical element. This coating can be formed of a Ta_2O_5 layer having a preferred thickness of between 5 and 50 nm. The optical element can be SiO_2, and has a thickness of between about 100-5,000 nm, preferably 400-1,000 nm.

Also included are a light source for directing a beam of light onto the first and second reflecting surfaces, and a detector unit that operates to detect a change in the optical thickness of the first reflecting layer resulting from binding of analyte to the analyte-binding molecules, when the assembly is placed in the solution of analyte. The optical thickness change at the first reflecting layer is related to a shift in a phase characteristic of the interference wave formed by the two light waves reflected from said first and second surfaces. This phase characteristic can be a shift in the spectral position(s) of one or more peaks and valleys of the interference wave, or by a change in the period of a full cycle of the wave.

The light source can include an optical fiber having a distal end adapted to be placed adjacent the second reflecting surface in the assembly, and the apparatus further includes an optical coupling for directing reflected light waves reflected from the assembly to the detector.

In a first embodiment, the optical assembly is fixedly mounted on the optical fiber, with the distal end of the optical fiber in contact with the second reflecting surface. In a second embodiment, the optical assembly further includes a second transparent optical element having an index of refraction less than that of the second coating and a thickness greater than about 100 nm, where the coating of high index of refraction material is sandwiched between the two transparent optical elements. In this latter embodiment, the assembly is removably attached to the distal end region of the fiber with a spacing of less than 100 nm or greater than 2 μm between the distal end of the fiber and the confronting face of the second transparent optical element in the assembly.

For detecting multiple analytes, such as multiple nucleic acid species, the layer of analyte-binding molecules can be composed of an array of discrete analyte-binding regions, such as single strands of nucleic acid. The regions are effective to bind different analytes. The optical fiber includes a plurality of individual fibers, each aligned with one of the regions, the detector includes a plurality of detection zones, and the optical coupling functions to couple each of the plurality of fibers with one of the zones.

The analyte-binding molecules in the assembly can be, for example, (i) an anti-species antibody molecules, for use in screening hybridoma libraries for the presence of secreted antibody, (ii) antigen molecules, for use in detecting the presence of antibodies specific against that antigen; (iii) protein molecules, for use in detecting the presence of a binding partner for that protein; (iv) protein molecules, for use in detecting the presence of multiple binding species capable of forming a multi-protein complex with the protein; or (v) single stranded nucleic acid molecules, for detecting the presence of nucleic acid binding molecules.

The detector can be a spectrometer for measuring reflected light intensity over a selected range of wavelengths. Alternatively, or in addition, the light source can include a plurality of light-emitting diodes, each with a characteristic spectral frequency, and the detector functions to record light intensity of reflected light at each of the different LED frequencies. In still another embodiment, the light source includes a white-light source and the detector is designed to record light intensity of reflected light at each of a plurality of different wavelengths.

In another aspect, the invention includes a method for detecting the presence or amount of an analyte in a sample solution. The method involves reacting the sample solution with a first reflecting surface formed by a layer of analyte-binding molecules carried on the distal surface of a transparent optical element having a thickness of at least 50 nm, thereby to increase the thickness of the first reflecting layer by the binding of analyte to the analyte-binding molecules in the layer. The change in thickness of the first reflecting layer is measured by detecting a shift in a phase characteristic of the interference wave formed by the two light waves reflected from the first layer and from a second reflecting layer that is formed on the opposite, proximal surface of the optical element and which has an index of refraction greater than that of the optical element.

The detecting step can include directing light from an optical fiber onto the two reflecting surfaces, and directing reflected light from the two surfaces onto a detector through an optical coupling. The detector can be a spectrometer, where the detecting includes measuring a shift in the spectral position of one or more of the interference extrema produced by the two reflecting lightwaves.

Where the method is used for measuring the rate of association of analyte to the second layer, the reacting step can be carried out until a near-maximum increase in thickness of the first reflecting layer is observed. Where the method is used for measuring the rate of dissociation of analyte to the second layer, the reacting steps can include immersing the second layer in a dissociation buffer for a period of time until a decrease in thickness of the first reflecting layer is observed. Where the method is used for measuring the amount of analyte present in the sample, the detecting is carried out over a period sufficient to measure the thickness of the first reflecting layer at a plurality of different time points.

Where the method is used measuring one or more of a plurality of analytes in a sample, the first reflecting layer is composed of an array of discrete analyte-binding regions, the different regions being effective to bind different analytes, and the detecting is effective to detect a change in the thickness of each of the regions resulting from binding of analyte to the analyte-binding molecules.

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

FIG. **1** shows the basic system setup for the bioprobe and its apparatus;

FIG. **2** shows an optical assembly formed accordance to one embodiment of the invention;

FIGS. **3**A and **3**B show a portion of an interference wave over 7 peak and valley orders (**3**A), and over in a visible portion of the spectrum (**3**B);

FIG. **4** shows an optical assembly constructed according to another embodiment of the invention;

FIG. **5** shows a disposable multi-analyte optical assembly having an analyte-binding array and constructed according to another embodiment of the invention;

FIG. **6** shows a sequential binding of three molecules;

FIG. **7** shows on and off curves generated from the association and dissociation of antibodies;

FIG. **8** shows the curves of two antibodies binding to their antigen at different concentrations;

FIG. **9** shows immobilization of bis amino PEG (MW 3300) specifically through an amide bond formation. The PEG (MW 8000) is used as a negative control to monitor non-specific binding of the PEG polymer; and

FIG. **10** shows a small molecule binding to a large molecule, negative controls and the base line measurement.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Terms used in the claims and specification are to be construed in accordance with their usual meaning as understood by one skilled in the art except and as defined as set forth below. Numeric ranges recited in the claims and specification are to be construed as including the limits bounding the recited ranges.

The term "in vivo" refers to processes that occur in a living organism.

An "analyte-binding" molecule refers to any molecule capable of participating in a specific binding reaction with an analyte molecule. Examples include but are not limited to, e.g., antibody-antigen binding reactions, and nucleic acid hybridization reactions.

A "specific binding reaction" refers to a binding reaction that is saturable, usually reversible, and that can be competed with an excess of one of the reactants. Specific binding reactions are characterized by complementarity of shape, charge, and other binding determinants as between the participants in the specific binding reaction.

An "antibody" refers to an immunoglobulin molecule having two heavy chains and two light chains prepared by any method known in the art or later developed and includes polyclonal antibodies such as those produced by inoculating a mammal such as a goat, mouse, rabbit, etc. with an immunogen, as well as monoclonal antibodies produced using the well-known Kohler Milstein hybridoma fusion technique. The term includes antibodies produced using genetic engineering methods such as those employing, e.g., SCID mice reconstituted with human immunoglobulin genes, as well as antibodies that have been humanized using art-known resurfacing techniques.

An "antibody fragment" refers to a fragment of an antibody molecule produced by chemical cleavage or genetic engineering techniques, as well as to single chain variable fragments (SCFvs) such as those produced using combinatorial genetic libraries and phage display technologies. Antibody fragments used in accordance with the present invention usually retain the ability to bind their cognate antigen and so include variable sequences and antigen combining sites.

A "small molecule" refers to an organic compound having a molecular weight less than about 500 daltons. Small molecules are useful starting materials for screening to identify drug lead compounds that then can be optimized through traditional medicinal chemistry, structure activity relationship studies to create new drugs. Small molecule drug compounds have the benefit of usually being orally bioavailable. Examples of small molecules include compounds listed in the following databases: MDL/ACD (http://www.mdli.com/), MDL/MDDR (http://www.mdli.com/), SPECS (http://www.specs.net/), the China Natural Product Database (CNPD) (http://www.neotrident.com/), and the compound sample database of the National Center for Drug Screening (http://www.screen.org.cn/).

Abbreviations used in this application include the following: "ss" refers to single-stranded; "SNP" refers to single nucleotide polymorphism; "PBS" refers to phosphate buffered saline (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4); "NHS" refers to N-hydroxysuccinimide; "MW" refers to molecular weight; "Sulfo-SMCC" refers to sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Advantages and Utility

The advantages and utility of the invention are illustrated by reference to the Figures and Examples as described in greater detail below. These include the ability to monitor in real time analyte binding reactions without the use of labels, diminishing cost and potential toxicity. A further advantage includes the ability to practice the method using visible wavelength light sources. Yet other advantages are provided by the fiber optic nature of the detector tip that allows binding reactions to be monitored in very small sample volumes, including in "in vitro" spaces, and to bundle fibers to carry out highly multiplexed analyses of binding reactions.

FIG. **1** shows, in schematic view, an interferometer apparatus **20** constructed in accordance with the invention. In its most basic elements, the apparatus includes a light source **22**, an optical assembly **26** that functions as a sensing element or detector tip and that will be detailed further with respect to FIGS. **2**, **4** and **5** below, and a detector unit **28** for detecting interference signals produced by interfering light waves reflected from the optical assembly **26**.

Light from source **22** is directed onto the optical assembly **26**, and reflected back to the detector through an optical coupling assembly indicated by dashed lines at **30**. In a preferred embodiment, the coupling assembly includes a first optical waveguide or fiber **32** extending from the light source to the optical assembly, a second optical waveguide or fiber **34** which carries reflected light from the optical assembly to the detector, and an optical coupler **36** which optically couples fibers **32**, **34**. Suitable optical fiber and coupling components are detailed in the above-cited '453 patent. One exemplary coupler is commercially available from many vendors including Ocean Optics (Dunedin, Fla.).

Alternatively, the coupling assembling can include a lens system constructed to focus a light beam onto the upper surface of the optical assembly and to direct reflected interfering light from the optical assembly to the detector. The latter system would not require optical fibers, but would impose relatively stringent requirements on the positioning of the lens elements used for the optical coupling.

The light source in the apparatus can be a white light source, such as a light emitting diode (LED) which produces light over a broad spectrum, e.g., 400 nm or less to 700 nm or greater, typically over a spectral range of at least 100 nm. Alternatively, the light source can be a plurality of sources each having a different characteristic wavelength, such as LEDs designed for light emission at different selected wavelengths in the visible light range. The same function can be achieved by a single light source, e.g., white light source, with suitable filters for directing light with different selected wavelengths onto the optical assembly.

The detector is preferably a spectrometer, such as charge-coupled device (CCD), capable of recording the spectrum of

the reflected interfering light from the optical assembly. Alternatively, where the light source operates to direct different selected wavelengths onto the optical assembly, the detector can be a simple photodetector for recording light intensity at each of the different irradiating wavelengths. In still another embodiment, the detector can include a plurality of filters which allows detection of light intensity, e.g., from a white-light source, at each of a plurality of selected wavelengths of the interference reflectance wave. Exemplary light source and detector configurations are described in the above-cited '453 patent, particularly with respect to FIGS. **8** and **10** of that patent, and it will be understood that these configurations are suitable for use in the present invention.

FIG. **2** shows an optical assembly **26** constructed in accordance with one embodiment of the invention, and an adjoining portion of the distal end region of an optical fiber **32** to which the optical assembly is fixedly attached. As seen, the assembly **26** includes a transparent optical element **38** having first and second reflecting surfaces **42**, **40** formed on its lower (distal) and upper (proximal) end faces, respectively. According to an important feature of the invention, the thickness "d" of the optical element between its distal and proximal surfaces, i.e., between the two reflecting surfaces, is at least 50 nm, and preferably at least 100 nm. An exemplary thickness is between about 100-5,000 nm, preferably 400-1,000 nm. The first reflecting surface **42** is formed of a layer of analyte-binding molecules, such as molecules **44**, which are effective to bind analyte molecules **46** specifically and with high affinity. That is, the analyte and anti-analyte molecules are opposite members of a binding pair of the type described above, which can include, without limitations, antigen-antibody pairs, complementary nucleic acids, and receptor-binding agent pairs.

The index of refraction of the optical element is preferably similar to that of the first reflecting surface, so that reflection from the lower distal end of the end optical assembly occurs predominantly from the layer formed by the analyte-binding molecules, rather than from the interface between the optical element and the analyte-binding molecules. Similarly, as analyte molecules bind to the lower layer of the optical assembly, light reflection form the lower end of the assembly occurs predominantly from the layer formed by the analyte-binding molecules and bound analyte, rather than from the interface region. One exemplary material forming the optical element is SiO_2, e.g., a high-quality quality glass having an index of refraction of about 1.4-1.5. The optical element can also be formed of a transparent polymer, such as polystyrene or polyethylene, having an index of refraction preferably in the 1.3-1.8 range.

The second reflecting surface in the optical assembly formed as a layer of transparent material having an index of refraction that is substantially higher than that of the optical element, such that this layer functions to reflect a portion of the light directed onto the optical assembly. Preferably, the second layer has a refractive index greater than 1.8. One exemplary material for the second layer is Ta_2O_5 with refractive index equal to 2.1. The layer is typically formed on the optical element by a conventional vapor deposition coating or layering process, to a layer thickness of less than 50 nm, typically between 5 and 30 nm.

The thickness of the first (analyte-binding) layer is designed to optimize the overall sensitivity based on specific hardware and optical components. Conventional immobilization chemistries are used in chemically, e.g., covalently, attaching a layer of analyte-binding molecules to the lower surface of the optical element. For example, a variety of bifunctional reagents containing a siloxane group for chemical attachment to SiO_2, and an hydroxyl, amine, carboxyl or other reaction group for attachment of biological molecules, such as proteins (e.g., antigens, antibodies), or nucleic acids. It is also well known to etch or otherwise treat glass a glass surface to increase the density of hydroxyl groups by which analyte-binding molecules can be bound. Where the optical element is formed of a polymer, such as polystyrene, a variety of methods are available for exposing available chemically-active surface groups, such as amine, hydroxyl, and carboxyl groups.

The analyte-binding layer is preferably formed under conditions in which the distal surface of the optical element is densely coated, so that binding of analyte molecules to the layer forces a change in the thickness of the layer, rather than filling in the layer. The analyte-binding layer can be either a monolayer or a multi-layer matrix.

The measurement of the presence, concentration, and/or binding rate of analyte to the optical assembly is enabled by the interference of reflected light beams from the two reflecting surfaces in the optical assembly. Specifically, as analyte molecules attach to or detach from the surface, the average thickness of the first reflecting layer changes accordingly. Because the thickness of all other layers remains the same, the interference wave formed by the light waves reflected from the two surfaces is phase shifted in accordance with this thickness change.

Assume that there are two reflected beams: The first beam is reflected from the first surface, which is the distal end interface between analyte-binding molecules and bound analyte and the surrounding medium; and the second beam is reflected from the second surface, which is the proximal interface between the optical element (the first layer) and the high-index of refraction layer (the second layer). The overall wavelength-dependent intensity of the interference wave is:

$$I = I_1 + I_2 + 2\sqrt{I_1 I_2}\cos\left(\frac{2\pi\Delta}{\lambda}\right)$$

where I is the intensity, I_1 and I_2 are the intensity of two interference beams, Δ is the optical path difference, and λ is the wavelength.

When $(2\pi\Delta/\lambda)=N\pi$, the curve is at its peak or valley if N is an integer 0, 1, 2, ...

The thickness of the first layer $d=\Delta/2n$. Therefore, $\lambda=4nd/N$ at peaks or valleys (extrema).

For the first several values of N, i.e., 0, 1, 2, . . . 7, and assuming a d of 770 nm, the equation gives:

N=0: $\lambda=\infty$ (peak)

N=1: $\lambda=4nd=4,496.80$ nm (Valley)

N=2: $\lambda=2nd=2,248.40$ nm (Peak)

N=3: $\lambda=4nd/3=1,498.9$ nm (Valley)

N=4: $\lambda=nd=1,124.20$ nm (Peak)

N=5: $\lambda=4nd/5=899.36$ nm (Valley)

N=6: $\lambda=2nd/3=749.47$ nm (Peak)

N=7: $\lambda=4nd/7=642$ nm (Valley)

N=8: $\lambda=nd/2=562$ nm (Peak)

N=9: $\lambda=4nd/9=499.64$ nm (Valley)

N=10: λ=4nd/10=449.6 nm (Peak)

As can be seen, and illustrated further in FIGS. **3**A and **3**B, at least three peaks/valleys (N=7-9) occur in the visible spectral range.

If the 7^{th} order valley is used to calculate the change in molecular layer thickness, when the molecular layer attached to the first layer increases from 0 nm to 10 nm, the 7^{th} order valley will shift to 650.74 nm. Therefore, the ratio between the actual the phase shift of the 7^{th} order valley and thickness change equals (650.74-642.40)/10=0.834.

By contrast, if the initial spacing between the two reflecting layers is made up entirely of the analyte-binding molecules on the end of the fiber, assuming a thickness of this layer of 25 nm, then the first order peak will occur at 146 nm, clearly out of the range of the visible spectrum, so that the device will only see a portion of the region between the O-order valley and the first order peak, but will not see any peaks, making a shift in the spectral characteristics of the interference wave difficult to measure accurately.

Not until the total thickness of the reflecting layer approaches about 100 nm will the first-order peak appear in the visible spectrum. Assuming a total thickness change of up to 50 nm, the thickness of the optical element can then be as small as 50 nm, but is preferably on the order of several hundred nm, so that the phase shift or change in periodicity of the interference wave can be measured readily by a shift in the spectral positions of higher-order peaks or valleys, e.g. where N=3-10.

The ratio between the actual thickness and the measured phase shift is considered as a key factor of measurement sensitivity. It can be appreciated how one can adjust the thickness of the optical element and its refractive index to improve and optimize the sensitivity to accommodate the electronics and optical designs.

FIG. **4** shows an optical assembly **50** that is removably carried on the distal end of an optical fiber **52** in the assay apparatus. The optical element includes a plurality of flexible gripping arms, such as arms **54**, that are designed to slide over the end of the fiber and grip the fiber by engagement of an annular rim or detente **56** on the fiber with complementary-shaped recesses formed in the arms, as shown. This attachment serves to position the optical assembly on the fiber to provide an air gap **58** between the distal end of the fiber and the confronting (upper) face of the assembly, of less than 100 nm or greater than 2 μm. With an air gap of greater than about 100 nm, but less that 2 μm, internal reflection from the upper surface of the optical assembly can contribute significantly to undesirable fringes that can adversely impact the detection accuracy.

With continued reference to FIG. **4**, the optical assembly includes a first optical element **60** similar to optical element **38** described above, and having first and second reflective layers **62**, **64**, respectively, corresponding to above-described reflective layers **40**, **42**, respectively. The assembly further includes a second optical element **66** whose thickness is preferably greater than 100 nm, typically at least 200 nm, and whose index of refraction is similar to that of first optical element **60**. Preferably, the two optical elements are constructed of the same glass or a polymeric material having an index of refraction of between about 1.4 and 1.6. Layer **64**, which is formed of a high index of refraction material, and has a thickness preferably less than about 30 nm, is sandwiched between the 2 optical elements as shown.

In operation, the optical assembly is placed over the distal fiber end and snapped into place on the fiber. The lower surface of the assembly is then exposed to a sample of analyte, under conditions that favor binding of sample analyte to the analyte-binding molecules forming reflective layer **62**. As analyte molecules bind to this layer, the thickness of the layer increases, increasing the distance "d" between reflective surfaces **62** and **64**. This produces a shift in the extrema of the interference wave produced by reflection from the two layers, as described above with reference to FIGS. **3**A and **3**B. This shift in extrema or wavelength, or wavelength period, in turn, is used to determine the change in thickness at the lower (distal-most) reflecting layer. After use, the optical assembly can be removed and discarded, and replaced with fresh element for a new assay, for assaying the same or a different analyte.

FIG. **5** illustrates an optical assembly and fiber bundle in an embodiment of the invention designed for detecting one or more of a plurality of analytes, e.g. different-sequence nucleic acid analytes, in a sample. A fiber bundle **72** is composed of an array, e.g., circular array, for individual optical fibers, such as fibers **74**. The optical assembly, indicated generally at **70**, is composed of the basic optical elements described above with reference to FIG. **4**, but in an array format. Specifically, a first optical element **80** in the element provides at its lower distal surface, an array of analyte-reaction regions, such as regions **84**, each containing a layer of analyte-binding molecules effective to bind to one of the different analytes in the sample. Each region forms a first reflective layer in the optical assembly. One preferred sensing provides an array of different-sequence nucleic acids, e.g., cDNAs or oligonucleotides, designed to hybridize specifically with different-sequence nucleic acid analyte species in a sample. That is, the array surface forms a "gene chip" for detecting each of a plurality of different gene sequences.

Also included in the optical assembly are a second optical element **78** and a layer **79** of high index of refraction material sandwiched between the two optical elements, and which provides the second reflecting surface in the optical assembly. The assembly is carried on the fiber bundle **72** by engagement between a pair of flexible support arm, such as arm **76** and an annular rim or detente **86** on the bundle. With the assembly placed on the fiber bundle, the lower distal ends of the fibers are spaced from the confronting surface of optical element **78** by an air gap **85** whose spacing is preferably less than 100 nm or greater than 2 μm. Further, each of the fibers is aligned with a corresponding assay region of the optical assembly, so that each fiber is directing light on, and receiving reflected light from, its aligned detection region. Similarly, the optical coupler in the apparatus, which serves to couple multiple fibers to the detector, preserves the alignment between the array regions and corresponding positions on an optical detector, e.g., two-dimensional CCD. The materials and thickness dimensions of the various optical-assembly components are similar to those described above with respect to FIG. **4**.

The apparatus described in this invention can be used more specifically for the following applications:

(i) with an anti-species antibody carried on the tip, for screening hybridoma expression lines for cell lines with high antibody expression;

(ii) with an antigen carried on the tip, to characterize high affinity antibodies against that antigen;

(iii) with a protein carried on the tip, for identifying and characterizing binding partners (DNA, RNA, proteins, carbohydrates, organic molecules) for that protein;

(iv) with a carbohydrate or glycosyl moiety carried on the tip, for identifying and characterizing binding partners (such as, e.g., DNA, RNA, proteins, carbohydrates, organic molecules) for that carbohydrate;

(v) with a protein thought to participate in a multi-protein complex carried on the tip, for characterizing the binding components and/or kinetics of complex formation;

(vi) with a small protein-binding molecule carried on the tip, for identifying and characterizing protein binders for that molecule;

(vii) with an antibody carried on the tip, for constructing a calibration curve for the analyte using a set of analytes standards. Using this calibration curve, one can then determine the concentration of the analyte in unknown solutions (cell culture supernatants, biological samples, process mixtures, etc).

(viii) with a single-stranded nucleic acid, e.g. ssDNA or RNA carried on the tip, for identifying and molecules that bind specifically to the nucleic acid.

Using a temperature control block, the apparatus and method can also be used to monitor the binding and characterize the binding of an immobilized ssDNA to an oligonucleotide in solution to perform SNP analysis.

The following examples illustrate various methods and applications of the invention, but are in no way intended to limit its scope.

EXAMPLES

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T. E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A. L. Lehninger, Biochemistry (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences,* 18th Edition (Easton, Pa.: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry* 3rd Ed. (Plenum Press) Vols A and B (1992).

Example 1

Small Molecule-Protein Binding Reaction

This example demonstrates the capability to detect the binding of protein to small molecule immobilized on a sensor tip and subsequent bindings of multiple antibodies. The two-layer configuration on the tip of an optic fiber is used for this test. The thickness of the first Ta_2O_5 layer is 25 nm and the thickness of the second SiO_2 layer is 770 nm. The fiber was purchased from Ocean Optics (Dunedin, Fla.). It was manually cut into segments that are 40 mm long. Both ends of these segments were polished to standard mirror surface quality. The polishing method used here was exactly the same as those for optical lenses and mirrors. One surface of these fiber segments was outsourced to an optical coating house for Ta_2O_5 layer and SiO_2 layer. This vendor employed an ion-beam assisted physical vapor deposition (IAPVD) coater made by Leybold. IAPVD is a commonly used coating technique for anti-reflection and optical filters. The experimental steps included the following (all steps are performed at room temperature unless otherwise noted):

The fiber tip was coated with a polymer monolayer derivatized with biotin. The polymer monolayer was prepared using a biotinylated lipid (custom). This lipid was using to form a lipid monolayer on the surface of water solution. The monolayer was cross linked using UV light for 15 minutes. Clean, dry fibers were then brought in contact with the floating thin film and the biotin polymer was adsorbed onto the fiber tip. The fibers were then dryed at 60° C. for 1 hour. The fiber were then stored under ambient conditions

The biosensor tip was immersed in 50 μg/ml streptavidin streptavidin (Pierce Biotechnology, Rockford Ill., cat #21122) in PBS (Invitrogen, Carlsbad, Calif.; cat #14190078) for 9 minutes and then rinsed briefly with PBS.

The same tip was dipped into 10 μg/ml rabbit-anti-streptavidin solution (AbCam, Cambridge, Mass.; cat #ab6676-1000) in PBS for 36 minutes and then washed with PBS briefly.

Finally, the tip was immersed in 50 μg/mL donkey-anti-rabbit antibody solution antibody (Jackson ImmunoResearch, West Grove, Pa.; cat# 711-005-152) in PBS for 25 minutes. A final 10 minute rinse was performed in PBS solution.

FIG. **6** shows the real-time response curve for this sequential binding test. The vertical axis is the 7^{th} order valley phase shift in nanometers. It clearly shows the binding of streptavidin to the biotin already immobilized on the tip, and subsequent bindings of anti-streptavidin antibody to streptavidin and a second antibody to this first antibody. The dissociation of the streptavidin layer from the tip was visible (a small reduction in the optical thickness) at 900 seconds.

Example 2

Biomolecular Interaction Analysis of Kinetics and Affinity of Biomolecular Interactions

This example illustrates use of the invention to carry out a biomolecular interaction analysis (BIA) measuring kinetics and affinity of biomolecular interactions. The same tip configuration as described in Example 1 was used. The experimental steps included the following (all steps are performed at room temperature unless otherwise noted):

Mercaptosilane coated tips were prepared using the following procedure. Clean, dry fibers were incubated in a mixture of Toluene: hexanoic acid: mercaptopropyltrioxysilane (10:2:1 volumetric ratio) at room temperature for 24 hours. The fibers were rinsed 2× with 10 mL toluene for 5 minutes each. The fibers were then rinsed 1× with 10 mL of ethanol and dried under a stream of argon and stored at ambient conditions.

The biosensor tip was first derivatized by immersion in a with 10 μg/ml solution of rabbit-IgG (Jackson ImmunoResearch, West Grove, Pa.; cat# 309-005-003) in PBS for 1 hour.

The coated tip was dipped into 10 μg/ml goat-anti-rabbit antibody solution (Jackson ImmunoResearch, West Grove, Pa.; cat# 111-005-003) in PBS and remained in it for 15 minutes.

The tip was removed and washed in PBS. To facilitate the dissociation of the second antibody from the first antibody, the PBS was agitated manually for 20 minutes.

The tip was then dipped into the same goat-anti-rabbit solution again to show the reproducible association of goat-anti-rabbit to rabbit-IgG.

FIG. **7** shows the on and off curves generated from the association and dissociation of rabbit-IgG and goat-anti-rab-

bit. The vertical axis is again the 7^{th} order valley phase shift. The phase shift is directly related to the average thickness with a ratio of 0.834. The ability to detect the on and off curves reliably is essential for measuring interaction kinetics and affinity.

Example 3

Calculating Affinity Constants from Antibody-Antigen Binding and Release Curves

This experiment demonstrates the calculation of affinity constants from measuring on and off curves for two antibodies and their antigen. The proprietary antibodies were labeled as Ab-1 and Ab-2. The molecular weight of the antigen was about 30 kilodaltons. The same tip configuration as described in Example 1 was used. The same mercaptosilane fiber preparation as described in Example 2 was used. The experimental steps included (all steps are performed at room temperature unless otherwise noted):

The fiber tip was activated for covalent attachment of the antigen. Mercaptosilane coated fibers were activated by immersing the sensor tips in 50 µL of a 50 mg/mL solution of sulfo-SMCC (Pierce Biotechnology, Rockford Ill.; cat #22322) in DMF (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #494488) at for 2 hours. The sensor tips were rinsed briefly in DMF and dried;

The antigen was covalently bound to the activated fiber tip by immersing the activated tip in a 20 µg/ml solution of antigen in PBS for 20 minutes. The tip was rinsed with PBS for 2 minutes. Following the PBS rinse, the tip was quenched with an aqueous solution of 100 µM ethanolamine pH 8.5 (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #E9508) for 5 minutes and then was rinsed again in PBS for 2 minutes.

The same tip was immersed in antibody for an association test and the real-time binding data were recorded for 9-15 minutes (depending on the antibody identity and concentration). Once those data were recorded, the tip was again immersed in PBS and agitated to measure the off curve (i.e., dissociation between the immobilized antigen and bound antibody) for 9-15 minutes. The binding (on curve) and dissociation (off curve) measurements were repeated using different concentrations of antibody (25 nM, 150 nM, and 430 nM) and with two different antibodies identified as Ab-1 and Ab-2.

FIG. **8** shows the association and dissociation curves at different concentrations. The test of 25 nM Ab-2 was not completed because the association was extremely slow at this concentration. These illustrated curves are plots of the raw data.

K_{on}, K_{off}, and K_D were derived from these curves by fitting the raw data with a first order exponential function. By averaging two sets of data, kinetic and affinity coefficients were obtained as follows:

Ab-1	Ab-2
$K_{on} = 1.35 \times 10^5$ ($M^{-1}S^{-1}$)	$K_{on} = 2.01 \times 10^5$ ($M^{-1}S^{-1}$)
$K_{off} = 5.55 \times 10^{-5}$ (S^{-1})	$K_{off} = 8.15 \times 10^{-5}$ (S^{-1})
$K_D = K_{off}/K_{on} = 3.99 \times 10^{-9}$ (M)	$K_D = K_{off}/K_{on} = 4.45 \times 10^{-9}$ (M)

Example 4

NHS-Ester Activated Tips

The same tip configuration as described in Example 1 was used. The same mercaptosilane fiber preparation as described in Example 2 was used. Mercaptosilane coated fibers were activated by immersing the sensor tips in 50 µL of a 50 mg/mL solution of sulfo-SMCC (Pierce Biotechnology, Rockford Ill.; cat #22322) in DMF (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #494488) at for 2 hours. The sensor tips were rinsed briefly in DMF and dried.

Amine containing molecules can be covalently bound to this surface through formation of a stable amide linkage. Molecules that do not contain free amines are not immobilized through the NHS moiety, but these molecules can still bind to the surface through non-specific binding. This non-specific binding can be multi-layered whereas the covalent immobilization through the NHS esters will be in a single layer controlled by the availability and accessibility of the NHS ester.

In this set of experiments, a bis amino PEG (MW 3300) (Shearwater Polymers, San Carlos, Calif.) was used as a test compound to covalently bind to the activated surface. A PEG (MW 8000) (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #04162) that contained no free amino groups was used as a negative control. This negative control was used to look for any non-specific or multi-layered binding that might be inherent to PEG polymers on this surface.

FIG. **9** shows the time course of the treatment of the activated mercaptosilane tip with the test molecules. The activated tip showed a distinct increase in optical thickness upon exposure to the 0.1 mg/mL bis amino PEG (MW 3300) in PBS. This increase is stopped when the bis amino PEG solution is replaced by the PBS buffer. The activated tip exposed to 0.1 mg/mL PEG (MW8000) in PBS, which contains no amines, shows a small initial increase in optical thickness but the trace quickly becomes flat. From this it can be concluded that the PEG polymer does not have intrinsic non-specific binding and that the binding seen for the bis amino PEG is attributed to the specific covalent immobilization of the amine group.

Example 5

Antibody Derivatized Tips Using NHS-Ester Chemistry

This example illustrates the binding of a low molecular weight molecule binding to an immobilized high molecular weight molecule. Using the same NHS ester terminated surface described in Example 4 and the same tip configuration as described in Example 1, an anti-biotin antibody was immobilized to 3 fibers. Immobilization of the antibody was accomplished by immersing the activated fiber in a 20 µg/mL solution of mouse anti-biotin antibody (Biodesign, Saco Minn.; cat #H61504M) in PBS for 1 hour at room temperature. The tip was rinsed with PBS for 2 minutes. Following the PBS rinse, the tip was quenched with an aqueous solution of 100 µM ethanolamine pH 8.5 (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #E9508) for 5 minutes and then was rinsed again in PBS for 2 minutes.

The first fiber was exposed to a solution of 200 µg/mL biotin (Pierce Biotechnology, Rockford Ill.; cat #29129) in PBS. Controls using a solution of sucrose (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #S8501) (2 mg/mL) and PBS were carried out on the second and the third fibers to

determine baseline noise. Data from these tests are shown in FIG. **10**. Biotin binding is seen as an increase in optical thickness, whereas exposure to sucrose shows no detectable increase over baseline (PBS).

Another negative control was carried out using an irrelevant antibody (anti-Lewis Y antibody from Calbiochem, San Diego Calif.; cat# 434636) immobilized in an identical fashion to the anti-biotin antibody above. This immobilized antibody was exposed to a solution of 200 μg/mL biotin. The lack of biotin binding to this antibody indicates that the biotin binding to the anti-biotin antibody is a result of specific interactions and not due to non-specific binding.

While the invention has been particularly shown and described with reference to a preferred embodiments and various alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the invention.

All references, issued patents and patent applications cited within the body of the instant specification are hereby incorporated by reference in their entirety, for all purposes.

What is claimed is:

1. An optical assembly for use in detecting an analyte in a sample based on interference, comprising:

an optical fiber having a tip; and

an optical element removably attached to the tip of the optical fiber and configured for receiving a beam of light from the optical fiber, the optical element comprising flexible gripping arms that slide over and grip the optical fiber to removably attach to optical element to the optical fiber,

said optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface separated from the first reflecting surface by the transparent material, said first and second reflecting surfaces separated by at least 50 nm,

wherein said first reflecting surface binds a layer of analyte binding molecules positioned so that interference between a beam of light reflected from the first reflecting surface into the optical fiber and a beam of light reflected from the second reflecting surface into the optical fiber varies as analyte binds to the layer of analyte binding molecules.

2. The assembly of claim **1**, wherein said second reflecting surface comprises a layer of material having an index of refraction greater than the refractive index of said transparent material.

3. The assembly of claim **1**, wherein the separation between said first and second reflecting surfaces is between 100 nm and 5,000 nm.

4. The assembly of claim **3**, wherein the separation between said first and second reflecting surfaces is between 400 nm and 1,000 nm.

5. The assembly of claim **1**, wherein the refractive index of said optical element transparent material is less than 1.8.

6. The assembly of claim **5**, wherein said optical element transparent material is a material selected from the group consisting of SiO_2 and a transparent polymer.

7. The assembly of claim **6**, wherein said transparent polymer comprises polystyrene or polyethylene.

8. The assembly of claim **1**, wherein the second reflecting surface comprises a layer of material having a refractive index greater than 1.8.

9. The assembly of claim **8**, wherein said second reflecting surface layer comprises Ta_2O_5.

10. The assembly of claim **9**, wherein the thickness of said second reflecting surface layer is between 5 nm and 50 nm.

11. The assembly of claim **1**, wherein said layer of analyte binding molecules comprises a molecule selected from the group consisting of a protein, a small molecule, a nucleic acid and a carbohydrate.

12. The assembly of claim **11**, wherein said protein is selected from the group consisting of an avidin, a streptavidin, an antibody, and an antibody fragment.

13. The assembly of claim **1**, wherein said optical assembly is adapted for coupling to said light source through a mechanical coupling that engages said optical element with said fiber.

14. The assembly of claim **1**, wherein said optical element is adapted for coupling to said light source through a coupling assembly that comprises a lens system.

15. The assembly of claim **1**, further comprising a second optical element overlaying said second reflecting surface.

16. The assembly of claim **15**, wherein the thickness of said second optical element is greater than 100 nm.

17. The assembly of claim **16**, wherein the thickness of said second optical element is greater than 200 nm.

18. The assembly of claim **15**, wherein said optical assembly is adapted for coupling to said light source through a mechanical coupling that engages said second optical element with said fiber.

19. The assembly of claim **18**, wherein said mechanical coupling provides an air gap between said second optical element and said fiber.

20. The assembly of claim **19**, wherein said air gap is less than 100 nm.

21. The assembly of claim **19**, wherein said air gap is greater than 2 μm.

22. The assembly of claim **1**, wherein the optical element is configured for detachment from the optical fiber and disposal after use.

23. A two dimensional array of the assemblies of claim **1**.

24. A two dimensional array of the assemblies of claim **15**.

25. An apparatus for detecting an analyte, comprising:

the assembly of claim **1**;

a light source for directing light onto said first and said second reflecting surfaces; and

a detector that receives light from said first and said second reflecting surfaces and detects a change in optical thickness of said first reflecting surface upon exposure of said first reflecting surface to said analyte.

26. An apparatus for detecting an analyte, comprising:

the assembly of claim **15**;

a light source for directing light onto said first and said second reflecting surfaces; and

a detector that receives light from said first and said second reflecting surfaces and detects a change in optical thickness of said first reflecting surface upon exposure of said first reflecting surface to said analyte.

27. An apparatus for detecting an analyte, comprising:

the two dimensional array of the assemblies of claim **23**;

a light source for directing light onto said first and said second reflecting surfaces; and

a detector that receives light from said first and said second reflecting surfaces and detects a change in optical thickness of said first reflecting surface upon exposure of said first reflecting surface to said analyte.

28. An apparatus for detecting an analyte, comprising:

the two dimensional array of the assemblies of claim **24**;

a light source for directing light onto said first and said second reflecting surfaces; and

a detector that receives light from said first and said second reflecting surfaces and detects a change in optical thick-

ness of said first reflecting surface upon exposure of said first reflecting surface to said analyte.

29. A kit, comprising:

an optical assembly comprising:

an optical fiber having a tip;

an optical element removably attached to the tip of the optical fiber and configured for receiving a beam of light from the optical fiber, the optical element comprising flexible gripping arms that slide over and grip the optical fiber to removably attach to optical element to the optical fiber,

said optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface, said second reflecting surface separated from the first reflecting surface by the transparent material, said first and second reflecting surfaces separated by at least 50 nm,

wherein said first reflecting surface binds a layer of analyte binding molecules positioned so that interference between a beam of light reflected from the first reflecting surface into the optical fiber and a beam of light reflected from the second reflecting surface into the optical fiber varies as analyte binds to the layer of analyte binding molecules, and said second reflecting surface comprises a layer of material having an index of refraction greater than the refractive index of said transparent material; and

instructions for binding said layer of analyte binding molecules to said first reflecting surface.

30. The kit of claim **29**, further comprising a reagent for chemically modifying said first surface and instructions for using said reagent.

31. The kit of claim **29**, wherein said optical assembly further comprises a second optical element overlaying said second reflecting surface.

32. A method for detecting analyte in a sample, comprising:

providing the apparatus of claim **25** and a sample;

exposing said first reflecting surface to said sample, and

determining whether said exposure results in a change in optical thickness of said first reflecting surface.

33. A method for detecting analyte in a sample, comprising:

providing the apparatus of claim **26** and a sample;

exposing said first reflecting surface to said sample, and

determining whether said exposure results in a change in optical thickness of said first reflecting surface.

34. A method for detecting analyte in a sample, comprising:

providing the apparatus of claim **27** and a sample;

exposing said first reflecting surface to said sample, and

determining whether said exposure results in a change in optical thickness of said first reflecting surface.

35. A method for detecting analyte in a sample, comprising:

providing the apparatus of claim **28** and a sample;

exposing said first reflecting surface to said sample, and

determining whether said exposure results in a change in optical thickness of said first reflecting surface.

* * * * *

EXHIBIT D

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United States Patent and Trademark Office

December 13, 2021

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

U.S. PATENT: ***8,305,585***
ISSUE DATE: ***November 06, 2012***

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

SYLVIA HOLLEY
Certifying Officer

(12) **United States Patent**
Tan et al.

(10) **Patent No.: US 8,305,585 B2**
(45) **Date of Patent: *Nov. 6, 2012**

(54) **FIBER-OPTIC ASSAY APPARATUS BASED ON PHASE-SHIFT INTERFEROMETRY**

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(73) Assignee: **Pall Corporation**, Port Washington, NY (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 66 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/790,736**

(22) Filed: **May 28, 2010**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. 12/099,751, filed on Apr. 8, 2008, now Pat. No. 7,728,982, which is a continuation of application No. 10/981,901, filed on Nov. 4, 2004, now Pat. No. 7,394,547.

(60) Provisional application No. 60/518,068, filed on Nov. 6, 2003, provisional application No. 60/558,381, filed on Mar. 31, 2004.

(51) **Int. Cl.**
G01B 9/02 (2006.01)

(52) **U.S. Cl.** **356/478**; 356/480

(58) **Field of Classification Search** 356/480, 356/519

See application file for complete search history.

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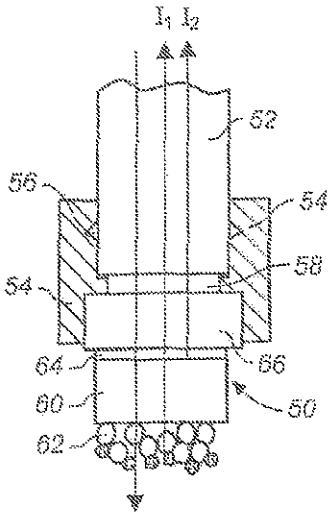
Primary Examiner — Gregory J Toatley
Assistant Examiner — Dominic J Bologna
(74) *Attorney, Agent, or Firm* — Leydig, Voit & Mayer, Ltd.

(57) **ABSTRACT**

Apparatus and method for detecting the presence or amount or rate of binding of an analyte in a sample solution is disclosed. The apparatus includes an optical assembly having first and second reflecting surfaces separated by a distance "d" greater than 50 nm, where the first surface is formed by a layer of analyte-binding molecules, and a light source for directing a beam of light onto said first and second reflecting surface. A detector in the apparatus operates to detect a change in the thickness of the first reflecting layer resulting from binding of analyte to the analyte-binding molecules, when the assembly is placed in the solution of analyte, by detecting a shift in phase of light waves reflected from the first and second surfaces.

19 Claims, 6 Drawing Sheets



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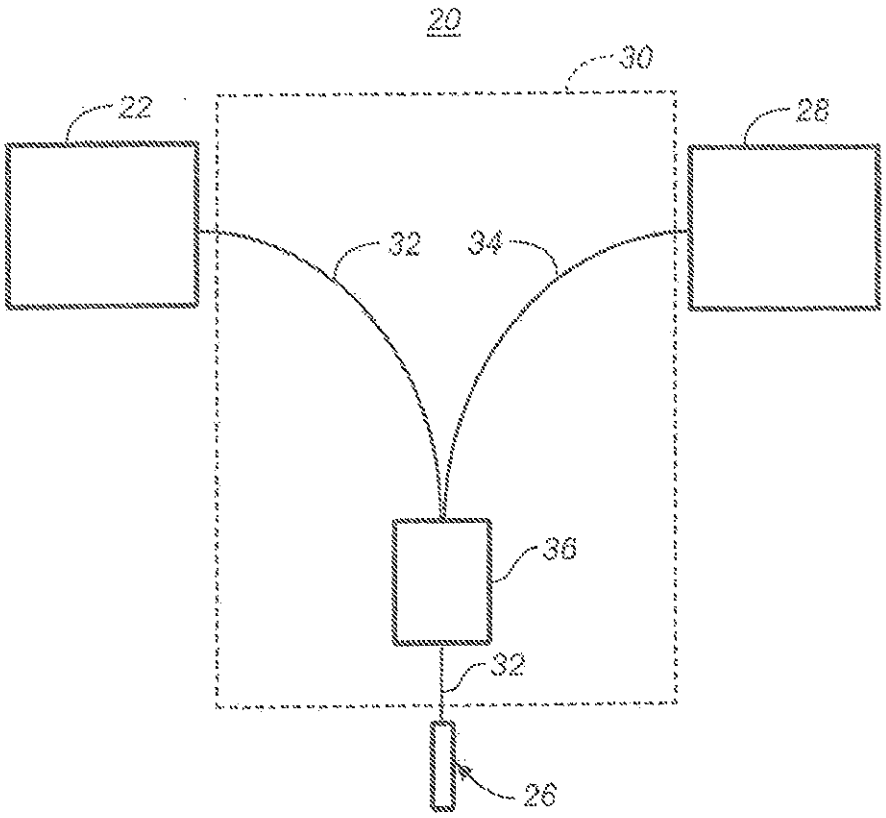

FIG. 1

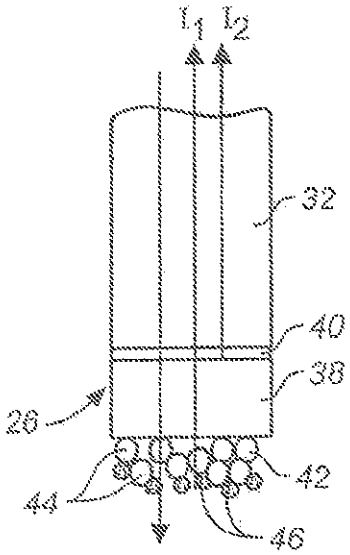

FIG. 2

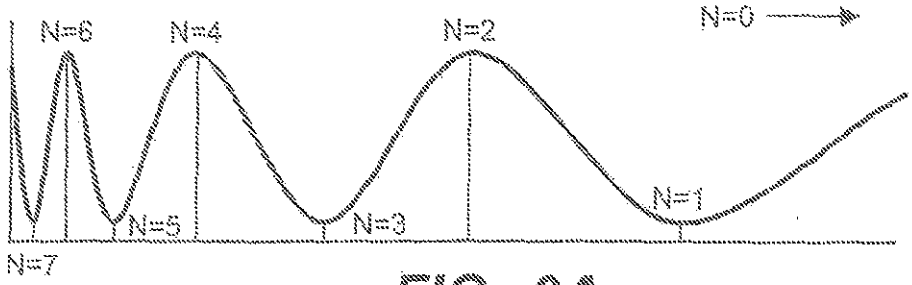

FIG. 3A

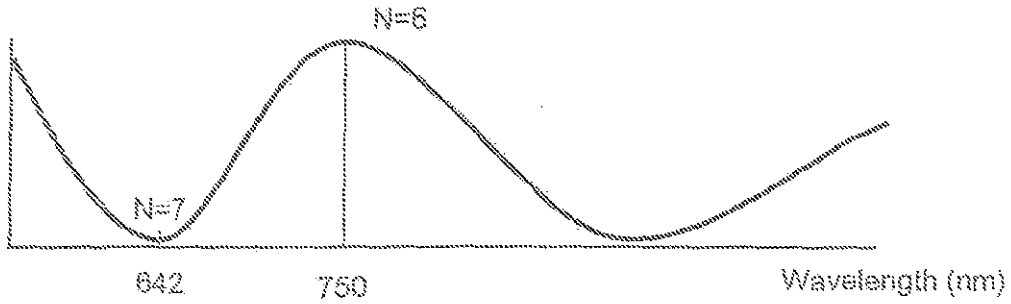

FIG. 3B

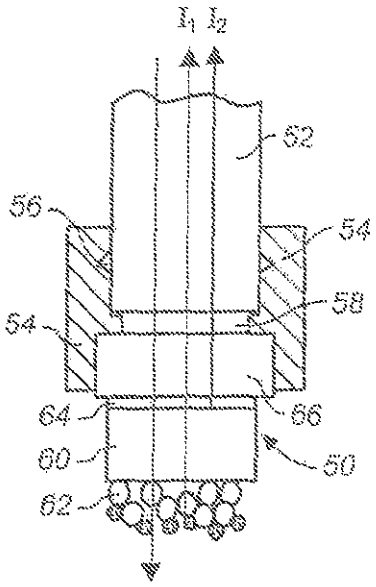

FIG. 4

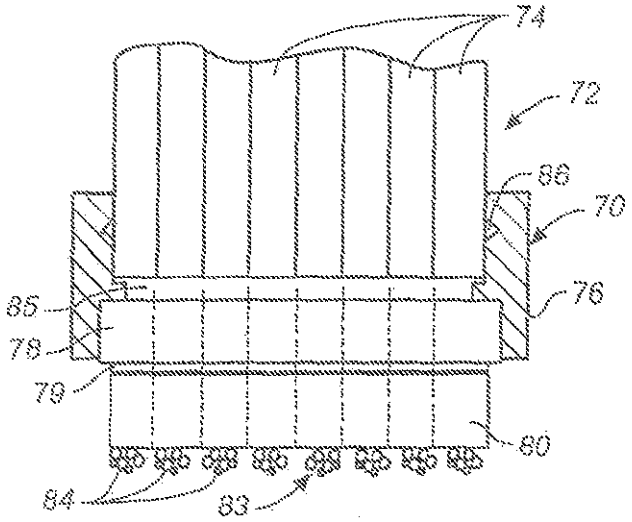

FIG. 5

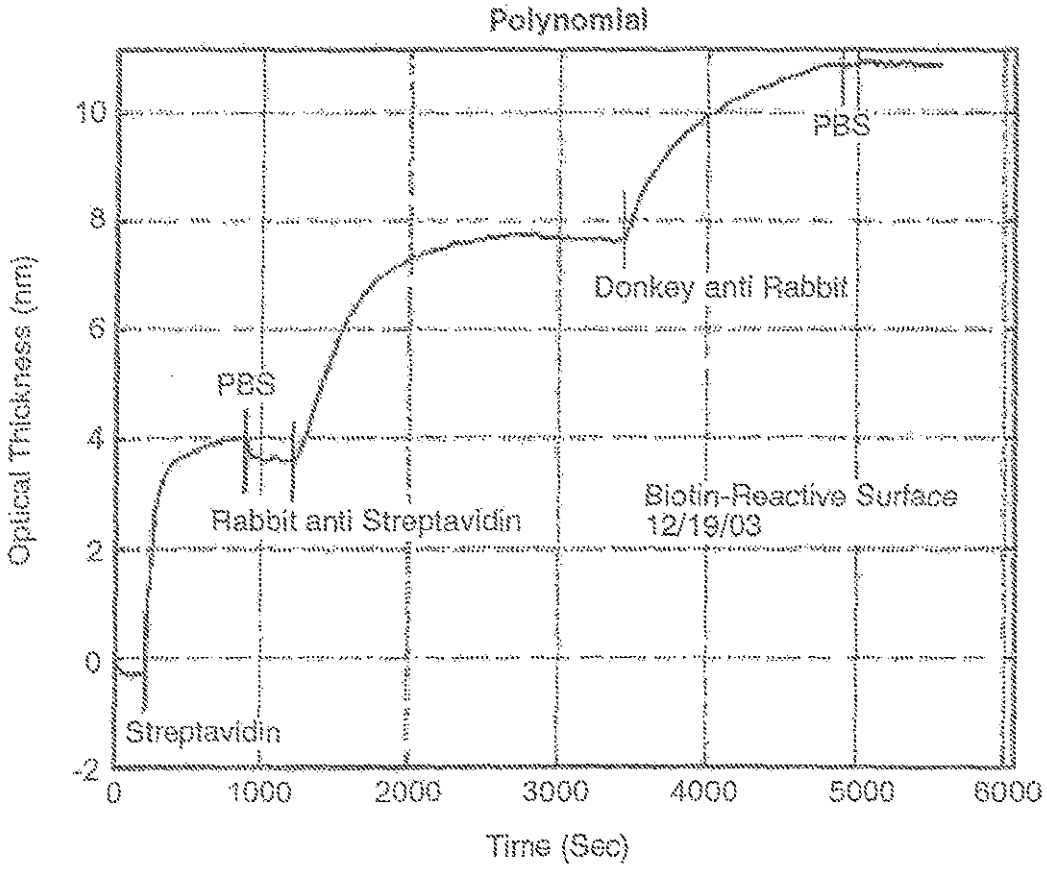

FIG. 6

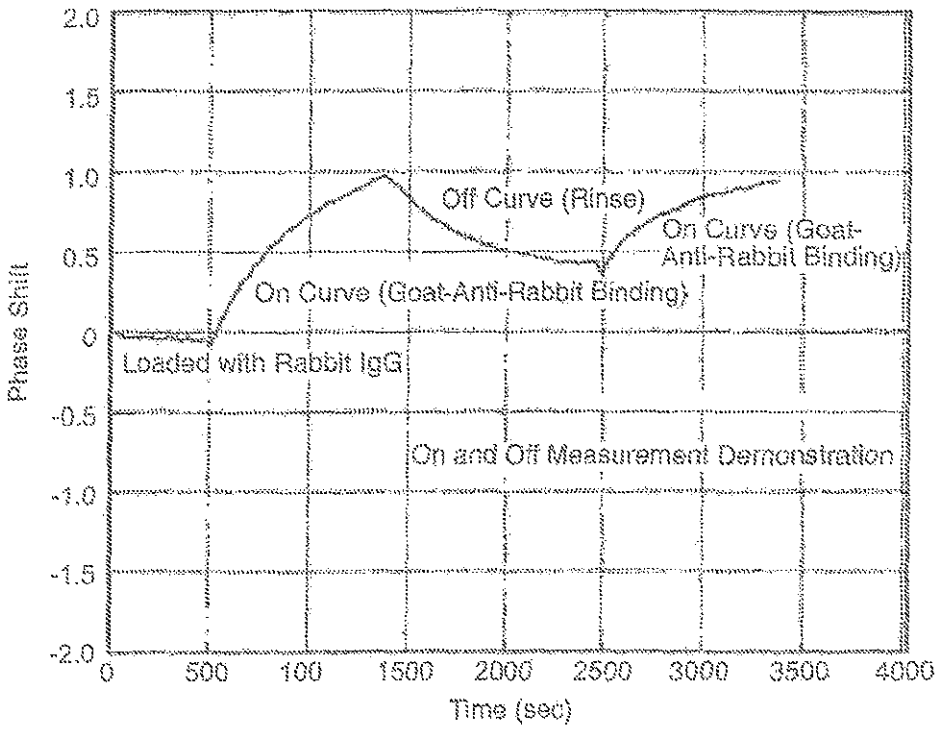

FIG. 7

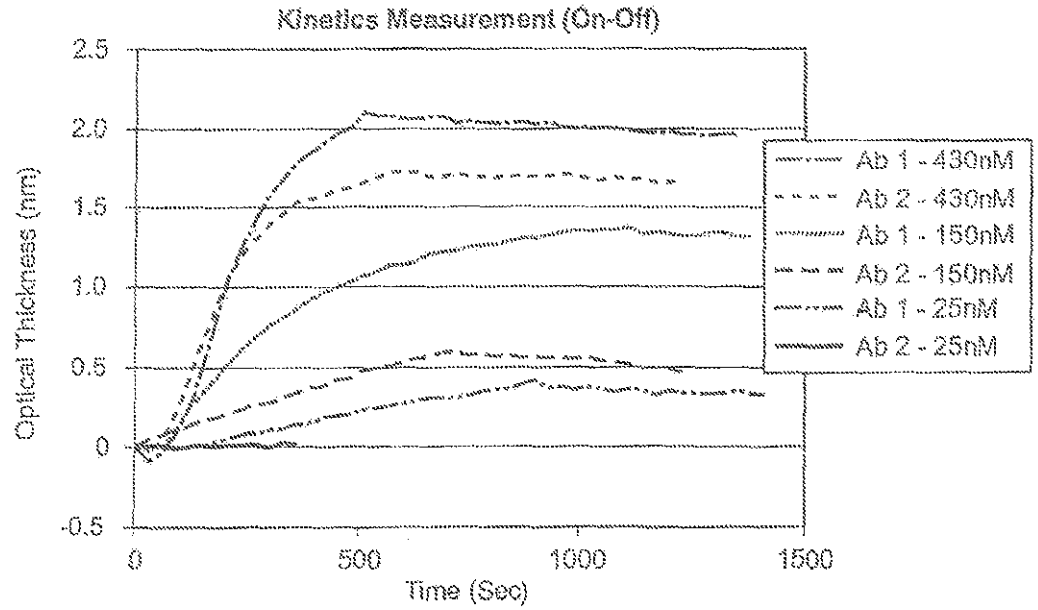

FIG. 8

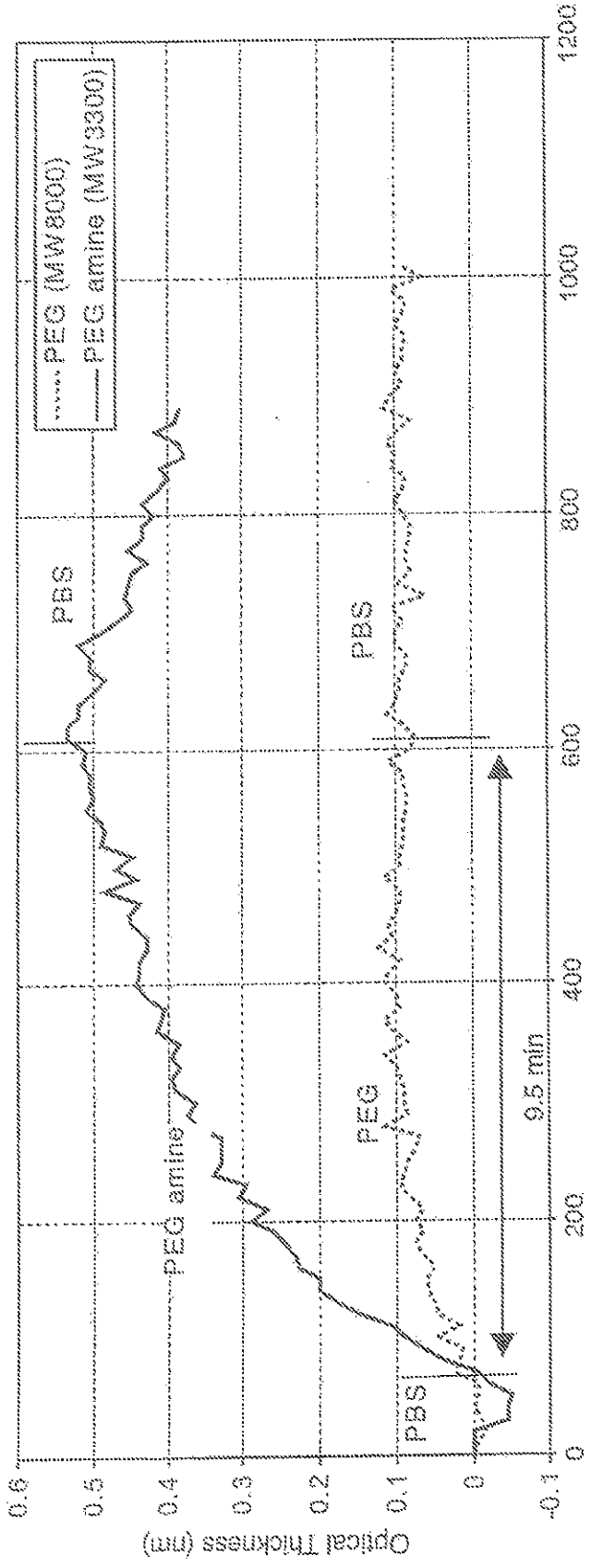

FIG. 9

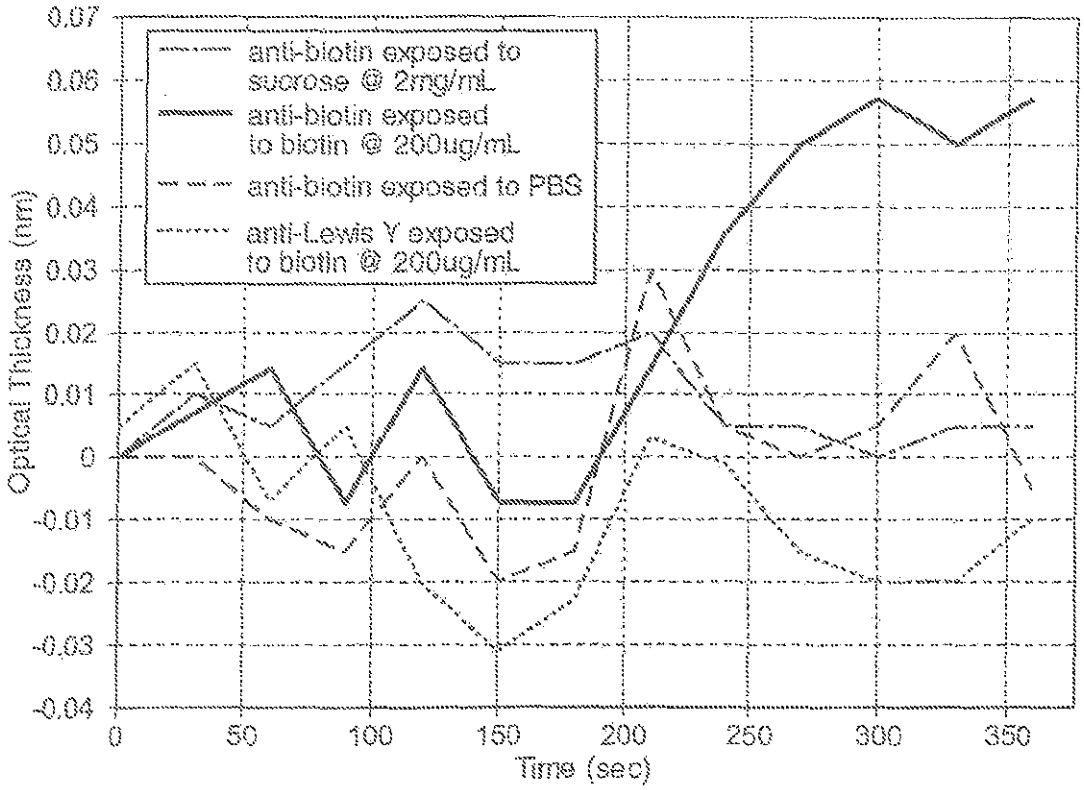

FIG. 10

FIBER-OPTIC ASSAY APPARATUS BASED ON PHASE-SHIFT INTERFEROMETRY

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 12/099,751, filed Apr. 8, 2008, which is a continuation of U.S. patent application Ser. No. 10/981,901, filed Nov. 4, 2004, which claims the benefit of (1) U.S. Provisional Application No. 60/518,068, filed Nov. 6, 2003, and (2) U.S. Provisional Application No. 60/558,381, filed Mar. 31, 2004, the entire disclosures of which are hereby incorporated by reference in their entireties, including any appendices or attachments thereof, for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

1. Field of the invention

The present invention relates to an apparatus and method for detecting the presence, amount, or rate of binding of one or more analytes in a sample, and in particular, to apparatus and method based on fiber optic interferometry.

2. Description of the Related Art

Diagnostic tests based on a binding event between members of an analyte-anti-analyte binding pair are widely used in medical, veterinary, agricultural and research applications. Typically, such methods are employed to detect the presence or amount or an analyte in a sample, and/or the rate of binding of the analyte to the anti-analyte. Typical analyte-anti-analyte pairs include complementary strands of nucleic acids, antigen-antibody pairs, and receptor-receptor binding agent, where the analyte can be either member of the pair, and the anti-analyte molecule, the opposite member.

Diagnostics methods of this type often employ a solid surface having immobilized anti-analyte molecules to which sample analyte molecules will bind specifically and with high affinity at a defined detection zone. In this type of assay, known as a solid-phase assay, the solid surface is exposed to the sample under conditions that promote analyte binding to immobilized anti-analyte molecules. The binding event can be detected directly, e.g., by a change in the mass, reflectivity, thickness, color or other characteristic indicative of a binding event. Where the analyte is pre-labeled, e.g., with a chromophore, or fluorescent or radiolabel, the binding event is detectable by the presence and/or amount of detectable label at the detection zone. Alternatively, the analyte can be labeled after it is bound at the detection zone, e.g., with a secondary, fluorescent-labeled anti-analyte antibody.

Co-owned U.S. Pat. No. 5,804,453, (the '453 patent) which is incorporated herein by reference, discloses a fiber-optic interferometer assay device designed to detect analyte binding to a fiber-optic end surface. Analyte detection is based on a change in the thickness at the end surface of the optical fiber resulting from the binding of analyte molecules to the surface, with greater amount of analyte producing a greater thickness-related change in the interference signal. The change in interference signal is due to a phase shift between light reflected from the end of the fiber and from the binding layer carried on the fiber end, as illustrated particularly in FIGS. 7*a* and 7*b* of the '453 patent. The device is simple to operate and provides a rapid assay method for analyte detection.

Ideally, an interferometer assay device will yield readily observable changes in spectral peak and valley (extrema) positions within the range of a conventional visible-light spectrometer, that is, in the visible light range between about 450-700 nm, such that relatively small optical thickness changes at the fiber end can be detected as significant changes in the spectral positions of interference wavelength peaks and valleys. One limitation which has been observed with the device described in the '453 patent is the absence of readily identified wavelength spectral extrema over this spectral range.

The present invention is designed to overcome this limitation, preserving the advantages of speed and simplicity of the earlier-disclosed device, but significantly enhancing sensitivity and accuracy. The present invention also provides a more convenient disposable-head format, as well as a multi-analyte array format, e.g., for gene-chip and protein-chip applications.

SUMMARY OF THE INVENTION

The invention includes, in one aspect, an apparatus for detecting an analyte in a sample, including detecting the presence of analyte, the amount of analyte or the rate of association and/or dissociation of analyte to analyte-binding molecules. The apparatus includes an optical element with a proximal reflecting surface and a distal reflecting surface separated by at least 50 nm. A beam of light from an optical fiber is directed to and reflected from the two reflecting surfaces. The reflected beams are coupled back into the optical fiber and interfere. The optical element also includes a layer of analyte binding molecules that is positioned so that the interference between the reflected beams varies as analyte binds to the layer of analyte binding molecules.

The change in interference can be caused by different physical phenomenon. For example, analyte binding can cause a change in the optical path length or in the physical distance between the two reflecting surfaces. Alternately, analyte binding can cause a change in the index or in the optical absorption of material located between the reflecting surfaces. Analyte binding can also cause the layer of analyte binding molecules to swell, resulting in a change in the interference.

In one particular design, the distal reflecting surface includes the layer of analyte binding molecules. As analyte binds to the layer of analyte binding molecules, the optical path length or the physical distance between the two reflecting surfaces may increase, for example. In another aspect of the invention, a transparent solid material is located between the reflecting surfaces and, optionally, the proximal reflecting surface includes a material with an index greater than that of the transparent solid material. Alternately, an air gap may be located between the reflecting surfaces. In yet another design, the distal reflecting surface is positioned between the proximately reflecting surface and the layer of analyte binding molecules. For example, analyte binding may cause the layer of analyte binding molecules to swell, moving the distal reflecting surface closer to the proximal reflecting surface. In yet another design, the layer of analyte binding molecules is positioned between the two reflecting surfaces. Analyte binding may cause the layer to swell or to change its index, thus changing the interference between the two reflected beams.

In another aspect, the apparatus includes an optical assembly having first and second reflecting surfaces separated by a distance "d" greater than 50 nm. The optical assembly is composed of a transparent optical element that can have a thickness defined between proximal and distal faces of the

element of at least 50 nm, preferably between 400-1,000 nm. The first reflecting surface is carried on the distal face of optical element, and is formed of a layer of analyte-binding molecules. The second reflecting surface is formed by a coating of transparent material having an index of refraction greater than that of the optical element. This coating can be formed of a Ta_2O_5 layer having a preferred thickness of between 5 and 50 nm. The optical element can be SiO_2, and has a thickness of between about 100-5,000 nm, preferably 400-1,000 nm.

Also included are a light source for directing a beam of light onto the first and second reflecting surfaces, and a detector unit that operates to detect a change in the optical thickness of the first reflecting layer resulting from binding of analyte to the analyte-binding molecules, when the assembly is placed in the solution of analyte. The optical thickness change at the first reflecting layer is related to a shift in a phase characteristic of the interference wave formed by the two light waves reflected from said first and second surfaces. This phase characteristic can be a shift in the spectral position(s) of one or more peaks and valleys of the interference wave, or by a change in the period of a full cycle of the wave.

The light source can include an optical fiber having a distal end adapted to be placed adjacent the second reflecting surface in the assembly, and the apparatus further includes an optical coupling for directing reflected light waves reflected from the assembly to the detector.

In a first embodiment, the optical assembly is fixedly mounted on the optical fiber, with the distal end of the optical fiber in contact with the second reflecting surface. In a second embodiment, the optical assembly further includes a second transparent optical element having an index of refraction less than that of the second coating and a thickness greater than about 100 nm, where the coating of high index of refraction material is sandwiched between the two transparent optical elements. In this latter embodiment, the assembly is removably attached to the distal end region of the fiber with a spacing of less than 100 nm or greater than 2 μm between the distal end of the fiber and the confronting face of the second transparent optical element in the assembly.

For detecting multiple analytes, such as multiple nucleic acid species, the layer of analyte-binding molecules can be composed of an array of discrete analyte-binding regions, such as single strands of nucleic acid. The regions are effective to bind different analytes. The optical fiber includes a plurality of individual fibers, each aligned with one of the regions, the detector includes a plurality of detection zones, and the optical coupling functions to couple each of the plurality of fibers with one of the zones.

The analyte-binding molecules in the assembly can be, for example, (i) an anti-species antibody molecules, for use in screening hybridoma libraries for the presence of secreted antibody, (ii) antigen molecules, for use in detecting the presence of antibodies specific against that antigen; (iii) protein molecules, for use in detecting the presence of a binding partner for that protein; (iv) protein molecules, for use in detecting the presence of multiple binding species capable of forming a multi-protein complex with the protein; or (v) single stranded nucleic acid molecules, for detecting the presence of nucleic acid binding molecules.

The detector can be a spectrometer for measuring reflected light intensity over a selected range of wavelengths. Alternatively, or in addition, the light source can include a plurality of light-emitting diodes, each with a characteristic spectral frequency, and the detector functions to record light intensity of reflected light at each of the different LED frequencies. In still another embodiment, the light source includes a white-light source and the detector is designed to record light intensity of reflected light at each of a plurality of different wavelengths.

In another aspect, the invention includes a method for detecting the presence or amount of an analyte in a sample solution. The method involves reacting the sample solution with a first reflecting surface formed by a layer of analyte-binding molecules carried on the distal surface of a transparent optical element having a thickness of at least 50 nm, thereby to increase the thickness of the first reflecting layer by the binding of analyte to the analyte-binding molecules in the layer. The change in thickness of the first reflecting layer is measured by detecting a shift in a phase characteristic of the interference wave formed by the two light waves reflected from the first layer and from a second reflecting layer that is formed on the opposite, proximal surface of the optical element and which has an index of refraction greater than that of the optical element.

The detecting step can include directing light from an optical fiber onto the two reflecting surfaces, and directing reflected light from the two surfaces onto a detector through an optical coupling. The detector can be a spectrometer, where the detecting includes measuring a shift in the spectral position of one or more of the interference extrema produced by the two reflecting lightwaves.

Where the method is used for measuring the rate of association of analyte to the second layer, the reacting step can be carried out until a near-maximum increase in thickness of the first reflecting layer is observed. Where the method is used for measuring the rate of dissociation of analyte to the second layer, the reacting steps can include immersing the second layer in a dissociation buffer for a period of time until a decrease in thickness of the first reflecting layer is observed. Where the method is used for measuring the amount of analyte present in the sample, the detecting is carried out over a period sufficient to measure the thickness of the first reflecting layer at a plurality of different time points.

Where the method is used measuring one or more of a plurality of analytes in a sample, the first reflecting layer is composed of an array of discrete analyte-binding regions, the different regions being effective to bind different analytes, and the detecting is effective to detect a change in the thickness of each of the regions resulting from binding of analyte to the analyte-binding molecules.

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, and accompanying drawings, where:

FIG. **1** shows the basic system setup for the bioprobe and its apparatus;

FIG. **2** shows an optical assembly formed accordance to one embodiment of the invention;

FIGS. **3**A and **3**B show a portion of an interference wave over 7 peak and valley orders (**3**A), and over in a visible portion of the spectrum (**3**B);

FIG. **4** shows an optical assembly constructed according to another embodiment of the invention;

FIG. **5** shows a disposable multi-analyte optical assembly having an analyte-binding array and constructed according to another embodiment of the invention;

FIG. **6** shows a sequential binding of three molecules;

FIG. **7** shows on and off curves generated from the association and dissociation of antibodies;

FIG. **8** shows the curves of two antibodies binding to their antigen at different concentrations;

FIG. **9** shows immobilization of bis amino PEG (MW 3300) specifically through an amide bond formation. The PEG (MW 8000) is used as a negative control to monitor non-specific binding of the PEG polymer; and

FIG. **10** shows a small molecule binding to a large molecule, negative controls and the base line measurement.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Terms used in the claims and specification are to be construed in accordance with their usual meaning as understood by one skilled in the art except and as defined as set forth below. Numeric ranges recited in the claims and specification are to be construed as including the limits bounding the recited ranges.

The term "in vivo" refers to processes that occur in a living organism.

An "analyte-binding" molecule refers to any molecule capable of participating in a specific binding reaction with an analyte molecule. Examples include but are not limited to, e.g., antibody-antigen binding reactions, and nucleic acid hybridization reactions.

A "specific binding reaction" refers to a binding reaction that is saturable, usually reversible, and that can be competed with an excess of one of the reactants. Specific binding reactions are characterized by complementarity of shape, charge, and other binding determinants as between the participants in the specific binding reaction.

An "antibody" refers to an immunoglobulin molecule having two heavy chains and two light chains prepared by any method known in the art or later developed and includes polyclonal antibodies such as those produced by inoculating a mammal such as a goat, mouse, rabbit, etc. with an immunogen, as well as monoclonal antibodies produced using the well-known Kohler Milstein hybridoma fusion technique. The term includes antibodies produced using genetic engineering methods such as those employing, e.g., SCID mice reconstituted with human immunoglobulin genes, as well as antibodies that have been humanized using art-known resurfacing techniques.

An "antibody fragment" refers to a fragment of an antibody molecule produced by chemical cleavage or genetic engineering techniques, as well as to single chain variable fragments (SCFvs) such as those produced using combinatorial genetic libraries and phage display technologies. Antibody fragments used in accordance with the present invention usually retain the ability to bind their cognate antigen and so include variable sequences and antigen combining sites.

A "small molecule" refers to an organic compound having a molecular weight less than about 500 daltons. Small molecules are useful starting materials for screening to identify drug lead compounds that then can be optimized through traditional medicinal chemistry, structure activity relationship studies to create new drugs. Small molecule drug compounds have the benefit of usually being orally bioavailable. Examples of small molecules include compounds listed in the following databases: MDL/ACD (http://www.mdli.com/), MDL/MDDR (http://www.mdli.com/), SPECS (http://www.specs.net/), the China Natural Product Database (CNPD) (http://www.neotrident.com/), and the compound sample database of the National Center for Drug Screening (http://www.screen.org.cn/).

Abbreviations used in this application include the following: "ss" refers to single-stranded; "SNP" refers to single nucleotide polymorphism; "PBS" refers to phosphate buffered saline (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4); "NHS" refers to N-hydroxysuccinimide; "MW" refers to molecular weight; "Sulfo-SMCC" refers to sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

Advantages and Utility

The advantages and utility of the invention are illustrated by reference to the Figures and Examples as described in greater detail below. These include the ability to monitor in real time analyte binding reactions without the use of labels, diminishing cost and potential toxicity. A further advantage includes the ability to practice the method using visible wavelength light sources. Yet other advantages are provided by the fiber optic nature of the detector tip that allows binding reactions to be monitored in very small sample volumes, including in "in vitro" spaces, and to bundle fibers to carry out highly multiplexed analyses of binding reactions.

FIG. **1** shows, in schematic view, an interferometer apparatus **20** constructed in accordance with the invention. In its most basic elements, the apparatus includes a light source **22**, an optical assembly **26** that functions as a sensing element or detector tip and that will be detailed further with respect to FIGS. **2**, **4** and **5** below, and a detector unit **28** for detecting interference signals produced by interfering light waves reflected from the optical assembly **26**.

Light from source **22** is directed onto the optical assembly **26**, and reflected back to the detector through an optical coupling assembly indicated by dashed lines at **30**. In a preferred embodiment, the coupling assembly includes a first optical waveguide or fiber **32** extending from the light source to the optical assembly, a second optical waveguide or fiber **34** which carries reflected light from the optical assembly to the detector, and an optical coupler **36** which optically couples fibers **32**, **34**. Suitable optical fiber and coupling components are detailed in the above-cited '453 patent. One exemplary coupler is commercially available from many vendors including Ocean Optics (Dunedin, Fla.).

Alternatively, the coupling assembling can include a lens system constructed to focus a light beam onto the upper surface of the optical assembly and to direct reflected interfering light from the optical assembly to the detector. The latter system would not require optical fibers, but would impose relatively stringent requirements on the positioning of the lens elements used for the optical coupling.

The light source in the apparatus can be a white light source, such as a light emitting diode (LED) which produces light over a broad spectrum, e.g., 400 nm or less to 700 nm or greater, typically over a spectral range of at least 100 nm. Alternatively, the light source can be a plurality of sources each having a different characteristic wavelength, such as LEDs designed for light emission at different selected wavelengths in the visible light range. The same function can be achieved by a single light source, e.g., white light source, with suitable filters for directing light with different selected wavelengths onto the optical assembly.

The detector is preferably a spectrometer, such as charge-coupled device (CCD), capable of recording the spectrum of

the reflected interfering light from the optical assembly. Alternatively, where the light source operates to direct different selected wavelengths onto the optical assembly, the detector can be a simple photodetector for recording light intensity at each of the different irradiating wavelengths. In still another embodiment, the detector can include a plurality of filters which allows detection of light intensity, e.g., from a white-light source, at each of a plurality of selected wavelengths of the interference reflectance wave. Exemplary light source and detector configurations are described in the above-cited '453 patent, particularly with respect to FIGS. **8** and **10** of that patent, and it will be understood that these configurations are suitable for use in the present invention.

FIG. **2** shows an optical assembly **26** constructed in accordance with one embodiment of the invention, and an adjoining portion of the distal end region of an optical fiber **32** to which the optical assembly is fixedly attached. As seen, the assembly **26** includes a transparent optical element **38** having first and second reflecting surfaces **42**, **40** formed on its lower (distal) and upper (proximal) end faces, respectively. According to an important feature of the invention, the thickness "d" of the optical element between its distal and proximal surfaces, i.e., between the two reflecting surfaces, is at least 50 nm, and preferably at least 100 nm. An exemplary thickness is between about 100-5,000 nm, preferably 400-1,000 nm. The first reflecting surface **42** is formed of a layer of analyte-binding molecules, such as molecules **44**, which are effective to bind analyte molecules **46** specifically and with high affinity. That is, the analyte and anti-analyte molecules are opposite members of a binding pair of the type described above, which can include, without limitations, antigen-antibody pairs, complementary nucleic acids, and receptor-binding agent pairs.

The index of refraction of the optical element is preferably similar to that of the first reflecting surface, so that reflection from the lower distal end of the end optical assembly occurs predominantly from the layer formed by the analyte-binding molecules, rather than from the interface between the optical element and the analyte-binding molecules. Similarly, as analyte molecules bind to the lower layer of the optical assembly, light reflection form the lower end of the assembly occurs predominantly from the layer formed by the analyte-binding molecules and bound analyte, rather than from the interface region. One exemplary material forming the optical element is SiO_2, e.g., a high-quality quality glass having an index of refraction of about 1.4-1.5. The optical element can also be formed of a transparent polymer, such as polystyrene or polyethylene, having an index of refraction preferably in the 1.3-1.8 range.

The second reflecting surface in the optical assembly formed as a layer of transparent material having an index of refraction that is substantially higher than that of the optical element, such that this layer functions to reflect a portion of the light directed onto the optical assembly. Preferably, the second layer has a refractive index greater than 1.8. One exemplary material for the second layer is Ta_2O_5 with refractive index equal to 2.1. The layer is typically formed on the optical element by a conventional vapor deposition coating or layering process, to a layer thickness of less than 50 nm, typically between 5 and 30 nm.

The thickness of the first (analyte-binding) layer is designed to optimize the overall sensitivity based on specific hardware and optical components. Conventional immobilization chemistries are used in chemically, e.g., covalently, attaching a layer of analyte-binding molecules to the lower surface of the optical element. For example, a variety of bifunctional reagents containing a siloxane group for chemical attachment to SiO_2, and an hydroxyl, amine, carboxyl or other reaction group for attachment of biological molecules, such as proteins (e.g., antigens, antibodies), or nucleic acids. It is also well known to etch or otherwise treat glass a glass surface to increase the density of hydroxyl groups by which analyte-binding molecules can be bound. Where the optical element is formed of a polymer, such as polystyrene, a variety of methods are available for exposing available chemically-active surface groups, such as amine, hydroxyl, and carboxyl groups.

The analyte-binding layer is preferably formed under conditions in which the distal surface of the optical element is densely coated, so that binding of analyte molecules to the layer forces a change in the thickness of the layer, rather than filling in the layer. The analyte-binding layer can be either a monolayer or a multi-layer matrix.

The measurement of the presence, concentration, and/or binding rate of analyte to the optical assembly is enabled by the interference of reflected light beams from the two reflecting surfaces in the optical assembly. Specifically, as analyte molecules attach to or detach from the surface, the average thickness of the first reflecting layer changes accordingly. Because the thickness of all other layers remains the same, the interference wave formed by the light waves reflected from the two surfaces is phase shifted in accordance with this thickness change.

Assume that there are two reflected beams: The first beam is reflected from the first surface, which is the distal end interface between analyte-binding molecules and bound analyte and the surrounding medium; and the second beam is reflected from the second surface, which is the proximal interface between the optical element (the first layer) and the high-index of refraction layer (the second layer). The overall wavelength -dependent intensity of the interference wave is:

$$I = I_1 + I_2 + 2\sqrt{I_1 I_2}\cos\left(\frac{2\pi\Delta}{\lambda}\right)$$

where I is the intensity, I_1 and I_2 are the intensity of two interference beams, Δ is the optical path difference, and λ is the wavelength.

When $(2\pi\Delta/\lambda)=N\pi$, the curve is at its peak or valley if N is an integer 0, 1, 2, . . .

The thickness of the first layer $d=\Delta/2n$. Therefore, $\lambda=4nd/N$ at peaks or valleys (extrema).

For the first several values of N, i.e., 0, 1, 2, . . . 7, and assuming a d of 770 nm, the equation gives:

N=0: $\lambda=\infty$ (peak)
N=1: $\lambda=4nd=4,496.80$ nm (Valley)
N=2: $\lambda=2nd=2,248.40$ nm (Peak)
N=3: $\lambda=4nd/3=1,498.9$ nm (Valley)
N=4: $\lambda=nd=1,124.20$ nm (Peak)
N=5: $\lambda=4nd/5=899.36$ nm (Valley)
N=6: $\lambda=2nd/3=749.47$ nm (Peak)
N=7: $\lambda=4nd/7=642$ nm (Valley)
N=8: $\lambda=nd/2=562$ nm (Peak)
N=9: $\lambda=4nd/9=499.64$ nm (Valley)
N=10: $\lambda=4nd/10=449.6$ nm (Peak)

As can be seen, and illustrated further in FIGS. **3**A and **3**B, at least three peaks/valleys (N=7-9) occur in the visible spectral range.

If the 7^{th} order valley is used to calculate the change in molecular layer thickness, when the molecular layer attached to the first layer increases from 0 nm to 10 nm, the 7^{th} order valley will shift to 650.74 nm. Therefore, the ratio between

the actual the phase shift of the 7th order valley and thickness change equals (650.74−642.40)/10=0.834.

By contrast, if the initial spacing between the two reflecting layers is made up entirely of the analyte-binding molecules on the end of the fiber, assuming a thickness of this layer of 25 nm, then the first order peak will occur at 146 nm, clearly out of the range of the visible spectrum, so that the device will only see a portion of the region between the 0-order valley and the first order peak, but will not see any peaks, making a shift in the spectral characteristics of the interference wave difficult to measure accurately.

Not until the total thickness of the reflecting layer approaches about 100 nm will the first-order peak appear in the visible spectrum. Assuming a total thickness change of up to 50 nm, the thickness of the optical element can then be as small as 50 nm, but is preferably on the order of several hundred nm, so that the phase shift or change in periodicity of the interference wave can be measured readily by a shift in the spectral positions of higher-order peaks or valleys, e.g., where N=3-10.

The ratio between the actual thickness and the measured phase shift is considered as a key factor of measurement sensitivity. It can be appreciated how one can adjust the thickness of the optical element and its refractive index to improve and optimize the sensitivity to accommodate the electronics and optical designs.

FIG. **4** shows an optical assembly **50** that is removably carried on the distal end of an optical fiber **52** in the assay apparatus. The optical element includes a plurality of flexible gripping arms, such as arms **54**, that are designed to slide over the end of the fiber and grip the fiber by engagement of an annular rim or detente **56** on the fiber with complementary-shaped recesses formed in the arms, as shown. This attachment serves to position the optical assembly on the fiber to provide an air gap **58** between the distal end of the fiber and the confronting (upper) face of the assembly, of less than 100 nm or greater than 2 μm. With an air gap of greater than about 100 nm, but less that 2 μm, internal reflection from the upper surface of the optical assembly can contribute significantly to undesirable fringes that can adversely impact the detection accuracy.

With continued reference to FIG. **4**, the optical assembly includes a first optical element **60** similar to optical element **38** described above, and having first and second reflective layers **62**, **64**, respectively, corresponding to above-described reflective layers **40**, **42**, respectively. The assembly further includes a second optical element **66** whose thickness is preferably greater than 100 nm, typically at least 200 nm, and whose index of refraction is similar to that of first optical element **60**. Preferably, the two optical elements are constructed of the same glass or a polymeric material having an index of refraction of between about 1.4 and 1.6. Layer **64**, which is formed of a high index of refraction material, and has a thickness preferably less than about 30 nm, is sandwiched between the 2 optical elements as shown.

In operation, the optical assembly is placed over the distal fiber end and snapped into place on the fiber. The lower surface of the assembly is then exposed to a sample of analyte, under conditions that favor binding of sample analyte to the analyte-binding molecules forming reflective layer **62**. As analyte molecules bind to this layer, the thickness of the layer increases, increasing the distance "d" between reflective surfaces **62** and **64**. This produces a shift in the extrema of the interference wave produced by reflection from the two layers, as described above with reference to FIGS. **3**A and **3**B. This shift in extrema or wavelength, or wavelength period, in turn, is used to determine the change in thickness at the lower (distal-most) reflecting layer. After use, the optical assembly can be removed and discarded, and replaced with fresh element for a new assay, for assaying the same or a different analyte.

FIG. **5** illustrates an optical assembly and fiber bundle in an embodiment of the invention designed for detecting one or more of a plurality of analytes, e.g., different-sequence nucleic acid analytes, in a sample. A fiber bundle **72** is composed of an array, e.g., circular array, for individual optical fibers, such as fibers **74**. The optical assembly, indicated generally at **70**, is composed of the basic optical elements described above with reference to FIG. **4**, but in an array format. Specifically, a first optical element **80** in the element provides at its lower distal surface, an array of analyte-reaction regions, such as regions **84**, each containing a layer of analyte-binding molecules effective to bind to one of the different analytes in the sample. Each region forms a first reflective layer in the optical assembly. One preferred sensing provides an array of different-sequence nucleic acids, e.g., cDNAs or oligonucleotides, designed to hybridize specifically with different-sequence nucleic acid analyte species in a sample. That is, the array surface forms a "gene chip" for detecting each of a plurality of different gene sequences.

Also included in the optical assembly are a second optical element **78** and a layer **79** of high index of refraction material sandwiched between the two optical elements, and which provides the second reflecting surface in the optical assembly. The assembly is carried on the fiber bundle **72** by engagement between a pair of flexible support arm, such as arm **76** and an annular rim or detente **86** on the bundle. With the assembly placed on the fiber bundle, the lower distal ends of the fibers are spaced from the confronting surface of optical element **78** by an air gap **85** whose spacing is preferably less than 100 nm or greater than 2 μm. Further, each of the fibers is aligned with a corresponding assay region of the optical assembly, so that each fiber is directing light on, and receiving reflected light from, its aligned detection region. Similarly, the optical coupler in the apparatus, which serves to couple multiple fibers to the detector, preserves the alignment between the array regions and corresponding positions on an optical detector, e.g., two-dimensional CCD. The materials and thickness dimensions of the various optical-assembly components are similar to those described above with respect to FIG. **4**.

The apparatus described in this invention can be used more specifically for the following applications:

(i) with an anti-species antibody carried on the tip, for screening hybridoma expression lines for cell lines with high antibody expression;

(ii) with an antigen carried on the tip, to characterize high affinity antibodies against that antigen;

(iii) with a protein carried on the tip, for identifying and characterizing binding partners (DNA, RNA, proteins, carbohydrates, organic molecules) for that protein;

(iv) with a carbohydrate or glycosyl moiety carried on the tip, for identifying and characterizing binding partners (such as, e.g., DNA, RNA, proteins, carbohydrates, organic molecules) for that carbohydrate;

(v) with a protein thought to participate in a multi-protein complex carried on the tip, for characterizing the binding components and/or kinetics of complex formation;

(vi) with a small protein-binding molecule carried on the tip, for identifying and characterizing protein binders for that molecule;

(vii) with an antibody carried on the tip, for constructing a calibration curve for the analyte using a set of analytes standards. Using this calibration curve, one can then

determine the concentration of the analyte in unknown solutions (cell culture supernatants, biological samples, process mixtures, etc).

(viii) with a single-stranded nucleic acid, e.g., ssDNA or RNA carried on the tip, for identifying and molecules that bind specifically to the nucleic acid.

Using a temperature control block, the apparatus and method can also be used to monitor the binding and characterize the binding of an immobilized ssDNA to an oligonucleotide in solution to perform SNP analysis.

The following examples illustrate various methods and applications of the invention, but are in no way intended to limit its scope.

EXAMPLES

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T. E. Creighton, *Proteins: Structures and Molecular Properties* (W.H. Freeman and Company, 1993); A. L. Lehninger, *Biochemistry* (Worth Publishers, Inc., current addition); Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Remington's Pharmaceutical Sciences,* 18th Edition (Easton, Pa.: Mack Publishing Company, 1990); Carey and Sundberg *Advanced Organic Chemistry* 3rd Ed. (Plenum Press) Vols A and B (1992).

Example 1

Small Molecule-protein Binding Reaction

This example demonstrates the capability to detect the binding of protein to small molecule immobilized on a sensor tip and subsequent bindings of multiple antibodies. The two-layer configuration on the tip of an optic fiber is used for this test. The thickness of the first Ta_2O_5 layer is 25 nm and the thickness of the second SiO_2 layer is 770 nm. The fiber was purchased from Ocean Optics (Dunedin, Fla.). It was manually cut into segments that are 40 mm long. Both ends of these segments were polished to standard mirror surface quality. The polishing method used here was exactly the same as those for optical lenses and mirrors. One surface of these fiber segments was outsourced to an optical coating house for Ta_2O_5 layer and SiO_2 layer. This vendor employed an ion-beam assisted physical vapor deposition (IAPVD) coater made by Leybold. IAPVD is a commonly used coating technique for anti-reflection and optical filters. The experimental steps included the following (all steps are performed at room temperature unless otherwise noted):

The fiber tip was coated with a polymer monolayer derivatized with biotin. The polymer monolayer was prepared using a biotinylated lipid (custom). This lipid was using to form a lipid monolayer on the surface of water solution. The monolayer was cross linked using UV light for 15minutes. Clean, dry fibers were then brought in contact with the floating thin film and the biotin polymer was adsorbed onto the fiber tip. The fibers were then dryed at 60° C. for 1 hour. The fiber were then stored under ambient conditions

The biosensor tip was immersed in 50 μg/ml streptavidin streptavidin (Pierce Biotechnology, Rockford Ill., cat #21122) in PBS (Invitrogen, Carlsbad, Calif.; cat #14190078) for 9 minutes and then rinsed briefly with PBS.

The same tip was dipped into 10 μg/ml rabbit-anti-streptavidin solution (AbCam, Cambridge, Mass.; cat #ab6676-1000) in PBS for 36 minutes and then washed with PBS briefly.

Finally, the tip was immersed in 50 μg/mL donkey-anti-rabbit antibody solution antibody (Jackson ImmunoResearch, West Grove, Pa.; cat #711-005-152) in PBS for 25 minutes. A final 10 minute rinse was performed in PBS solution.

FIG. **6** shows the real-time response curve for this sequential binding test. The vertical axis is the 7th order valley phase shift in nanometers. It clearly shows the binding of streptavidin to the biotin already immobilized on the tip, and subsequent bindings of anti-streptavidin antibody to streptavidin and a second antibody to this first antibody. The dissociation of the streptavidin layer from the tip was visible (a small reduction in the optical thickness) at 900 seconds.

Example 2

Biomolecular Interaction Analysis of Kinetics and Affinity of Biomolecular Interactions

This example illustrates use of the invention to carry out a biomolecular interaction analysis (BIA) measuring kinetics and affinity of biomolecular interactions. The same tip configuration as described in Example 1 was used. The experimental steps included the following (all steps are performed at room temperature unless otherwise noted):

Mercaptosilane coated tips were prepared using the following procedure. Clean, dry fibers were incubated in a mixture of Toluene: hexanoic acid: mercaptopropyltrioxysilane (10:2:1 volumetric ratio) at room temperature for 24 hours. The fibers were rinsed 2× with 10 mL toluene for 5 minutes each. The fibers were then rinsed 1× with 10 mL of ethanol and dried under a stream of argon and stored at ambient conditions.

The biosensor tip was first derivatized by immersion in a with 10 μg/ml solution of rabbit-IgG (Jackson ImmunoResearch, West Grove, Pa.; cat #309-005-003) in PBS for 1 hour.

The coated tip was dipped into 10 μg/ml goat-anti-rabbit antibody solution (Jackson ImmunoResearch, West Grove, Pa.; cat #111-005-003) in PBS and remained in it for 15 minutes.

The tip was removed and washed in PBS. To facilitate the dissociation of the second antibody from the first antibody, the PBS was agitated manually for 20 minutes.

The tip was then dipped into the same goat-anti-rabbit solution again to show the reproducible association of goat-anti-rabbit to rabbit-IgG.

FIG. **7** shows the on and off curves generated from the association and dissociation of rabbit-IgG and goat-anti-rabbit. The vertical axis is again the 7th order valley phase shift. The phase shift is directly related to the average thickness with a ratio of 0.834. The ability to detect the on and off curves reliably is essential for measuring interaction kinetics and affinity.

Example 3

Calculating Affinity Constants from Antibody-antigen Binding and Release Curves

This experiment demonstrates the calculation of affinity constants from measuring on and off curves for two antibodies and their antigen. The proprietary antibodies were labeled as Ab-1 and Ab-2. The molecular weight of the antigen was about 30 kilodaltons. The same tip configuration as described in Example 1 was used. The same mercaptosilane fiber preparation as described in Example 2 was used. The experimental steps included (all steps are performed at room temperature unless otherwise noted):

The fiber tip was activated for covalent attachment of the antigen. Mercaptosilane coated fibers were activated by immersing the sensor tips in 50 μL of a 50 mg/mL solution of sulfo-SMCC (Pierce Biotechnology, Rockford Ill.; cat #22322) in DMF (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #494488) at for 2 hours. The sensor tips were rinsed briefly in DMF and dried;

The antigen was covalently bound to the activated fiber tip by immersing the activated tip in a 20 μg/ml solution of antigen in PBS for 20 minutes. The tip was rinsed with PBS for 2 minutes. Following the PBS rinse, the tip was quenched with an aqueous solution of 100 μM ethanolamine pH 8.5 (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #E9508) for 5 minutes and then was rinsed again in PBS for 2 minutes.

The same tip was immersed in antibody for an association test and the real-time binding data were recorded for 9-15 minutes (depending on the antibody identity and concentration). Once those data were recorded, the tip was again immersed in PBS and agitated to measure the off curve (i.e., dissociation between the immobilized antigen and bound antibody) for 9-15 minutes. The binding (on curve) and dissociation (off curve) measurements were repeated using different concentrations of antibody (25 nM, 150 nM, and 430 nM) and with two different antibodies identified as Ab-1 and Ab-2.

FIG. **8** shows the association and dissociation curves at different concentrations. The test of 25 nM Ab-2 was not completed because the association was extremely slow at this concentration. These illustrated curves are plots of the raw data.

K_{on}, K_{off}, and K_D were derived from these curves by fitting the raw data with a first order exponential function. By averaging two sets of data, kinetic and affinity coefficients were obtained as follows:

Ab-1	Ab-2
$K_{on} = 1.35 \times 10^5$ ($M^{-1}S^{-1}$)	$K_{on} = 2.01 \times 10^5$ ($M^{-1}S^{-1}$)
$K_{off} = 5.55 \times 10^{-5}$ (S^{-1})	$K_{off} = 8.15 \times 10^{-5}$ (S^{-1})
$K_D = K_{off}/K_{on} = 3.99 \times 10^{-9}$ (M)	$K_D = K_{off}/K_{on} = 4.45 \times 10^{-9}$ (M)

Example 4

NHS-ester Activated Tips

The same tip configuration as described in Example 1 was used. The same mercaptosilane fiber preparation as described in Example 2 was used. Mercaptosilane coated fibers were activated by immersing the sensor tips in 50 μL of a 50 mg/mL solution of sulfo-SMCC (Pierce Biotechnology, Rockford Ill.; cat #22322) in DMF (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #494488) at for 2 hours. The sensor tips were rinsed briefly in DMF and dried.

Amine containing molecules can be covalently bound to this surface through formation of a stable amide linkage. Molecules that do not contain free amines are not immobilized through the NHS moiety, but these molecules can still bind to the surface through non-specific binding. This non-specific binding can be multi-layered whereas the covalent immobilization through the NHS esters will be in a single layer controlled by the availability and accessibility of the NHS ester.

In this set of experiments, a bis amino PEG (MW 3300) (Shearwater Polymers, San Carlos, Calif.) was used as a test compound to covalently bind to the activated surface. A PEG (MW 8000) (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #04162) that contained no free amino groups was used as a negative control. This negative control was used to look for any non-specific or multi-layered binding that might be inherent to PEG polymers on this surface.

FIG. **9** shows the time course of the treatment of the activated mercaptosilane tip with the test molecules. The activated tip showed a distinct increase in optical thickness upon exposure to the 0.1 mg/mL bis amino PEG (MW 3300) in PBS. This increase is stopped when the bis amino PEG solution is replaced by the PBS buffer. The activated tip exposed to 0.1 mg/mL PEG (MW8000) in PBS, which contains no amines, shows a small initial increase in optical thickness but the trace quickly becomes flat. From this it can be concluded that the PEG polymer does not have intrinsic non-specific binding and that the binding seen for the bis amino PEG is attributed to the specific covalent immobilization of the amine group.

Example 5

Antibody Derivatized Tips Using NHS-ester Chemistry

This example illustrates the binding of a low molecular weight molecule binding to an immobilized high molecular weight molecule. Using the same NHS ester terminated surface described in Example 4 and the same tip configuration as described in Example 1, an anti-biotin antibody was immobilized to 3 fibers Immobilization of the antibody was accomplished by immersing the activated fiber in a 20 μg/mL solution of mouse anti-biotin antibody (Biodesign, Saco Minn.; cat #H61504M) in PBS for 1 hour at room temperature. The tip was rinsed with PBS for 2 minutes. Following the PBS rinse, the tip was quenched with an aqueous solution of 100 μM ethanolamine pH 8.5 (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #E9508) for 5 minutes and then was rinsed again in PBS for 2 minutes.

The first fiber was exposed to a solution of 200 μg/mL biotin (Pierce Biotechnology, Rockford Ill.; cat #29129) in PBS. Controls using a solution of sucrose (Sigma-Aldrich Chemical Company, St Louis, Mo.; cat #S8501) (2 mg/mL) and PBS were carried out on the second and the third fibers to determine baseline noise. Data from these tests are shown in FIG. **10**. Biotin binding is seen as an increase in optical thickness, whereas exposure to sucrose shows no detectable increase over baseline (PBS).

Another negative control was carried out using an irrelevant antibody (anti-Lewis Y antibody from Calbiochem, San Diego Calif.; cat #434636) immobilized in an identical fashion to the anti-biotin antibody above. This immobilized anti-

body was exposed to a solution of 200 μg/mL biotin. The lack of biotin binding to this antibody indicates that the biotin binding to the anti-biotin antibody is a result of specific interactions and not due to non-specific binding.

While the invention has been particularly shown and described with reference to a preferred embodiment and various alternate embodiments, it will be understood by persons skilled in the relevant art that various changes in form and details can be made therein without departing from the spirit and scope of the invention.

All references, issued patents and patent applications cited within the body of the instant specification are hereby incorporated by reference in their entirety, for all purposes.

What is claimed is:

1. An optical assembly for use in detecting an analyte in a sample based on interference, comprising:

an optical fiber having a tip; and

an optical element removably attached to the tip of the optical fiber and configured for receiving a beam of light from the optical fiber,

said optical element comprising a transparent material, a first reflecting surface, and a second reflecting surface separated from the first reflecting surface by the transparent material, said first and second reflecting surfaces separated by at least 50 nm,

wherein said first reflecting surface binds a layer of analyte binding molecules positioned so that interference between a beam of light reflected from the first reflecting surface into the optical fiber and a beam of light reflected from the second reflecting surface into the optical fiber varies as analyte binds to the layer of analyte binding molecules.

2. The assembly of claim **1**, wherein said second reflecting surface comprises a layer of material having an index of refraction greater than the refractive index of said transparent material.

3. The assembly of claim **1**, wherein the separation between said first and second reflecting surfaces is between 100 nm and 5,000 nm.

4. The assembly of claim **3**, wherein the separation between said first and second reflecting surfaces is between 400 nm and 1,000 nm.

5. The assembly of claim **1**, wherein the refractive index of said optical element transparent material is less than 1.8.

6. The assembly of claim **5**, wherein said optical element transparent material is a material selected from the group consisting of SiO_2 and a transparent polymer.

7. The assembly of claim **6**, wherein said transparent polymer comprises polystyrene or polyethylene.

8. The assembly of claim **1**, wherein the second reflecting surface comprises a layer of material having a refractive index greater than 1.8.

9. The assembly of claim **8**, wherein said second reflecting surface layer comprises Ta_2O_5.

10. The assembly of claim **9**, wherein the thickness of said second reflecting surface layer is between 5 nm and 50 nm.

11. The assembly of claim **1**, wherein said layer of analyte binding molecules comprises a molecule selected from the group consisting of a protein, a small molecule, a nucleic acid and a carbohydrate.

12. The assembly of claim **11**, wherein said protein is selected from the group consisting of an avidin, a streptavidin, an antibody, and an antibody fragment.

13. The assembly of claim **1**, wherein said optical assembly is adapted for coupling to said light source through a mechanical coupling that engages said optical element with said fiber.

14. The assembly of claim **1**, wherein said optical element is adapted for coupling to said light source through a coupling assembly that comprises a lens system.

15. The assembly of claim **1**, further comprising a second optical element overlaying said second reflecting surface.

16. The assembly of claim **15**, wherein the thickness of said second optical element is greater than 100 nm.

17. The assembly of claim **16**, wherein the thickness of said second optical element is greater than 200 nm.

18. The assembly of claim **15**, wherein said optical assembly is adapted for coupling to said light source through a mechanical coupling that engages said second optical element with said fiber.

19. The assembly of claim **18**, wherein said mechanical coupling provides an air gap between said second optical element and said fiber.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,305,585 B2
APPLICATION NO. : 12/790736
DATED : November 6, 2012
INVENTOR(S) : Hong Tan et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page, item [73]:

The assignee is listed "Pall Corporation, Port Washington, NY (US)"

The assignee should appear "Pall Fortebio Corporation, Menlo Park, California (US)"

Signed and Sealed this
Second Day of April, 2013

Teresa Stanek Rea

Teresa Stanek Rea
Acting Director of the United States Patent and Trademark Office