in embodiments of the invention is an antibody, e.g., a monoclonal antibody, that reacts with free cardiac troponin I (cTnI) a.a. 87-91 and cTnI forming complexes with other troponin components. Preferably, this antibody is not affected by heparin, phosphorylation, oxidation and troponin complex formation, and does not cross-react with skeletal muscle troponin I. An exemplary antibody of this type is Monoclonal Antibody Clone Number A34440228P, available from BiosPacific, Everyville, CA. It will be appreciated that antibodies identified herein as useful as a capture antibody may also be useful as detection antibodies, and vice versa.

[0061] The attachment of the binding partner, e.g., antibody, to the solid support may be covalent or noncovalent. In some embodiments, the attachment is noncovalent. An example of a noncovalent attachment well-known in the art is biotin-avidin/streptavidin interactions. Thus, in some embodiments, a solid support, e.g., a microtiter plate or a paramagnetic bead, is attached to the capture binding partner, e.g., antibody, through noncovalent attachment, e.g., biotin-avidin/streptavidin interactions. In some embodiments, the attachment is covalent. Thus, in some embodiments, a solid support, e.g., a microtiter plate or a paramagnetic bead, is attached to the capture binding partner, e.g., antibody, through covalent attachment. Covalent attachment in which the orientation of the capture antibody is such that capture of the molecule of interest is optimized is especially useful. For example, in some embodiments a solid support, e.g., a microtiter plate or a paramagnetic microparticle, may be used in which the attachment of the binding partner, e.g., antibody, is an oriented attachment, e.g., a covalent oriented attachment.

[0062] An exemplary protocol for oriented attachment of an antibody to a solid support is as follows: IgG is dissolved in 0.1M sodium acetate buffer, pH 5.5 to a final concentration of 1 mg/ml. An equal volume of ice-cold 20 mM sodium periodate in 0.1 M sodium acetate, pH 5.5 is added. The IgG is allowed to oxidize for ½ hour on ice. Excess periodate reagent is quenched by the addition of 0.15 volume of 1 M glycerol. Low molecular weight byproducts of the oxidation reaction are removed by ultrafiltration. The oxidized IgG fraction is diluted to a suitable concentration (typically 0.5 micrograms IgG per ml) and reacted with hydrazide-activated multiwell plates for at least two hours at room temperature. Unbound IgG is removed by washing the multiwell plate with borate buffered saline or another suitable buffer. The plate may be dried for storage, if desired. A similar protocol may be followed for microbeads if the material of the microbead is suitable for such attachment.

[0063] In some embodiments, the solid support is a microtiter plate. In some embodiments, the solid support is a paramagnetic bead. An exemplary paramagnetic bead is Streptavidin C1(Dynal, 650.01-03). Other suitable beads will be apparent to those of skill in the art. Methods for attachment of antibodies to paramagnetic beads are well-known in the art. One example is given in the Examples.

[0064] The cardiac troponin of interest is contacted with the capture binding partner, e.g., capture antibody immobilized on a solid support. Some sample preparation may be used; e.g., preparation of serum from blood samples or concentration procedures before the sample is contacted with the capture antibody. Protocols for binding of proteins in immunoassays are well-known in the art and are included in the Examples.

[0065] The time allowed for binding will vary depending on the conditions; it will be apparent that shorter binding times are desirable in some settings, especially in a clinical setting. The use of, e.g., paramagnetic beads can reduce the time required for binding. In some embodiments, the time allowed for binding of the protein of interest to the capture binding partner, e.g., antibody, is less that about 12, 10, 8, 6, 4, 3, 2, or 1 hours, or less than about 60, 50, 40, 30, 25, 20, 15, 10, or 5 minutes. In some embodiments, the time allowed for binding of the protein of interest to the capture binding partner, e.g., antibody, is less than about 60 minutes. In some embodiments, the time allowed for binding of the protein of interest to the capture binding partner, e.g., antibody, is less that about 40 minutes. In some embodiments, the time allowed for binding of the protein of interest to the capture binding partner, e.g., antibody, is less that about 30 minutes. In some embodiments, the time allowed for binding of the protein of interest to the

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capture binding partner, e.g., antibody, is less that about 20 minutes. In some embodiments, the time allowed for binding of the protein of interest to the capture binding partner, e.g., antibody, is less that about 15 minutes. In some embodiments, the time allowed for binding of the protein of interest to the capture binding partner, e.g., antibody, is less that about 10 minutes. In some embodiments, the time allowed for binding of the protein of interest to the capture binding partner, e.g., antibody, is less that about 5 minutes.

[0066] In some embodiments, following the binding of the troponin particles to the capture binding partner, e.g., capture antibody, particles that may have bound nonspecifically, as well as other unwanted substances in the sample, are washed away leaving substantially only specifically bound troponin particles. In other embodiments, no wash is used between additions of sample and label; it will be appreciated that this reduces sample preparation time even further. Thus, in some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less that about 12, 10, 8, 6, 4, 3, 2, or 1 hours, or less than about 60, 50, 40, 30, 25, 20, 15, 10, or 5 minutes. In some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less that about 60 minutes. In some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less than about 40 minutes. In some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less than about 30 minutes. In some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less than about 20 minutes. In some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less than about 15 minutes. In some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less than about 10 minutes. In some embodiments, the time allowed for both binding of the protein of interest to the capture binding partner, e.g., antibody, and binding of the label to the protein of interest, is less than about 5 minutes.

[0067] Some immunoassay diagnostic reagents including the capture and signal antibodies used to measure the target analytes may be derived from the sera of animals. Endogenous human heterophilic antibodies, or human anti-animal antibodies, which have the ability to bind to immunoglobulins of other species, are present in the serum or plasma of more than 10% of patients. These circulating heterophile antibodies may interfere with immunoassay measurements. In sandwich immunoassays, these heterophilic antibodies can either bridge the capture and detection (diagnostic) antibodies, thereby producing a false-positive signal, or they may block the binding of the diagnostic antibodies, thereby producing a false-negative signal. In competitive immunoassays, the heterophile antibodies may bind to the analytic antibody and inhibit its binding to the troponin. They also may either block or augment the separation of the antibody-troponin complex from free troponin, especially when antispecies antibodies are used in the separation systems. Therefore, the impact of these heterophile antibody interferences are difficult to predict. Thus, it would be advantageous to block the binding of any heterophilic antibodies. In some embodiments of the invention, the immunoassay includes the step of depleting the sample of heterophile antibodies using one or more heterophile antibody blockers. Methods for removing heterophile antibodies from samples that are to be tested in immunoassays are known and include: heating the specimen in a sodium acetate buffer, pH 5.0, for 15 minutes at 90°C and centrifuging at 1200g for 10 minutes, or the heterophile antibodies can be precipitated using polyethylene glycol (PEG); immunoextracting the interfering heterophile immunoglobulins from the specimen using protein A or protein G; or adding nonimmune mouse IgG. Embodiments of the methods of the invention contemplate preparing

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the sample prior to analysis with the single molecule detector. The appropriateness of the method of pretreatment may be determined. Biochemicals to minimize immunoassay interference caused by heterophile antibodies are commercially available. For example, a product called MAK33, which is an IgG1 monoclonal antibody to h-CK-MM, may be obtained from Boehringer Mannheim. The MAK33 plus product contains a combination of IgG1 and IgG1-Fab. The polyMAK33 contains IgG1-Fab polymerized with IgG1, and the polyMAC 2b/2a contains IgG2a-Fab polymerized with IgG2b. A second commercial source of biochemicals to neutralize heterophile antibodies is Immunoglobulin Inhibiting Reagent marketed by Bioreclamation Inc, East Meadow, NY. This product is a preparation of immunoglobulins (IgG and IgM) from multiple species, mainly murine IgG2a, IgG2b, and IgG3 from Balb/c mice. In some embodiments the heterophile antibody may be immunoextracted from the sample using methods known in the art e.g. depleting the sample of the heterophile antibody by binding the interfering antibody to protein A or G. In some embodiments, the heterophile antibody is neutralized using one or more heterophile antibody blockers. Heterophile blockers may be selected from the group consisting of anti-isotype heterophile antibody blockers, anti-idiotype heterophile antibody blockers, and anti-anti-idiotype heterophile antibody blockers. In some embodiments a combination of heterophile antibody blockers may be used.

[0068] Label is added either with or following the addition of sample and washing. Protocols for binding of antibody and other immunolabels to proteins and other molecules are well-known in the art. If the label binding step is separate from capture binding, the time allowed for label binding can be important, e.g., in the clinical setting. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 12, 10, 8, 6, 4, 3, 2, or 1 hours, or less than about 60, 50, 40, 30, 25, 20, 15, 10, or 5 minutes. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 60 minutes. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 40 minutes. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 30 minutes. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 20 minutes. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 15 minutes. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 10 minutes. In some embodiments, the time allowed for binding of the protein of interest to the label, e.g., antibody-dye, is less than about 5 minutes. Excess label is removed by washing.

[0069] Label is then eluted from the protein of interest. Preferred elution buffers are effective in releasing the label without generating significant background. It is also useful if the elution buffer is bacteriostatic. Elution buffers of use in the invention include a chaotrope, e.g., urea or a guanidinium compound; a buffer, e.g., borate buffered saline; a protein carrier, e.g., an albumin, such as human, bovine, or fish albumin, or an IgG, to coat the wall of the capillary tube in the detection instrument; and a surfactant, e.g., an ionic or nonionic detergent, selected so as to produce a relatively low background, e.g., Tween 20, Triton X-100, or SDS.

[0070] The elution buffer/label aliquot that is sampled into the single molecule detector is referred to as the "processing sample," to distinguish it from the original sample which was obtained from an individual.

[0071] In another embodiment, the solid phase binding assay may employ a competitive binding assay format. One such method comprises a) competitively binding to a capture antibody immobilized on a binding surface i) a troponin particle in a sample and ii) a labeled analog of the troponin particle comprising a detectable label (the detection reagent) and b) measuring the amount of the label using a single particle analyzer. Another such method comprises a) competitively binding to an antibody having a detectable label (the detection reagent) i) a troponin particle in a sample and ii) an analog of troponin particle that is immobilized on a binding surface (the capture

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reagent) and b) measuring the amount of the label using a single particle analyzer. An "analog of a troponin" refers, herein, to a species that competes with troponin for binding to a capture antibody. Examples of competitive immunoassays are disclosed in U.S. Pat. No. 4,235,601 to Deutsch et al., U.S. Pat. No. 4,442,204 to Liotta, and U.S. Pat. No. 5,208,535 to Buechler et al., all of which are incorporated herein by reference.

C. Detection of troponin and determination of concentration

[0072] Following elution, the label is run through a single molecule detector in e.g., the elution buffer. A processing sample may contain no label, a single label, or a plurality of labels. The number of labels corresponds or is proportional to (if dilutions or fractions of samples are used) the number of molecules of cardiac troponin captured during the capture step.

[0073] Any suitable single molecule detector capable of detecting the label used with the protein of interest may be used. Suitable single molecule detectors are described herein. Typically the detector will be part of a system that includes an automatic sampler for sampling prepared samples, and, optionally, a recovery system to recover samples.

[0074] In some embodiments, the processing sample is analyzed in a single molecule analyzer that utilizes a capillary flow system, and that includes a capillary flow cell, a laser to illuminate an interrogation space in the capillary through which processing sample is passed, a detector to detect radiation emitted from the interrogation space, and a source of motive force to move a processing sample through the interrogation space. In some embodiments, the single molecule analyzer further comprises a microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space, e.g., a high numerical aperture microscope objective. In some embodiments, the laser and detector are in a confocal arrangement. In some embodiments, the laser is a continuous wave laser. In some embodiments, the detector is a avalanche photodiode detector. In some embodiments, the source of motive force is a pump to provide pressure. In some embodiments, the invention provides an analyzer system that includes a sampling system capable of automatically sampling a plurality of samples providing a fluid communication between a sample container and the interrogation space. In some embodiments, the interrogation space has a volume of between about 0.001 and 500 pL, or between about 0.01 pL and 100 pL, or between about 0.01 pL and 10 pL, or between about 0.01 pL and 5 pL, or between about 0.01 pL and 0.5 pL, or between about 0.02 pL and about 300 pL, or between about 0.02 pL and about 50 pL or between about 0.02 pL and about 5 pL or between about 0.02 pL and about 0.5 pL or between about 0.02 pL and about 2 pL, or between about 0.05 pL and about 50 pL, or between about 0.05 pL and about 5 pL, or between about 0.05 pL and about 0.5 pL, or between about 0.05 pL and about 0.2 pL ,or between about 0.1 pL and about 25 pL. In some embodiments, the interrogation space has a volume between about 0.004 pL and 100 pL. In some embodiments, the interrogation space has a volume between about 0.02 pL and 50 pL. In some embodiments, the interrogation space has a volume between about 0.001 pL and 10 pL. In some embodiments, the interrogation space has a volume between about 0.001 pL and 10 pL. In some embodiments, the interrogation space has a volume between about 0.01 pL and 5 pL. In some embodiments, the interrogation space has a volume between about 0.02 pL and about 5 pL. In some embodiments, the interrogation space has a volume between about 0.05 pL and 5 pL In some embodiments, the interrogation space has a volume between about 0.05 pL and 10 pL. In some embodiments, the interrogation space has a volume between about 0.5 pL and about 5 pL. In some embodiments, the interrogation space has a volume between about 0.02 pL and about 0.5 pL.

[0075] In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the

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capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.02 pL and about 50 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.004 pL and about 100 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.05 pL and about 10 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.05 pL and about 5 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.5 pL and about 5 pL.

[0076] In some embodiments, the single molecule detector is capable of determining a concentration for a molecule of interest in a sample where sample may range in concentration over a range of at least about 100-fold, or 1000-fold, or 10,000-fold, or 100,000-fold, or 300,00-fold, or 1,000,000-fold, or 10,000,000-fold, or 30,000,000-fold.

[0077] In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 50%, 40%, 30%, 20%, 15%, or 10% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 5, 4, 3, 2, or 1 ul, and wherein the analyte is present at a concentration of less than about 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 5, 4, 3, 2, or 1 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 50% in concentration of an analyte between a first sample

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and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 100 ul, and wherein the analyte is present at a concentration of less than about 100 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 40% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 50 ul, and wherein the analyte is present at a concentration of less than about 50 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 20% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 20 ul, and wherein the analyte is present at a concentration of less than about 20 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 20% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 10 ul, and wherein the analyte is present at a concentration of less than about 10 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 20% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 5 ul, and wherein the analyte is present at a concentration of less than about 5 femtomolar.

[0078] The single molecule detector and systems are described in more detail below. Further embodiments of single molecule analyzers useful in the methods of the invention, such as detectors with more than one interrogation window, detectors utilize electrokinetic or electrophoretic flow, and the like, may be found in U.S. Patent Application No. 11/048,660, incorporated by reference herein in its entirety.

[0079] Between runs the instrument may be washed. A wash buffer that maintains the salt and surfactant concentrations of the sample may be used in some embodiments to maintain the conditioning of the capillary; i.e., to keep the capillary surface relatively constant between samples to reduce variability.

[0080] A feature that contributes to the extremely high sensitivity of the instruments and methods of the invention is the method of detecting and counting labels, which, in some embodiments, are attached to single molecules to be detected or, more typically, correspond to a single molecule to be detected. Briefly, the processing sample flowing through the capillary is effectively divided into a series of detection events, by subjecting a given interrogation space of the capillary to EM radiation from a laser that emits light at an appropriate excitation wavelength for the fluorescent moiety used in the label for a predetermined period of time, and detecting photons emitted during that time. Each predetermined period of time is a "bin." If the total number of photons detected in a given bin exceeds a predetermined threshold level, a detection event is registered for that bin, i.e., a label has been detected. If the total number of photons is not at the predetermined threshold level, no detection event is registered. In some embodiments, processing sample concentration is dilute enough that, for a large percentage of detection events, the detection event represents only one label passing through the window, which corresponds to a single molecule of interest in the original sample, that is, few detection events represent more than one label in a single bin. In some embodiments, further refinements are applied to allow greater concentrations of label in the processing sample to be detected accurately, i.e., concentrations at which the probability of two or more labels being detected as a single detection event is no longer insignificant.

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[0081] Although other bin times may be used without departing from the scope of the present invention, in some embodiments the bin times are selected in the range of about 1 microsecond to about 5 ms. In some embodiments, the bin time is more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 250, 300, 400, 500, 600, 700, 750, 800, 900, 1000, 2000, 3000, 4000, or 5000 microseconds. . In some embodiments, the bin time is less than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 250, 300, 400, 500, 600, 700, 750, 800, 900, 1000, 2000, 3000, 4000, or 5000 microseconds. In some embodiments, the bin time is about 1 to 1000 microseconds. In some embodiments, the bin time is about 1 to 750 microseconds. In some embodiments, the bin time is about 1 to 500 microseconds. In some embodiments, the bin time is about 1 to 250 microseconds. In some embodiments, the bin time is about 1 to 100 microseconds. In some embodiments, the bin time is about 1 to 50 microseconds. In some embodiments, the bin time is about 1 to 40 microseconds. In some embodiments, the bin time is about 1 to 30 microseconds. In some embodiments, the bin time is about 1 to 500 microseconds. In some embodiments, the bin time is about 1 to 20 microseconds. In some embodiments, the bin time is about 1 to 10 microseconds. In some embodiments, the bin time is about 1 to 500 microseconds. In some embodiments, the bin time is about 1 to 5 microseconds. In some embodiments, the bin time is about 5 to 500 microseconds. In some embodiments, the bin time is about 5 to 250 microseconds. In some embodiments, the bin time is about 5 to 100 microseconds. In some embodiments, the bin time is about 5 to 50microseconds. In some embodiments, the bin time is about 5 to 20 microseconds. In some embodiments, the bin time is about 5 to 10 microseconds. In some embodiments, the bin time is about 10 to 500 microseconds. In some embodiments, the bin time is about 10 to 250 microseconds. In some embodiments, the bin time is about 10 to 100 microseconds. In some embodiments, the bin time is about 10 to 50 microseconds. In some embodiments, the bin time is about 10 to 30 microseconds. In some embodiments, the bin time is about 10 to 20 microseconds. In some embodiments, the bin time is about 5 microseconds. In some embodiments, the bin time is about 5 microseconds. In some embodiments, the bin time is about 6 microseconds. In some embodiments, the bin time is about 7 microseconds. In some embodiments, the bin time is about 8 microseconds. In some embodiments, the bin time is about 9 microseconds. In some embodiments, the bin time is about 10 microseconds. In some embodiments, the bin time is about 11 microseconds. In some embodiments, the bin time is about 12 microseconds. In some embodiments, the bin time is about 13 microseconds. In some embodiments, the bin time is about 14 microseconds. In some embodiments, the bin time is about 5 microseconds. In some embodiments, the bin time is about 15 microseconds. In some embodiments, the bin time is about 16 microseconds. In some embodiments, the bin time is about 17 microseconds. In some embodiments, the bin time is about 18 microseconds. In some embodiments, the bin time is about 19 microseconds. In some embodiments, the bin time is about 20 microseconds. In some embodiments, the bin time is about 25 microseconds. In some embodiments, the bin time is about 30 microseconds. In some embodiments, the bin time is about 40 microseconds. In some embodiments, the bin time is about 50 microseconds. In some embodiments, the bin time is about 100 microseconds. In some embodiments, the bin time is about 250 microseconds. In some embodiments, the bin time is about 500 microseconds. In some embodiments, the bin time is about 750 microseconds. In some embodiments, the bin time is about 1000 microseconds.

[0082] In some embodiments, the background noise level is determined from the mean noise level, or the root-mean-square noise. In other cases, a typical noise value or a statistical value is chosen. In most cases, the noise is expected to follow a Poisson distribution. Thus, in some embodiments, determining the concentration of a particle-label complex in a sample comprises determining the background noise level.

[0083] Thus, as a label flows through the capillary flow cell, it is irradiated by the laser beam to generate a burst of photons. The photons emitted by the label are discriminated from background light or background noise emission

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by considering only the bursts of photons that have energy above a predetermined threshold energy level which accounts for the amount of background noise that is present in the sample. Background noise typically comprises low frequency emission produced, for example, by the intrinsic fluorescence of non-labeled particles that are present in the sample, the buffer or diluent used in preparing the sample for analysis, Raman scattering and electronic noise. In some embodiments, the value assigned to the background noise is calculated as the average background signal noise detected in a plurality of bins, which are measurements of photon signals that are detected in an interrogation space during a predetermined length of time. Thus in some embodiments, background noise is calculated for each sample as a number specific to that sample.

[0084] Given the value for the background noise, the threshold energy level can be assigned. As discussed above, the threshold value is determined to discriminate true signals (due to fluorescence of a label) from the background noise. Care must be taken in choosing a threshold value such that the number of false positive signals from random noise is minimized while the number of true signals which are rejected is also minimized. Methods for choosing a threshold value include determining a fixed value above the noise level and calculating a threshold value based on the distribution of the noise signal. In one embodiment, the threshold is set at a fixed number of standard deviations above the background level. Assuming a Poisson distribution of the noise, using this method one can estimate the number of false positive signals over the time course of the experiment. In some embodiments, the threshold level is calculated as a value of 4 sigma above the background noise. For example, given an average background noise level of 200 photons, the analyzer system establishes a threshold level of $4\sqrt{200}$ above the average background/noise level of 200 photons to be 256 photons. Thus, in some embodiments, determining the concentration of a label in a sample includes establishing the threshold level above which photon signals represent the presence of a label. Conversely, photon signals that have an energy level that is not greater than that of the threshold level indicate the absence of a label.

[0085] Many bin measurements are taken to determine the concentration of a sample, and the absence or presence of a label is ascertained for each bin measurement. Typically, 60,000 measurements or more can made in one minute (e.g., in embodiments in which the bin size is 1ms—for smaller bin sizes the number of measurements is correspondingly larger, e.g., 6,000,000 measurements per minute for a bin size of 10 microseconds). Thus, no single measurement is crucial and the method provides for a high margin of error. The bins that are determined not to contain a label ("no" bins) are discounted and only the measurements made in the bins that are determined to contain label ("yes" bins) are accounted in determining the concentration of the label in the processing sample. Discounting measurements made in the "no" bins or bins that are devoid of label increases the signal to noise ratio and the accuracy of the measurements. Thus, in some embodiments, determining the concentration of a label in a sample comprises detecting the bin measurements that reflect the presence of a label.

[0086] The signal to noise ratio or the sensitivity of the analyzer system can be increased by minimizing the time that background noise is detected during a bin measurement in which a particle-label complex is detected. For example, in a bin measurement lasting 1 millisecond during which one particle-label complex is detected when passing across an interrogation space within 250 microseconds, 750 microseconds of the 1 millisecond are spent detecting background noise emission. The signal to noise ratio can be improved by decreasing the bin time. In some embodiments, the bin time is 1 millisecond. In other embodiments, the bin time is 750, 500, 250 microseconds, 100 microseconds, 50 microseconds, 25 microseconds or 10 microseconds. Other bin times are as described herein.

[0087] Other factors that affect measurements are the brightness or dimness of the fluorescent moiety, the flow rate, and the power of the laser. Various combinations of the relevant factors that allow for detection of label will be

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apparent to those of skill in the art. In some embodiments, the bin time is adjusted without changing the flow rate. It will be appreciated by those of skill in the art that as bin time decreases, laser power output directed at the interrogation space must increase to maintain a constant total energy applied to the interrogation space during the bin time. For example, if bin time is decreased from 1000 microseconds to 250 microseconds, as a first approximation, laser power output must be increased approximately four-fold. These settings allow for the detection of the same number of photons in a 250μs as the number of photons counted during the 1000 μs given the previous settings, and allow for faster analysis of sample with lower backgrounds and thus greater sensitivity. In addition, flow rates may be adjusted in order to speed processing of sample. These numbers are merely exemplary, and the skilled practitioner can adjust the parameters as necessary to achieve the desired result.

[0088] In some embodiments, the interrogation space encompasses the entire cross-section of the sample stream. When the interrogation space encompasses the entire cross-section of the sample stream, only the number of labels counted and the volume passing through a cross-section of the sample stream in a set length of time are needed to calculate the concentration of the label in the processing sample. In some embodiments, the interrogation space can be defined to be smaller than the cross-sectional area of sample stream by, for example, the interrogation space is defined by the size of the spot illuminated by the laser beam. In some embodiments, the interrogation space can be defined by adjusting the apertures 306 (Figure 1A) or 358 and 359 (Figure 1B) of the analyzer and reducing the illuminated volume that is imaged by the objective lens to the detector. In the embodiments when the interrogation space is defined to be smaller than the cross-sectional area of sample stream, the concentration of the label can be determined by interpolation of the signal emitted by the complex from a standard curve that is generated using one or more samples of known standard concentrations. In yet other embodiments, the concentration of the label can be determined by comparing the measured particles to an internal label standard. In embodiments when a diluted sample is analyzed, the dilution factor is accounted in calculating the concentration of the molecule of interest in the starting sample.

[0089] As discussed above, when the interrogation space encompasses the entire cross-section of the sample stream, only the number of labels counted passing through a cross-section of the sample stream in a set length of time (bin) and the volume of sample that was interrogated in the bin are needed to calculate the concentration the sample. The total number of labels contained in the "yes" bins is determined and related to the sample volume represented by the total number of bins used in the analysis to determine the concentration of labels in the processing sample. Thus, in one embodiment, determining the concentration of a label in a processing sample comprises determining the total number of labels detected "yes" bins and relating the total number of detected labels to the total sample volume that was analyzed. The total sample volume that is analyzed is the sample volume that is passed through the capillary flow cell and across the interrogation space in a specified time interval. Alternatively, the concentration of the label complex in a sample is determined by interpolation of the signal emitted by the label in a number of bins from a standard curve that is generated by determining the signal emitted by labels in the same number of bins by standard samples containing known concentrations of the label.

[0090] In some embodiments, the number of individual labels that are detected in a bin is related to the relative concentration of the particle in the processing sample. At relatively low concentrations, for example at concentrations below about 10 -16 M the number of labels is proportional to the photon signal that is detected in a bin. Thus, at low concentrations of label the photon signal is provided as a digital signal. At relatively higher concentrations, for example at concentrations greater than about 10 -16 M, the proportionality of photon signal to a label is lost as the likelihood of two or more labels crossing the interrogation space at about the same time and being

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counted as one becomes significant. Thus, in some embodiments, individual particles in a sample of a concentration greater than about 10^{-16} M are resolved by decreasing the length of time of the bin measurement.

[0091] Alternatively, in other embodiments, the total the photon signal that is emitted by a plurality of particles that are present in any one bin is detected. These embodiments allow for single molecule detectors of the invention wherein the dynamic range is at least 3, 3.5, 4, 4.5, 5.5, 6, 6.5, 7, 7.5, 8, or more than 8 logs.

[0092] "Dynamic range," as that term is used herein, refers to the range of sample concentrations that may be quantitated by the instrument without need for dilution or other treatment to alter the concentration of successive samples of differing concentrations, where concentrations are determined with an accuracy appropriate for the intended use. For example, if a microtiter plate contains a sample of 1 femtomolar concentration for an analyte of interest in one well, a sample of 10,000 femtomolar concentration for an analyte of interest in another well, and a sample of 100 femtomolar concentration for the analyte in a third well, an instrument with a dynamic range of at least 4 logs and a lower limit of quantitation of 1 femtomolar is able to accurately quantitate the concentration of all the samples without the need for further treatment to adjust concentration, e.g., dilution. Accuracy may be determined by standard methods, e.g., using a series of standards of concentrations that span the dynamic range and constructing a standard curve. Standard measures of fit of the resulting standard curve may be used as a measure of accuracy, e.g., an r^2 greater than about 0.7, 0.75, 0.8, 0.85, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, or 0.99.

[0093] Increased dynamic range is achieved by altering the manner in which data from the detector is analyzed, and/or by the use of an attenuator between the detector and the interrogation space. At the low end of the range, where processing sample is sufficiently dilute that each detection event, i.e., each burst of photons above a threshold level in a bin (the "event photons"), likely represents only one label, the data is analyzed to count detection events as single molecules. I.e., each bin is analyzed as a simple "yes" or "no" for the presence of label, as described above. For a more concentrated processing sample, where the likelihood of two or more labels occupying a single bin becomes significant, the number of event photons in a significant number of bins is found to be substantially greater than the number expected for a single label, e.g., the number of event photons in a significant number of bins corresponds to two-fold, three-fold, or more, than the number of event photons expected for a single label. For these samples, the instrument changes its method of data analysis to one of integrating the total number of event photons for the bins of the processing sample. This total will be proportional to the total number of labels that were in all the bins. For an even more concentrated processing sample, where many labels are present in most bins, background noise becomes an insignificant portion of the total signal from each bin, and the instrument changes its method of data analysis to one of counting total photons per bin (including background). An even further increase in dynamic range can be achieved by the use of an attenuator between the flow cell and the detector, when concentrations are such that the intensity of light reaching the detector would otherwise exceed the capacity of the detector for accurately counting photons, i.e., saturate the detector.

[0094] The instrument may include a data analysis system that receives input from the detector and determines the appropriate analysis method for the sample being run, and outputs values based on such analysis. The data analysis system may further output instructions to use or not use an attenuator, if an attenuator is included in the instrument.

[0095] By utilizing such methods, the dynamic range of the instrument can be dramatically increased. Thus, in some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than about 1000 (3 log), 10,000 (4 log), 100,000 (5 log), 350,000 (5.5 log), 1,000,000 (6 log), 3,500,000 (6.5 log), 10,000,000 (7 log), 35,000,000 (7.5 log), or 100,000,000 (8 log). In some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than about 100,000 (5 log). In some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than

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about 1,000,000 (6 log). In some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than about 10,000,000 (7 log). In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 1000; 10,000; 100,000; 350,000; 1,000,000; 3,500,000; 10,000,000, or 35,000,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 10,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 100,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 1,000,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 10,000,000.

[0096] In some embodiments, an analyzer or analyzer system of the invention is capable of detecting an analyte, e.g., a biomarker at a limit of detection of less than 1 nanomolar, or 1 picomolar, or 1 femtomolar, or 1 attomolar, or 1 zeptomolar. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte, or of multiple analytes, e.g., a biomarker or biomarkers, from one sample to another sample of less than about 0.1, 1, 2, 5, 10, 20, 30, 40, 50, 60, or 80% when the biomarker is present at a concentration of less than 1 nanomolar, or 1 picomolar, or 1 femtomolar, or 1 attomolar, or 1 zeptomolar, in the samples, and when the size of each of the sample is less than about 100, 50, 40, 30, 20, 10, 5, 2, 1, 0.1, 0.01, 0.001, or 0.0001 ul. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 1 picomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 100 femtomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 50 femtomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 5 femtomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 5 femtomolar, and when the size of each of the samples is less than about 5 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 1 femtomolar, and when the size of each of the samples is less than about 5 µl.

V. Instruments and Systems Suitable for Highly Sensitive Analysis of Troponin

[0097] The methods of the invention utilize analytical instruments of high sensitivity, e.g., single molecule detectors. Such single molecule detectors include embodiments as hereinafter described.

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A. Apparatus/System

[0098] In one aspect, the methods described herein utilize an analyzer system capable of detecting a single particle in a sample. In one embodiment, the analyzer system is capable of single particle detection of a fluorescently labeled particle wherein the analyzer system detects energy emitted by an excited fluorescent label in response to exposure by an electromagnetic radiation source when the single particle is present in an interrogation space defined within a capillary flow cell fluidly connected to the sampling system of the analyzer system. In a further embodiment of the analyzer system, the single particle moves through the interrogation space of the capillary flow cell by means of a motive force. In another embodiment of the analyzer system, an automatic sampling system may be included in the analyzer system for introducing the sample into the analyzer system. In another embodiment of the analyzer system, a sample preparation system may be included in the analyzer system for preparing a sample. In a further embodiment, the analyzer system may contain a sample recovery system for recovering at least a portion of the sample after analysis is complete.

[0099] In one aspect, the analyzer system consists of an electromagnetic radiation source for exciting a single particle labeled with a fluorescent label. In one embodiment, the electromagnetic radiation source of the analyzer system is a laser. In a further embodiment, the electromagnetic radiation source is a continuous wave laser.

[00100] In a typical embodiment, the electromagnetic radiation source excites a fluorescent moiety attached to a label as the label passes through the interrogation space of the capillary flow cell. In some embodiments, the fluorescent label moiety inclues one or more fluorescent dye molecules. In some embodiments, the fluorescent label moiety is a quantum dot. Any fluorescent moiety as described herein may be used in the label.

[00101] A label is exposed to electromagnetic radiation when the label passes through an interrogation space located within the capillary flow cell. The interrogation space is typically fluidly connected to a sampling system. In some embodiments the label passes through the interrogation space of the capillary flow cell due to a motive force to advance the label through the analyzer system. The interrogation space is positioned such that it receives electromagnetic radiation emitted from the radiation source. In some embodiments, the sampling system is an automated sampling system capable of sampling a plurality of samples without intervention from a human operator.

[00102] The label passes through the interrogation space and emits a detectable amount of energy when excited by the electromagnetic radiation source. In one embodiment, an electromagnetic radiation detector is operably connected to the interrogation space. The electromagnetic radiation detector is capable of detecting the energy emitted by the label, e.g., by the fluorescent moiety of the label.

[00103] In a further embodiment of the analyzer system, the system further includes a sample preparation mechanism where a sample may be partially or completely prepared for analysis by the analyzer system. In some embodiments of the analyzer system, the sample is discarded after it is analyzed by the system. In other embodiments, the analyzer system further includes a sample recovery mechanism whereby at least a portion, or alternatively all or substantially all, of the sample may be recovered after analysis. In such an embodiment, the sample can be returned to the origin of the sample. In some embodiments, the sample can be returned to microtiter wells on a sample microtiter plate. The analyzer system typically further consists of a data acquisition system for collecting and reporting the detected signal.

B. Single Particle Analyzer

[00104] As shown in Figure 1A, described herein is one embodiment of an analyzer system 300. The analyzer system 300 includes an electromagnetic radiation source 301, a mirror 302, a lens 303, a capillary flow cell 313, a

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microscopic objective lens 305, an aperture 306, a detector lens 307, a detector filter 308, a single photon detector 309, and a processor 310 operatively connected to the detector.

[00105] In operation the electromagnetic radiation source 301 is aligned so that its output 311 is reflected off of a front surface 312 of mirror 302. The lens 303 focuses the beam 311 onto a single interrogation space (an illustrative example of an interrogation space 314 is shown in Figure 2A) in the capillary flow cell 313. The microscope objective lens 305 collects light from sample particles and forms images of the beam onto the aperture 306. The aperture 306 affects the fraction of light emitted by the specimen in the interrogation space of the capillary flow cell 313 that can be collected. The detector lens 307 collects the light passing through the aperture 306 and focuses the light onto an active area of the detector 309 after it passes through the detector filters 308. The detector filters 308 minimize aberrant noise signals due to light scatter or ambient light while maximizing the signal emitted by the excited fluorescent moiety bound to the particle. The processor 310 processes the light signal from the particle according to the methods described herein.

[00106] In one embodiment, the microscope objective lens 305 is a high numerical aperture microscope objective. As used herein, "high numerical aperture lens" include a lens with a numerical aperture of equal to or greater than 0.6. The numerical aperture is a measure of the number of highly diffracted image-forming light rays captured by the objective. A higher numerical aperture allows increasingly oblique rays to enter the objective lens and thereby produce a more highly resolved image. Additionally, the brightness of an image increases with a higher numerical aperture. High numerical aperture lenses are commercially available from a variety of vendors, and any one lens having a numerical aperture of equal to or greater than approximately 0.6 may be used in the analyzer system. In some embodiments, the lens has a numerical aperture of about 0.6 to about 1.3. In some embodiments, the lens has a numerical aperture of about 0.6 to about 1.0. In some embodiments, the lens has a numerical aperture of about 0.7 to about 1.2. In some embodiments, the lens has a numerical aperture of about 0.7 to about 1.0. In some embodiments, the lens has a numerical aperture of about 0.7 to about 0.9. In some embodiments, the lens has a numerical aperture of about 0.8 to about 1.3. In some embodiments, the lens has a numerical aperture of about 0.8 to about 1.2. In some embodiments, the lens has a numerical aperture of about 0.8 to about 1.0. In some embodiments, the lens has a numerical aperture of at least about 0.6. In some embodiments, the lens has a numerical aperture of at least about 0.7. In some embodiments, the lens has a numerical aperture of at least about 0.8. In some embodiments, the lens has a numerical aperture of at least about 0.9. In some embodiments, the lens has a numerical aperture of at least about 1.0. In some embodiments, the aperture of the microscope objective lens 305 is approximately 1.25. In an embodiment where a microscope objective lens 305 of 0.8 is used, a Nikon 60X/0.8 NA Achromat lens (Nikon, Inc., USA) can be used.

[00107] In some embodiments, the electromagnetic radiation source 301 is a laser that emits light in the visible spectrum. In all embodiments, the electromagnetic radiation source is set such that wavelength of the laser is set such that it is of a sufficient wavelength to excite the fluorescent label attached to the particle. In some embodiments, the laser is a continuous wave laser with a wavelength of 639 nm. In other embodiments, the laser is a continuous wave laser with a wavelength of 532 nm. In other embodiments, the laser is a continuous wave laser with a wavelength of 422 nm. In other embodiments, the laser is a continuous wave laser with a wavelength of 405 nm. Any continuous wave laser with a wavelength suitable for exciting a fluorescent moiety as used in the methods and compositions of the invention may be used without departing from the scope of the invention.

[00108] In a single particle analyzer system 300, as each particle passes through the beam 311 of the electromagnetic radiation source, the particle enters into an excited state. When the particle relaxes from its excited state, a detectable burst of light is emitted. The excitation-emission cycle is repeated many times by each particle in

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the length of time it takes for it to pass through the beam allowing the analyzer system 300 to detect tens to thousands of photons for each particle as it passes through an interrogation space 314. Photons emitted by fluorescent particles are registered by the detector 309 (Figure 1A) with a time delay indicative of the time for the particle label complex to pass through the interrogation space. The photon intensity is recorded by the detector 309 and sampling time is divided into bins, which are uniform, arbitrary, time segments with freely selectable time channel widths. The number of signals contained in each bin evaluated. One or a combination of several statistical analytical methods are employed in order to determine when a particle is present. Such methods include determining the baseline noise of the analyzer system and setting a signal strength for the fluorescent label at a statistical level above baseline noise to eliminate false positive signals from the detector.

[00109] The electromagnetic radiation source 301 is focused onto a capillary flow cell 313 of the analyzer system 300 where the capillary flow cell 313 is fluidly connected to the sample system. An interrogation space 314 is shown in Figure 2A. The beam 311 from the continuous wave electromagnetic radiation source 301 of Figure 1A is optically focused to a specified depth within the capillary flow cell 313. The beam 311 is directed toward the sample-filled capillary flow cell 313 at an angle perpendicular to the capillary flow cell 313. The beam 311 is operated at a predetermined wavelength that is selected to excite a particular fluorescent label used to label the particle of interest. The size or volume of the interrogation space 314 is determined by the diameter of the beam 311 together with the depth at which the beam 311 is focused. Alternatively, the interrogation space can be determined by running a calibration sample of known concentration through the analyzer system.

[00110] When single molecules are detected in the sample concentration, the beam size and the depth of focus required for single molecule detection are set and thereby define the size of the interrogation space 314. The interrogation space 314 is set such that, with an appropriate sample concentration, only one particle is present in the interrogation space 314 during each time interval over which time observations are made. It will be appreciated that the detection interrogation volume as defined by the beam is not perfectly spherically shaped, and typically is a "bow-tie" shape. However, for the purposes of definition, "volumes" of interrogation spaces are defined herein as the volume encompassed by a sphere of a diameter equal to the focused spot diameter of the beam. The focused spot of the beam 311 may have various diameters without departing from the scope of the present invention. In some embodiments, the diameter of the focused spot of the beam is about 1 to about 5, 10, 15, or 20 microns, or about 5 to about 10, 15, or 20 microns, or about 10 to about 20 microns, or about 10 to about 15 microns. In some embodiments, the diameter of the focused spot of the beam is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 microns. In some embodiments, the diameter of the focused spot of the beam is about 5 microns. In some embodiments, the diameter of the focused spot of the beam is about 10 microns. In some embodiments, the diameter of the focused spot of the beam is about 12 microns. In some embodiments, the diameter of the focused spot of the beam is about 13 microns. In some embodiments, the diameter of the focused spot of the beam is about 14 microns. In some embodiments, the diameter of the focused spot of the beam is about 15 microns. In some embodiments, the diameter of the focused spot of the beam is about 16 microns. In some embodiments, the diameter of the focused spot of the beam is about 17 microns. In some embodiments, the diameter of the focused spot of the beam is about 18 microns. In some embodiments, the diameter of the focused spot of the beam is about 19 microns. In some embodiments, the diameter of the focused spot of the beam is about 20 microns.

[00111] In an alternate embodiment of the single particle analyzer system, more than one electromagnetic radiation source can be used to excite particles labeled with fluorescent labels of different wavelengths. In another alternate embodiment, more than one interrogation space in the capillary flow cell can be used. In another alternate embodiment, multiple detectors can be employed to detect different emission wavelengths from the fluorescent

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labels. An illustration incorporating each of these alternative embodiments of an analyzer system is shown in Figure 1B. These embodiments are incorporated by reference from previous U.S. Pat. App. No. 11/048,660.

[00112] In some embodiments of the analyzer system 300, a motive force is required to move a particle through the capillary flow cell 313 of the analyzer system 300. In one embodiment, the motive force can be a form of pressure. The pressure used to move a particle through the capillary flow cell can be generated by a pump. In some embodiments, a Scivex, Inc. HPLC pump can be used. In some embodiments where a pump is used as a motive force, the sample can pass through the capillary flow cell at a rate of 1 μL/min to about 20 μL/min, or about 5 μL/min to about 20 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 5 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 10 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 15 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 20 μL/min. In some embodiments, an electrokinetic force can be used to move the particle through the analyzer system. Such a method has been previously disclosed and is incorporated by reference from previous U.S. Pat. App. No. 11/048,660.

[00113] In one aspect of the analyzer system 300, the detector 309 of the analyzer system detects the photons emitted by the fluorescent label. In one embodiment, the photon detector is a photodiode. In a further embodiment, the detector is an avalanche photodiode detector. In some embodiments, the photodiodes can be silicon photodiodes with a wavelength detection of 190 nm and 1100 nm. When germanium photodiodes are used, the wavelength of light detected is between 400 nm to 1700 nm. In other embodiments, when an indium gallium arsenide photodiode is used, the wavelength of light detected by the photodiode is between 800 nm and 2600 nm. When lead sulfide photodiodes are used as detectors, the wavelength of light detected is between 1000 nm and 3500 nm.

[00114]

[00115] In some embodiments, the optics of the electromagnetic radiation source 301 and the optics of the detector 309 are arranged in a conventional optical arrangement. In such an arrangement, the electromagnetic radiation source and the detector are aligned on different focal planes. The arrangement of the laser and the detector optics of the analyzer system as shown in Figures 1A and 1B is that of a conventional optical arrangement.

[00116] In some embodiments, the optics of the electromagnetic radiation source and the optics of the detector are arranged in a confocal optical arrangement. In such an arrangement, the electromagnetic radiation source 301 and the detector 309 are aligned on the same focal plane. The confocal arrangement renders the analyzer more robust because the electromagnetic radiation source 301 and the detector optics 309 do not need to be realigned if the analyzer system is moved. This arrangement also makes the use of the analyzer more simplified because it eliminates the need to realign the components of the analyzer system. The confocal arrangement for the analyzer 300 (Figure 1A) and the analyzer 355 (Figure 1B) are shown in Figures 3A and 3B respectively. Figure 3A shows that the beam 311 from an electromagnetic radiation source 301 is focused by the microscope objective 315 to form one interrogation space 314 (Figure 2A) within the capillary flow cell 313. A dichroic mirror 316, which reflects laser light but passes fluorescent light, is used to separate the fluorescent light from the laser light. Filter 317 that is positioned in front of the detector eliminates any non-fluorescent light at the detector. In some embodiments, an analyzer system configured in a confocal arrangement can comprise two or more interrogations spaces. Such a method has been previously disclosed and is incorporated by reference from previous U.S. Pat. App. No. 11/048,660.

[00117] The laser can be a tunable dye laser, such as a helium-neon laser. The laser can be set to emit a wavelength of 632.8 nm. Alternatively, the wavelength of the laser can be set to emit a wavelength of 543.5 nm or 1523 nm. Alternatively, the electromagnetic laser can be an argon ion laser. In such an embodiment, the argon ion

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laser can be operated as a continuous gas laser at about 25 different wavelengths in the visible spectrum, the wavelength set between 408.9 and 686.1 nm but at its optimum performance set between 488 and 514.5 nm.

1 Electromagnetic Radiation Source

[00118] In some embodiments of the analyzer system a chemiluminescent label may be used. In such an embodiment, it may not be necessary to utilize an EM source for detection of the particle. In another embodiment, the extrinsic label or intrinsic characteristic of the particle is a light-interacting label or characteristic, such as a fluorescent label or a light-scattering label. In such an embodiment, a source of EM radiation is used to illuminate the label and/or the particle. EM radiation sources for excitation of fluorescent labels are preferred.

[00119] In some embodiments, the analyzer system consists of an electromagnetic radiation source 301. Any number of radiation sources may be used in any one analyzer system 300 without departing from the scope of the invention. Multiple sources of electromagnetic radiation have been previously disclosed and are incorporated by reference from previous U.S. Pat. App. No. 11/048,660. In some embodiments, all the continuous wave electromagnetic (EM) radiation sources emit electromagnetic radiation at the same wavelengths. In other embodiments, different sources emit different wavelengths of EM radiation.

[00120] In one embodiment, the EM source(s) 301, 351, 352 are continuous wave lasers producing wavelengths of between 200 nm and 1000 nm. Such EM sources have the advantage of being small, durable and relatively inexpensive. In addition, they generally have the capacity to generate larger fluorescent signals than other light sources. Specific examples of suitable continuous wave EM sources include, but are not limited to: lasers of the argon, krypton, helium-neon, helium-cadmium types, as well as, tunable diode lasers (red to infrared regions), each with the possibility of frequency doubling. The lasers provide continuous illumination with no accessory electronic or mechanical devices, such as shutters, to interrupt their illumination. In an embodiment where a continuous wave laser is used, an electromagnetic radiation source of 3 mW may be of sufficient energy to excite a fluorescent label. A beam from a continuous wave laser of such energy output may be between 2 to 5 μm in diameter. The time of exposure of the particle to laser beam in order to be exposed to 3mW may be a time period of about 1 msec. In alternate embodiments, the time of exposure to the laser beam may be equal to or less than about 500 μsec. In an alternate embodiment, the time of exposure may be equal to or less than about 100 μsec. In an alternate embodiment, the time of exposure may be equal to or less than about 50 μsec. In an alternate embodiment, the time of exposure may be equal to or less than about 10 μsec.

[00121] LEDs are another low-cost, high reliability illumination source. Recent advances in ultra-bright LEDs and dyes with high absorption cross-section and quantum yield support the applicability of LEDs to single particle detection. Such lasers could be used alone or in combination with other light sources such as mercury arc lamps, elemental arc lamps, halogen lamps, arc discharges, plasma discharges, light-emitting diodes, or combination of these.

[00122] In other embodiments, the EM source could be in the form of a pulse wave laser. In such an embodiment, the pulse size of the laser is an important factor. In such an embodiment, the size, focus spot, and the total energy emitted by the laser is important and must be of sufficient energy as to be able to excite the fluorescent label. When a pulse laser is used, a pulse of longer duration may be required. In some embodiments a laser pulse of 2 nanoseconds may be used. In some embodiments a laser pulse of 5 nanoseconds may be used. In some embodiments a pulse of between 2 to 5 nanoseconds may be used.

[00123] The optimal laser intensity depends on the photo bleaching characteristics of the single dyes and the length of time required to traverse the interrogation space (including the speed of the particle, the distance between

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interrogation spaces if more than one is used and the size of the interrogation space(s)). To obtain a maximal signal, it is desirable to illuminate the sample at the highest intensity which will not result in photo bleaching a high percentage of the dyes. The preferred intensity is one such that no more that 5% of the dyes are bleached by the time the particle has traversed the interrogation space.

[00124] The power of the laser is set depending on the type of dye molecules that need to be stimulated and the length of time the dye molecules are stimulated, and/or the speed with which the dye molecules pass through the capillary flow cell. Laser power is defined as the rate at which energy is delivered by the beam and is measured in units of Joules/second, or Watts. It will be appreciated that the greater the power output of the laser, the shorter the time that the laser illuminates the particle may be, while providing a constant amount of energy to the interrogation space while the particle is passing through the space. Thus, in some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is more than about 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is less than about 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or 110 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 0.1 and 100 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 1 and 100 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 1 and 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 2 and 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 60 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 40 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 30 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 1 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 3 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 5 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 10 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 15 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 20 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about

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30 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 40 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 60 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 70 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 80 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 90 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 100 microJoule.

[00125] In some embodiments, the laser power output is set to at least about 1 mW, 2 mW, 3mW, 4mW, 5 mW, 6, mw, 7 mW, 8 mW, 9 mW, 10 mW, 13 mW, 15 mW, 20 mW, 25 mW, 30 mW, 40 mW, 50 mW, 60 mW, 70 mW, 80 mW, 90 mW, 100 mW, or more than 100 mW. In some embodiments, the laser power output is set to at least about 1 mW. In some embodiments, the laser power output is set to at least about 3 mW. In some embodiments, the laser power output is set to at least about 5 mW. In some embodiments, the laser power output is set to at least about 10 mW. In some embodiments, the laser power output is set to at least about 20 mW. In some embodiments, the laser power output is set to at least about 30 mW. In some embodiments, the laser power output is set to at least about 40 mW. In some embodiments, the laser power output is set to at least about 50 mW. In some embodiments, the laser power output is set to at least about 60 mW. In some embodiments, the laser power output is set to at least about 90 mW.

[00126] The time that the laser illuminates the interrogation space can be set to no less than about 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 150, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 microseconds. The time that the laser illuminates the interrogation space can be set to no more than about 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 150, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1500, or 2000 microseconds. The time that the laser illuminates the interrogation space can be set between about 1 and 1000 microseconds. The time that the laser illuminates the interrogation space can be set between about 5 and 500 microseconds. The time that the laser illuminates the interrogation space can be set between about 5 and 100 microseconds. The time that the laser illuminates the interrogation space can be set between about 10 and 100 microseconds. The time that the laser illuminates the interrogation space can be set between about 10 and 50 microseconds. The time that the laser illuminates the interrogation space can be set between about 10 and 20 microseconds. The time that the laser illuminates the interrogation space can be set between about 5 and 50 microseconds. The time that the laser illuminates the interrogation space can be set between about 1 and 100 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 1 microsecond. In some embodiments, the time that the laser illuminates the interrogation space is about 5 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 10 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 25 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 50 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 100 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 250

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microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 500 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 1000 microseconds.

[00127] For example, the time that the laser illuminates the interrogation space can be set to 1 millisecond, 250 microseconds, 100 microseconds, 50 microseconds, 25 microseconds or 10 microseconds with a laser that provides a power output of 3 mW, 4 mw, 5 mW, or more than 5 mW. In some embodiments, a label is illuminated with a laser that provides a power output of 3mW and illuminates the label for about 1000 microseconds. In other embodiments, a label is illuminated for less than 1000 milliseconds with a laser providing a power output of not more than about 20 mW. In other embodiments, the label is illuminated with a laser power output of 20 mW for less than or equal to about 250 microseconds. In some embodiments, the label is illuminated with a laser power output of about 5 mW for less than or equal to about 1000 microseconds.

2. Capillary Flow Cell

[00128] The capillary flow cell is fluidly connected to the sample system. In one embodiment, the interrogation space 314 of an analyzer system, is determined by the cross sectional area of the corresponding beam 311 and by a segment of the beam within the field of view of the detector 309. In one embodiment of the analyzer system, the interrogation space 314 has a volume, as defined herein, of between about between about 0.01 and 500 pL, or between about 0.01 pL and 100 pL, or between about 0.01 pL and 10 pL, or between about 0.01 pL and 1 pL, or between about 0.01 pL and 0.5 pL, or between about 0.02 pL and about 300 pL, or between about 0.02 pL and about 50 pL or between about 0.02 pL and about 5 pL or between about 0.02 pL and about 0.5 pL or between about 0.02 pL and about 2 pL, or between about 0.05 pL and about 50 pL, or between about 0.05 pL and about 5 pL, or between about 0.05 pL and about 0.5 pL, or between about 0.05 pL and about 0.2 pL ,or between about 0.1 pL and about 25 pL. In some embodiments, the interrogation space has a volume between about 0.01 pL and 10 pL. In some embodiments, the interrogation space 314 has a volume between about 0.01 pL and 1 pL. In some embodiments, the interrogation space 314 has a volume between about 0.02 pL and about 5 pL. In some embodiments, the interrogation space 314 has a volume between about 0.02 pL and about 0.5 pL. In some embodiments, the interrogation space 314 has a volume between about 0.05 pL and about 0.2 pL. In some embodiments, the interrogation space 314 has a volume of about 0.1 pL. Other useful interrogation space volumes are as described herein. It should be understood by one skilled in the art that the interrogation space 314 can be selected for maximum performance of the analyzer. Although very small interrogation spaces have been shown to minimize the background noise, large interrogation spaces have the advantage that low concentration samples can be analyzed in a reasonable amount of time. In embodiments in which two interrogation spaces 370 and 371 are used, volumes such as those described herein for a single interrogation space 314 may be used.

[00129] In one embodiment of the present invention, the interrogation spaces are large enough to allow for detection of particles at concentrations ranging from about 1000 femtomolar (fM) to about 1 zeptomolar (zM). In one embodiment of the present invention, the interrogation spaces are large enough to allow for detection of particles at concentrations ranging from about 1000 fM to about 1 attomolar (aM). In one embodiment of the present invention, the interrogation spaces are large enough to allow for detection of particles at concentrations ranging from about 10 fM to about 1 attomolar (aM). In many cases, the large interrogation spaces allow for the detection of particles at concentrations of less than about 1 fM without additional pre-concentration devices or techniques. One skilled in the art will recognize that the most appropriate interrogation space size depends on the

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brightness of the particles to be detected, the level of background signal, and the concentration of the sample to be analyzed.

[00130] The size of the interrogation space 314 can be limited by adjusting the optics of the analyzer. In one embodiment, the diameter of the beam 311 can be adjusted to vary the volume of the interrogation space 314. In another embodiment, the field of view of the detector 309 can be varied. Thus, the source 301 and the detector 309 can be adjusted so that single particles will be illuminated and detected within the interrogation space 314. In another embodiment, the width of aperture 306 (Figure 1A) that determine the field of view of the detector 309 is variable. This configuration allows for altering the interrogation space, in near real time, to compensate for more or less concentrated samples, ensuring a low probability of two or more particles simultaneously being within an interrogation space. Similar alterations for two or more interrogation spaces, 370 and 371, may performed.

[00131] In another embodiment, the interrogation space can be defined through the use of a calibration sample of known concentration that is passed through the capillary flow cell prior to the actual sample being tested. When only one single particle is detected at a time in the calibration sample as the sample is passing through the capillary flow cell, the depth of focus together with the diameter of the beam of the electromagnetic radiation source determines the size of the interrogation space in the capillary flow cell.

[00132] Physical constraints to the interrogation spaces can also be provided by a solid wall. In one embodiment, the wall is one or more of the walls of a flow cell 313 (Figure 2A), when the sample fluid is contained within a capillary. In one embodiment, the cell is made of glass, but other substances transparent to light in the range of about 200 to about 1,000 nm or higher, such as quartz, fused silica, and organic materials such as Teflon, nylon, plastics, such as polyvinylchloride, polystyrene, and polyethylene, or any combination thereof, may be used without departing from the scope of the present invention. Although other cross-sectional shapes (e.g., rectangular, cylindrical) may be used without departing from the scope of the present invention, in one embodiment the capillary flow cell 313 has a square cross section. In another embodiment, the interrogation space may be defined at least in part by a channel (not shown) etched into a chip (not shown). Similar considerations apply to embodiments in which two interrogation spaces are used (370 and 371 in Fig. 2B).

[00133] The interrogation space is bathed in a fluid. In one embodiment, the fluid is aqueous. In other embodiments, the fluid is non-aqueous or a combination of aqueous and non-aqueous fluids. In addition the fluid may contain agents to adjust pH, ionic composition, or sieving agents, such as soluble macroparticles or polymers or gels. It is contemplated that valves or other devices may be present between the interrogation spaces to temporarily disrupt the fluid connection. Interrogation spaces temporarily disrupted are considered to be connected by fluid.

[00134] In another embodiment of the invention, an interrogation space is the single interrogation space present within the flow cell 313 which is constrained by the size of a laminar flow of the sample material within a diluent volume, also called sheath flow. In these and other embodiments, the interrogation space can be defined by sheath flow alone or in combination with the dimensions of the illumination source or the field of view of the detector. Sheath flow can be configured in numerous ways, including: The sample material is the interior material in a concentric laminar flow, with the diluent volume in the exterior; the diluent volume is on one side of the sample volume; the diluent volume is on two sides of the sample material; the diluent volume is on multiple sides of the sample material, but not enclosing the sample material completely; the diluent volume completely surrounds the sample material; the diluent volume completely surrounds the sample material concentrically; the sample material is the interior material in a discontinuous series of drops and the diluent volume completely surrounds each drop of sample material.

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[00135] In some embodiments, single molecule detectors of the invention comprise no more than one interrogation space. In some embodiments, multiple interrogation spaces are used. Multiple interrogation spaces have been previously disclosed and are incorporated by reference from U.S. Pat. App. No. 11/048,660. One skilled in the art will recognize that in some cases the analyzer will contain 2, 3, 4, 5, 6 or more distinct interrogation spaces.

3. Motive Force

[00136] In one embodiment of the analyzer system, the particles are moved through the interrogation space by a motive force. In some embodiments, the motive force for moving particles is pressure. In some embodiments, the pressure is supplied by a pump, and air pressure source, a vacuum source, a centrifuge, or a combination thereof. In some embodiments, the motive force for moving particles is an electrokinetic force. The use of an electrokinetic force as a motive force has been previously disclosed in a prior application and is incorporated by reference from U.S. Pat. App. No. 11/048,660.

[00137] In one embodiment, pressure can be used as a motive force to move particles through the interrogation space of the capillary flow cell. In a further embodiment, pressure is supplied to move the sample by means of a pump. Suitable pumps are known in the art. In one embodiment, pumps manufactured for HPLC applications, such as those made by Scivax, Inc. can be used as a motive force. In other embodiments, pumps manufactured for microfluidics applications can be used when smaller volumes of sample are being pumped. Such pumps are described in U.S. Pat. Nos. 5,094,594, 5,730,187, 6,033,628, and 6,533,553, which discloses devices which can pump fluid volumes in the nanoliter or picoliter range. Preferably all materials within the pump that come into contact with sample are made of highly inert materials, e.g., polyetheretherketone (PEEK), fused silica, or sapphire.

[00138] A motive force is necessary to move the sample through the capillary flow cell to push the sample through the interrogation space for analysis. A motive force is also required to push a flushing sample through the capillary flow cell after the sample has been passed through. A motive force is also required to push the sample back out into a sample recovery vessel, when sample recovery is employed. Standard pumps come in a variety of sizes, and the proper size may be chosen to suit the anticipated sample size and flow requirements. In some embodiments, separate pumps are used for sample analysis and for flushing of the system. The analysis pump may have a capacity of approximately 0.000001 mL to approximately 10 mL, or approximately 0.001 mL to approximately 1 mL, or approximately 0.01 mL to approximately 0.2 mL, or approximately 0.005, 0.01, 0.05, 0.1, or 0.5 mL. Flush pumps may be of larger capacity than analysis pumps. Flush pumps may have a volume of about 0.01 mL to about 20 mL, or about 0.1 mL to about 10 mL, or about 0.1 mL to about 2 mL, or about or about 0.05, 0.1, 0.5, 1, 5, or 10 mL. These pump sizes are illustrative only, and those of skill in the art will appreciate that the pump size may be chosen according to the application, sample size, viscosity of fluid to be pumped, tubing dimensions, rate of flow, temperature, and other factors well known in the art. In some embodiments, pumps of the system are driven by stepper motors, which are easy to control very accurately with a microprocessor.

[00139] In preferred embodiments, the flush and analysis pumps are used in series, with special check valves to control the direction of flow. The plumbing is designed so that when the analysis pump draws up the maximum sample, the sample does not reach the pump itself. This is accomplished by choosing the ID and length of the tubing between the analysis pump and the analysis capillary such that the tubing volume is greater than the stroke volume of the analysis pump.

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4. Detectors

[00140] In one embodiment, light (e.g., light in the ultra-violet, visible or infrared range) emitted by a fluorescent label after exposure to electromagnetic radiation is detected. The detector 309 (Figure 1A), or detectors (364, 365, Figure 1B), is capable of capturing the amplitude and duration of photon bursts from a fluorescent label-moiety complex, and further converting the amplitude and duration of the photon burst to electrical signals. Detection devices such as CCD cameras, video input module cameras, and Streak cameras can be used to produce images with contiguous signals. In another embodiment, devices such as a bolometer, a photodiode, a photodiode array, avalanche photodiodes, and photomultipliers which produce sequential signals may be used. Any combination of the aforementioned detectors may also be used. In one embodiment, avalanche photodiodes are used for detecting photons.

[00141] Using specific optics between an interrogation space 314 (Figure 2A) and its corresponding detector 309 (Figure 1A), several distinct characteristics of the emitted electromagnetic radiation can be detected including: emission wavelength, emission intensity, burst size, burst duration, and fluorescence polarization. In some embodiments, the detector 309 is a photodiode that is used in reverse bias. A photodiode set in reverse bias usually has an extremely high resistance. This resistance is reduced when light of an appropriate frequency shines on the P/N junction. Hence, a reverse biased diode can be used as a detector by monitoring the current running through it. Circuits based on this effect are more sensitive to light than ones based on zero bias.

[00142] In one embodiment of the analyzer system, the photodiode can be an avalanche photodiode, which can be operated with much higher reverse bias than conventional photodiodes, thus allowing each photo-generated carrier to be multiplied by avalanche breakdown, resulting in internal gain within the photodiode, which increases the effective responsiveness (sensitivity) of the device. The choice of photodiode is determined by the energy or emission wavelength emitted by the fluorescently labeled particle. In some embodiments, the photodiode is a silicon photodiode that detects energy in the range of 190-1100 nm; in another embodiment the photodiode is a germanium photodiode that detects energy in the range of 800-1700 nm; in another embodiment the photodiode is an indium gallium arsenide photodiode that detects energy in the range of 800-2600 nm; and in yet other embodiments, the photodiode is a lead sulfide photodiode that detects energy in the range of between less than 1000 nm to 3500 nm. In some embodiments, the avalanche photodiode is a single-photon detector designed to detect energy in the 400 nm to 1100 nm wavelength range. Single photon detectors are commercially available (for example Perkin Elmer, Wellesley, MA).

[00143] In some embodiments the detector is a avalanche photodiode detector that detects energy between 300 nm and 1700 nm. In one embodiment, silicon avalanche photodiodes can be used to detect wavelengths between 300 nm and 1100 nm. Indium gallium arsenic photodiodes can be used to detect wavelengths between 900nm and 1700 nm. In some embodiments, an analyzer system can comprise at least one detector; in other embodiments, the analyzer system can comprise at least two detectors, and each detector can be chosen and configured to detect light energy at a specific wavelength range. For example, two separate detectors can be used to detect particles that have been tagged with different labels, which upon excitation with an EM source, will emit photons with energy in different spectra. In one embodiment, an analyzer system can comprise a first detector that can detect fluorescent energy in the range of 450-700 nm such as that emitted by a green dye (e.g. Alexa 546); and a second detector that can detect fluorescent energy in the range of 620-780 nm such as that emitted by a far-red dye (e.g. Alexa 647). Detectors for detecting fluorescent energy in the range of 400-600 nm such as that emitted by blue dyes (e.g.

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Hoechst 33342), and for detecting energy in the range of 560-700 nm such as that emitted by red dyes (Alexa 546 and Cy3) can also be used.

[00144] A system comprising two or more detectors can be used to detect individual particles that are each tagged with two or more labels that emit light in different spectra. For example, two different detectors can detect an antibody that has been tagged with two different dye labels. Alternatively, an analyzer system comprising two detectors can be used to detect particles of different types, each type being tagged with a different dye molecules, or with a mixture of two or more dye molecules. For example, two different detectors can be used to detect two different types of antibodies that recognize two different proteins, each type being tagged with a different dye label or with a mixture of two or more dye label molecules. By varying the proportion of the two or more dye label molecules, two or more different particle types can be individually detected using two detectors. It is understood that three or more detectors can be used without departing from the scope of the invention.

[00145] It should be understood by one skilled in the art that one or more detectors can be configured at each interrogation space, whether one or more interrogation spaces are defined within a flow cell, and that each detector may be configured to detect any of the characteristics of the emitted electromagnetic radiation listed above. The use of multiple detectors, e.g., for multiple interrogation spaces, has been previously disclosed in a prior application and is incorporated by reference here from U.S. Pat. App. No. 11/048,660. Once a particle is labeled to render it detectable (or if the particle possesses an intrinsic characteristic rendering it detectable), any suitable detection mechanism known in the art may be used without departing from the scope of the present invention, for example a CCD camera, a video input module camera, a Streak camera, a bolometer, a photodiode, a photodiode array, avalanche photodiodes, and photomultipliers producing sequential signals, and combinations thereof. Different characteristics of the electromagnetic radiation may be detected including: emission wavelength, emission intensity, burst size, burst duration, fluorescence polarization, and any combination thereof.

C. Sampling System

[00146] In a further embodiment, the analyzer system may include a sampling system to prepare the sample for introduction into the analyzer system. The sampling system included is capable of automatically sampling a plurality of samples and providing a fluid communication between a sample container and a first interrogation space.

[00147] In some embodiments, the analyzer system of the invention includes a sampling system for introducing an aliquot of a sample into the single particle analyzer for analysis. Any mechanism that can introduce a sample may be used. Samples can be drawn up using either a vacuum suction created by a pump or by pressure applied to the sample that would push liquid into the tube, or by any other mechanism that serves to introduce the sample into the sampling tube. Generally, but not necessarily, the sampling system introduces a sample of known sample volume into the single particle analyzer; in some embodiments where the presence or absence of a particle or particles is detected, precise knowledge of the sample size is not critical. In preferred embodiments the sampling system provides automated sampling for a single sample or a plurality of samples. In embodiments where a sample of known volume is introduced into the system, the sampling system provides a sample for analysis of more than about 0.0001, 0.001, 0.01, 0.1, 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, 1000, 1500, or 2000 μl. In some embodiments the sampling system provides a sample for analysis of less than about 2000, 1000, 500, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.1, 0.01, or 0.001 μl. In some embodiments the sampling system provides a sample for analysis of between about 0.01 and 1500 μl, or about 0.1 and 1000 μl, or about 1 and 500 μl, or about 1 and 100 μl, or about 1 and 50 μl, or about 1 and 20 μl. In some embodiments, the sampling system

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provides a sample for analysis between about 5 µl and 200 µl, or about 5 µl and about 100 µl, or about 5 µl and 50 µl. In some embodiments, the sampling system provides a sample for analysis between about 10 µl and 200 µl, or between about 10 µl and 100 ul, or between about 10 µl and 50 µl. In some embodiments, the sampling system provides a sample for analysis between about 0.5 µl and about 50 µl.

[00148] In some embodiments, the sampling system provides a sample size that can be varied from sample to sample. In these embodiments, the sample size may be any one of the sample sizes described herein, and may be changed with every sample, or with sets of samples, as desired.

[00149] Sample volume accuracy, and sample to sample volume precision of the sampling system, is required for the analysis at hand. In some embodiments, the precision of the sampling volume is determined by the pumps used, typically represented by a CV of less than about 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.1, 0.05, or 0.01% of sample volume. In some embodiments, the sample to sample precision of the sampling system is represented by a CV of less than about 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.1, 0.05, or 0.01%. In some embodiments, the intra-assay precision of the sampling system is represented by a CV of less than about 10, 5, 1, 0.5, or 0.1%. In some embodiments, the intra-assay precision of the sampling system shows a CV of less than about 5%. In some embodiments, the interassay precision of the sampling system is represented by a CV of less than about 10, 5, or 1%. In some embodiments, the interassay precision of the sampling system shows a CV of less than about 5%.

[00150] In some embodiments, the sampling system provides low sample carryover, advantageous in that an additional wash step is not required between samples. Thus, in some embodiments, sample carryover is less than about 1, 0.5, 0.1, 0.05, 0.04, 0.03, 0.02, 0.01, 0.005, or 0.001%. In some embodiments, sample carryover is less than about 0.02%. In some embodiments, sample carryover is less than about 0.01%.

[00151] In some embodiments the sampler provides a sample loop. In these embodiments, multiple samples are drawn into tubing sequentially and each is separated from the others by a "plug" of buffer. The samples typically are read one after the other with no flushing in between. Flushing is done once at the end of the loop. In embodiments where a buffer "plug" is used, the plug may be recovered ejecting the buffer plug into a separate well of a microtiter plate.

[00152] The sampling system may be adapted for use with standard assay equipment, for example, a 96-well microtiter plate, or, preferably, a 384-well plate. In some embodiments the system includes a 96 well plate positioner and a mechanism to dip the sample tube into and out of the wells, e.g., a mechanism providing movement along the X, Y, and Z axes. In some embodiments, the sampling system provides multiple sampling tubes from which samples may be stored and extracted from, when testing is commenced. In some embodiments, all samples from the multiple tubes are analyzed on one detector. In other embodiments, multiple single molecule detectors may be connected to the sample tubes. Samples may be prepared by steps that include operations performed on sample in the wells of the plate prior to sampling by the sampling system, or sample may be prepared within the analyzer system, or some combination of both.

D. **Sample preparation system**

[00153] Sample preparation includes the steps necessary to prepare a raw sample for analysis. These steps can involve, by way of example, one or more steps of: separation steps such as centrifugation, filtration, distillation, chromatography; concentration, cell lysis, alteration of pH, addition of buffer, addition of diluents, addition of reagents, heating or cooling, addition of label, binding of label, cross-linking with illumination, separation of unbound label, inactivation and/or removal of interfering compounds and any other steps necessary for the sample to be prepared for analysis by the single particle analyzer. In some embodiments, blood is treated to separate out

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plasma or serum. Additional labeling, removal of unbound label, and/or dilution steps may also be performed on the serum or plasma sample.

[00154] In some embodiments, the analyzer system includes a sample preparation system that performs some or all of the processes needed to provide a sample ready for analysis by the single particle analyzer. This system may perform any or all of the steps listed above for sample preparation. In some embodiments samples are partially processed by the sample preparation system of the analyzer system. Thus, in some embodiments, a sample may be partially processed outside the analyzer system first. For example, the sample may be centrifuged first. The sample may then be partially processed inside the analyzer by a sample preparation system. Processing inside the analyzer includes labeling the sample, mixing the sample with a buffer and other processing steps that will be known to one in the art. In some embodiments, a blood sample is processed outside the analyzer system to provide a serum or plasma sample, which is introduced into the analyzer system and further processed by a sample preparation system to label the particle or particles of interest and, optionally, to remove unbound label. In other embodiments preparation of the sample can include immunodepletion of the sample to remove particles that are not of interest or to remove particles that can interfere with sample analysis. In yet other embodiments, the sample can be depleted of particles that can interfere with the analysis of the sample. For example, sample preparation can include the depletion of heterophilic antibodies, which are known to interfere with immunoassays that use non-human antibodies to directly or indirectly detect a particle of interest. Similarly, other proteins that interfere with measurements of the particles of interest can be removed from the sample using antibodies that recognize the interfering proteins.

[00155] In some embodiments, the sample can be subjected to solid phase extraction prior to being assayed and analyzed. For example, a serum sample that is assayed for cAMP can first be subjected to solid phase extraction using a c18 column to which it binds. Other proteins such as proteases, lipases and phosphatases are washed from the column, and the cAMP is eluted essentially free of proteins that can degrade or interfere with measurements of cAMP. Solid phase extraction can be used to remove the basic matrix of a sample, which can diminish the sensitivity of the assay. In yet other embodiments, the particles of interest present in a sample may be concentrated by drying or lyophilizing a sample and solubilizing the particles in a smaller volume than that of the original sample. For example, a sample of exhaled breath condensate (EBC) can be dried and resuspended in a small volume of a suitable buffer solution to enhance the detection of the particle of interest.

[00156] In some embodiments the analyzer system provides a sample preparation system that provides complete preparation of the sample to be analyzed on the system, such as complete preparation of a blood sample, a saliva sample, a urine sample, a cerebrospinal fluid sample, a lymph sample, a BAL sample, an exhaled breath condensate sample (EBC), a biopsy sample, a forensic sample, a bioterrorism sample, and the like. In some embodiments the analyzer system provides a sample preparation system that provides some or all of the sample preparation. In some embodiments, the initial sample is a blood sample that is further processed by the analyzer system. In some embodiments, the sample is a serum or plasma sample that is further processed by the analyzer system. The serum or plasma sample may be further processed by, e.g., contacting with a label that binds to a particle or particles of interest; the sample may then be used with or without removal of unbound label.

[00157] In some embodiments, sample preparation is performed, either outside the analysis system or in the sample preparation component of the analysis system, on one or more microtiter plates, such as a 96-well plate. Reservoirs of reagents, buffers, and the like can be in intermittent fluid communication with the wells of the plate by means of tubing or other appropriate structures, as are well-known in the art. Samples may be prepared separately in 96 well plates or tubes. Sample isolation, label binding and, if necessary, label separation steps may be done on one plate.

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In some embodiments, prepared particles are then released from the plate and samples are moved into tubes for sampling into the sample analysis system. In some embodiments, all steps of the preparation of the sample are done on one plate and the analysis system acquires sample directly from the plate. Although this embodiment is described in terms of a 96-well plate, it will be appreciated that any vessel for containing one or more samples and suitable for preparation of sample may be used. For example, standard microtiter plates of 384 or 1536 wells may be used. More generally, in some embodiments, the sample preparation system is capable of holding and preparing more than about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 500, 1000, 5000, or 10,000 samples. In some embodiments, multiple samples may be sampled for analysis in multiple analyzer systems. Thus, in some embodiments, 2 samples, or more than about 2, 3, 4, 5, 7, 10, 15 20, 50, or 100 samples are sampled from the sample preparation system and run in parallel on multiple sample analyzer systems.

[00158] Microfluidics systems may also be used for sample preparation and as sample preparation systems that are part of analyzer systems, especially for samples suspected of containing concentrations of particles high enough that detection requires smaller samples. Principles and techniques of microfluidic manipulation are known in the art. See, e.g., U.S. Patent Nos. 4,979,824; 5,770,029; 5,755,942; 5,746,901; 5,681,751; 5,658,413; 5,653,939; 5,653,859; 5,645,702; 5,605,662; 5,571,410; 5,543,838; 5,480,614, 5,716,825; 5,603,351; 5,858,195; 5,863,801; 5,955,028; 5,989,402; 6,041,515; 6,071,478; 6355,420; 6,495,104; 6,386,219; 6,606,609; 6,802,342;6, 749,734; 6,623,613; 6,554,744; 6,361,671; 6,143,152; 6,132,580; 5,274,240; 6,689,323; 6,783,992; 6,537,437; 6,599,436; 6,811,668 and published PCT patent application no. WO9955461(A1). Samples may be prepared in series or in parallel, for use in a single or multiple analyzer systems.

[00159] Preferably, the sample comprises a buffer. The buffer may be mixed with the sample outside the analyzer system, or it may be provided by the sample preparation mechanism. While any suitable buffer can be used, the preferable buffer has low fluorescence background, is inert to the detectably labeled particle, can maintain the working pH and, in embodiments wherein the motive force is electrokinetic, has suitable ionic strength for electrophoresis. The buffer concentration can be any suitable concentration, such as in the range from about 1 to about 200 mM. Any buffer system may be used as long as it provides for solubility, function, and delectability of the molecules of interest. Preferably, for application using pumping, the buffer is selected from the group consisting of phosphate, glycine, acetate, citrate, acidulate, carbonate/bicarbonate, imidazole, triethanolamine, glycine amide, borate, MES, Bis-Tris, ADA, aces, PIPES, MOPSO, Bis-Tris Propane, BES, MOPS, TES, HEPES, DIPSO, MOBS, TAPSO, Trizma, HEPPSO, POPSO, TEA, EPPS, Tricine, Gly-Gly, Bicine, HEPBS, TAPS, AMPD, TABS, AMPSO, CHES, CAPSO, AMP, CAPS, and CABS. The buffer can also be selected from the group consisting of Gly-Gly, bicine, tricine, 2-morpholine ethanesulfonic acid (MES), 4-morpholine propanesulfonic acid (MOPS) and 2-amino-2-methyl-1-propanol hydrochloride (AMP). A useful buffer is 2 mM Tris/borate at pH 8.1, but Tris/glycine and Tris/HCl are also acceptable. Other buffers are as described herein.

[00160] Buffers useful for electrophoresis are disclosed in a prior application and are incorporated by reference herein from U.S. Pat. App. No. 11/048,660.

E. Sample recovery

[00161] One highly useful feature of embodiments of the analyzers and analysis systems of the invention is that the sample can be analyzed without consuming it. This can be especially important when sample materials are limited. Recovering the sample also allows one to do other analyses or reanalyze it. The advantages of this feature for applications where sample size is limited and/or where the ability to reanalyze the sample is desirable, e.g., forensic, drug screening, and clinical diagnostic applications, will be apparent to those of skill in the art.

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[00162] Thus, in some embodiments, the analyzer system of the invention further provides a sample recovery system for sample recovery after analysis. In these embodiments, the system includes mechanisms and methods by which the sample is drawn into the analyzer, analyzed and then returned, e.g., by the same path, to the sample holder, e.g., the sample tube. Because no sample is destroyed and because it does not enter any of the valves or other tubing, it remains uncontaminated. In addition, because all the materials in the sample path are highly inert, e.g., PEEK, fused silica, or sapphire, there is little contamination from the sample path. The use of the stepper motor controlled pumps (particularly the analysis pump) allows precise control of the volumes drawn up and pushed back out. This allows complete or nearly complete recovery of the sample with little if any dilution by the flush buffer. Thus, in some embodiments, more than about 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 99.9% of the sample is recovered after analysis. In some embodiments, the recovered sample is undiluted. In some embodiments, the recovered sample is diluted less than about 1.5-fold, 1.4-fold, 1.3-fold, 1.2-fold, 1.1-fold, 1.05-fold, 1.01-fold, 1.005-fold, or 1.001-fold.

[00163] For sampling and/or sample recovery, any mechanism for transporting a liquid sample from a sample vessel to the analyzer may be used. In some embodiments the inlet end of the analysis capillary has attached a short length of tubing, e.g., PEEK tubing that can be dipped into a sample container, e.g. a test tube or sample well, or can be held above a waste container. When flushing, to clean the previous sample from the apparatus, this tube is positioned above the waste container to catch the flush waste. When drawing a sample in, the tube is put into the sample well or test tube. Typically the sample is drawn in quickly, and then pushed out slowly while observing particles within the sample. Alternatively, in some embodiments, the sample is drawn in slowly during at least part of the draw-in cycle; the sample may be analyzed while being slowly drawn in. This can be followed by a quick return of the sample and a quick flush. In some embodiments, the sample may be analyzed both on the inward (draw-in) and outward (pull out) cycle, which improves counting statistics, e.g., of small and dilute samples, as well as confirming results, and the like. If it is desired to save the sample, it can be pushed back out into the same sample well it came from, or to another. If saving the sample is not desired, the tubing is positioned over the waste container.

VI. Methods Using Highly Sensitive Analysis of Cardiac Troponin

[00164] The methods of the present invention make possible measurement of cardiac troponin levels at concentrations far lower than previously measured. Although cardiac troponin is an accepted marker for cardiac muscle damage, its usefulness has been limited by the fact that, with current methods of analysis, it is only detectable after considerable damage to cardiac muscle has occurred, because of the lack of sensitivity of current methods. The Joint European Society of Cardiology/American College of Cardiology committee for the Redefinition of Myocardial Infarction has recommended that an increased concentration of cardiac troponin be defined as a measurement exceeding the 99th percentile of the distribution of cardiac troponin concentrations in the reference group, a very low threshold. A total imprecision (CV) at this decision limit of <10% is recommended. However, the analytical imprecision obtained with presently available immunoassays for cardiac troponins is not uniform, mainly at the low concentration range. In addition, the assays that are currently available lack sufficient sensitivity for detecting troponin levels in nonclinical (normal) subjects, and a true baseline or a level of troponin defined in a normal population, has not been defined. The analyzer systems of the invention have been shown to be able to consistently detect levels of cTnI at concentrations of less than 10 pg/ml with a total imprecision of less than 10% (See Examples). Thus, the invention provides methods for diagnosis, prognosis, or methods of treatment based on the highly sensitive detection of cardiac troponin in individuals.

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[00165] In some embodiments, the invention provides a method for determining a diagnosis, prognosis, or method of treatment in an individual by i) determining a concentration of cardiac troponin in a sample or determining the concentrations of cardiac troponin in a series of samples from the individual, where the concentration is determined by a cardiac troponin assay with a limit of detection for the cardiac troponin in said sample of less than about 50, 40, 30, 10, 5, 4,3,2 or 1 pg/ml, e.g., less than about 20 pg/ml; and ii) determining a diagnosis, prognosis, or method of treatment in said individual, based on the concentration in the sample, or on the concentrations in the series of samples. The method of determining the concentration of cardiac troponin includes any suitable method with the requisite sensitivity, e.g., the methods descried herein. In some embodiments, the methods utilize a method of determining a concentration of cardiac troponin in the sample where the method comprises detecting single molecules of troponin, or complexes or fragments thereof.

[00166] In some embodiments, the threshold concentration of troponin is determined by analyzing samples, e.g., blood, serum, or plasma samples, from an apparently healthy population for cardiac troponin, e.g., cardiac troponin I, and determining the level at which 80, 90, 95, 96, 97, 98, 99, 99.5, or 99.9% of the population fall below that level (concentration). This value is the threshold value. In some embodiments, the threshold value is set at the 99th percentile. In some embodiments, the analyzing is performed using a method with a level of detection for the cardiac troponin of less than about 50, 20, 10, 5, or 1 pg/ml, e.g., less than about 5 pg/ml.

[00167] In some embodiments, the invention provides a method for determining a diagnosis, prognosis, or method of treatment in an individual by comparing a value for a concentration of cardiac troponin in a sample from the individual with a normal value or a range of normal values for cardiac troponin, where the normal vale or range of normal values is determined by a cardiac troponin assay with a limit of detection for the cardiac troponin in said sample of less than about 50, 40, 30, 10, 5, 4,3,2 or 1 pg/ml, e.g., less than about 20 pg/ml; and ii) determining a diagnosis, prognosis, or method of treatment in said individual, based on comparison.

[00168] In some embodiments, the cardiac troponin is cardiac troponin I or cardiac troponin T. In some embodiments, the cardiac troponin is cardiac troponin T. In some embodiments, the cardiac troponin is cardiac troponin I. The method may use total troponin, e.g., total cTnI, or cTnT, or total cTnI + cTnT, as described herein, in determining a diagnosis, prognosis, or method of treatment. In some embodiments, the method may use the concentration of free, complexed, or fragments of the cardiac troponin, or a comparison of these (e.g., a ratio), to determine a diagnosis, prognosis, or method of treatment.

A. Samples

[00169] The sample or series of samples may be any suitable sample; in some embodiments, the sample(s) will be blood, serum, or plasma. In some embodiments, the sample or series of samples are serum samples. The individual may be an animal, e.g., mammal, e.g., human.

[00170] A single sample may be taken, or a series of samples may be taken. If a series of samples is taken, they may be taken at any suitable interval, e.g., intervals of minutes, hours, days, weeks, months, or years. In an acute clinical setting, typically a series of samples will be taken over the course of hours and days, with the samples separated by a matter of hours. When an individual is followed for longer periods, sample intervals may be months or years. Diagnosis, prognosis, or method of treatment may be determined from a single sample, or from one or more of a series of samples, or from changes in the series of samples, e.g., an increase in concentration at a certain rate may indicate a severe condition whereas increase at a slower rate or no increase may indicate a relatively benign or less serious condition. The rate of change may be measured over the course of hours, days, weeks, months, or years. Rate of change in a given individual may, in some cases, be more relevant than an absolute value. In a acute

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setting, an extremely rapid rate of change, e.g., a "spike", can indicate an imminent, ongoing, or recent cardiac event. In other settings, a rise in values over a period of days, weeks, months or years in an individual can indicate ongoing and worsening cardiac damage, e.g., cardiac damage due to a cardiac condition (e.g., cardiac hypertrophy or congestive heart failure) or cardiac damage due to a non-cardiac condition (e.g., toxicity from drug exposure).

[00171] In some embodiments, at least one sample is taken during or after a cardiac stress test. E.g., a sample may be taken before the stress test, and one or more samples taken during the test. Deviations in cardiac troponin levels between the sample before the test and the sample(s) taken during the test can provide diagnostic or prognostic information, e.g., indicate the likelihood of coronary artery disease or other pathology associated with the cardiac muscle. Other comparisons may be done as well, such as comparisons of any of the samples to normal or threshold levels, or determination of a rate of change in the concentration of cardiac troponin in the samples, all of which may yield useful information regarding cardiac and cardiovascular health, as well as other conditions as described herein.

[00172] In some embodiments, at least one sample is taken at or near the time the individual presents to a health professional with one or more symptoms indicative of a condition that may involve cardiac damage. Settings in which an individual may present to a health care professional include, but are not limited to ambulatory, urgent care, critical care, intensive care, monitoring unit, inpatient, outpatient, physician office, medical clinic, emergency response setting, including an ambulance, and health screening settings. In some embodiments, one or more samples are taken from the individual and are assayed for cardiac troponin locally, i.e., at or near the setting at which the samples are taken. For example, an individual who presents at a hospital may have one or more samples takent that are assayed for cardiac troponin within the hospital. For example, an individual who presents at a hospital may have one or more samples takent that are assayed for cardiac troponin within the hospital. In some embodiments, one or more samples are taken from the individual and are assayed for cardiac troponin in a CLIA laboratory. In some embodiments, the individual displays one or more symptoms consistent with acute coronary syndrome. In some embodiments, the individual displays one or more symptoms consistent with AMI. Such symptoms include, but are not limited to, chest pain, chest pressure, arm pain, abnormal EKG, abnormal enzyme levels, and shortness of breath.

B. Determination of diagnosis, prognosis, or method of treatment

[00173] In some embodiments, step ii) includes comparing said concentration or series of concentrations to a normal value for said concentration, comparing said concentration or series of concentrations to a predetermined threshold level, comparing said concentration or series of concentrations to a baseline value, or determining a rate of change of concentration for said series of concentrations.

[00174] In some embodiments, step ii) comprises comparing said concentration of troponin in said sample with a predetermined threshold concentration, and determining a diagnosis, prognosis, or method of treatment if the sample concentration is greater than the threshold level. The threshold concentration can be determined by, e.g., determining the 99th percentile concentration of troponin in a group of individuals, and setting said threshold concentration at said 99th percentile concentration. An example of this is given in Examples.

[00175] Normal values, threshold values, rates of change, ratios of values, and other useful diagnostic and prognostic indicators may be established by methods well-known in the art. For example, these values may be determined by comparing samples from a case population and a control population, where the case population exhibits the biological state for which diagnosis, prognosis, or method of treatment is desired, and the control population does not exhibit the biological state. In some embodiments, a longitudinal study may be done, e.g., the case population may be a subset of the control population that, over time, exhibits the biological state. It will be

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appreciated that data from a plurality of studies may be used to determine a consensus value or range of values for normal, and for prognostic or diagnostic levels.

[00176] In developing diagnostic or prognostic test, data for one or more potential markers may be obtained from a group of subjects. The group of subjects is divided into at least two sets, and preferably the first set and the second set each have an approximately equal number of subjects. The first set includes subjects who have been confirmed as having a disease or, more generally, being in a first condition state. For example, this first set of patients may be those that have recently had a disease incidence, or may be those having a specific type of disease, such as AMI. The confirmation of the condition state may be made through a more rigorous and/or expensive testing such as MRI or CT. Hereinafter, subjects in this first set will be referred to as "diseased". The second set of subjects is simply those who do not fall within the first set. Subjects in this second set may be "non-diseased;" that is, normal subjects. Alternatively, subjects in this second set may be selected to exhibit one symptom or a constellation of symptoms that mimic those symptoms exhibited by the "diseased" subjects. In still another alternative, this second set may represent those at a different time point from disease incidence. Preferably, data for the same set of markers is available for each patient. This set of markers may include all candidate markers which may be suspected as being relevant to the detection of a particular disease or condition. Actual known relevance is not required. Embodiments of the compositions, methods and systems described herein may be used to determine which of the candidate markers are most relevant to the diagnosis of the disease or condition. The levels of each marker in the two sets of subjects may be distributed across a broad range, e.g., as a Gaussian distribution. However, no distribution fit is required.

1. Acute myocardial infarct

[00177] The methods of the invention are especially useful in diagnosis, prognosis, and/or treatment selection in patients suspected of acute myocardial infarct (AMI). Single or serial cardiac troponin measurements in patients suspected of AMI provide incremental prognostic information that improves the prognosis and indicates appropriate and early therapeutic intervention to minimize the risk of adverse outcomes.

[00178] Thus, the invention provides a method of diagnosing, predicting, and/or preventing or treating AMI in an individual by assaying a sample from the individual, e.g., a blood sample, plasma sample, and/or serum sample, for cardiac troponin, e.g., cTnI, and detecting a concentration of cardiac troponin in the sample at a limit of detection of less than about 50, 40, 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 pg/ml, e.g., less than about 20 pg/ml, wherein the concentration of cardiac troponin in the sample indicates or predicts AMI. The cardiac troponin may be cTnI or cTnT, and may be total troponin or a measure of a particular form, e.g., free, complexed, or fragment; in some embodiments, a ratio of one or more forms of the troponin is used, as described herein. In some embodiments, total cTnI is measured in the sample or series of samples. In some embodiments, total cTnT is measured in the sample or series of samples. In some embodiments, total cTnI + cTnT is measured in the sample or series of samples. In some embodiments, the cardiac troponin level is determined at or near the time the individual presents to a health professional with symptoms indicative of AMI. Such symptoms include, but are not limited to, chest pain, chest pressure, arm pain, abnormal EKG, abnormal enzyme levels, and shortness of breath.

[00179] In some embodiments, a series of measurements is taken, and a spike in the cardiac troponin concentration in the samples indicates, predicts, or provides a basis for prognosis of AMI. In some embodiments, a spike of over 50%, over 100%, over 150%, over 200%, over 250%, over 300%, over 400%, or over 500% of baseline indicates, predicts, or provides a basis for prognosis of AMI. In some embodiments, a cardiac troponin level of over about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 pg/ml in a single sample indicates, predicts, or provides a

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basis for prognosis of AMI, regardless of baseline levels, if obtained. In some embodiments, a cardiac troponin level of about 1-10, or about 5-15, or about 10-50, about 10-200, about 10-100, or about 10-40, or about 15-50, or about 15-40, or about 20-200, about 20-150, about 20-100, about 20-50, or about 20-40, or about 20-30 pg/ml indicates, predicts, or provides a basis for prognosis of AMI.

[00180] In some embodiments diagnosis or prognosis includes stratification for the individual, based on cardiac troponin concentration in the sample or series of samples. Such stratification may be based on the concentration of cardiac troponin in single samples, presence of spikes and/or size of spikes from baseline in a series of samples, ratios of different forms of the cardiac troponin, absolute values for different forms of cardiac troponin, rate of change in concentration for the cardiac troponin or for one or more forms of the cardiac troponin in a series of samples, change in ratios of different forms of cardiac troponin over time in a series of samples, and any other information based at least in part on cardiac troponin concentration in the sample or series of samples. Stratification may be based on values obtained from populations of normal and diseased subjects, as described herein. Appropriate treatment may also be determined based on the stratification of the individual.

[00181] In some embodiments, concentration of cardiac troponin is determined in combination with one or more other markers, e.g., markers of myocardial ischemia, myocardial infarct or markers of stroke, and the concentrations of each marker are considered in determining the diagnosis, prognosis, or method of treatment. Other clinical indications typically will also be taken into account, e.g., EKG, symptoms, history, and the like, as will be apparent to those of skill in the art. Appropriate algorithms for diagnosis, prognosis, or treatment may be constructed based on the combinations of such markers and clinical indications in combination with troponin levels.

[00182] Markers useful in combination with cardiac troponin in the methods of the invention include but are not limited to creatine kinase (CK) and its myocardial fraction CK myocardial band (MB), aspartate aminotransferase, lactate dehydrogenase (LDH), α-hydroxybutyrate dehaydrogenase, myoglobin, glutamate oxaloacetate transaminase, glycogen phosphorylase BB, unbound free fatty acids, heart fatty acid binding protein (H-FABP), ischemia-modified albumin, myosin light chain 1, myosin light chain 2. Markers of inflammation and plaque instability useful in combination with cardiac troponin in the methods of the invention include but are not limited to C-reactive protein, white blood cell count, soluble CD40 ligand, myeloperoxidase, monocyte chemoattractant protein-1, whole blood choline, and pregnancy-associated plasma protein A. Other markers of inflammation may be detected, and include combinations of Il-8, IL-1β, IL6, IL10, TNF, and IL-12p70, as well as other cytokines or markers that will be apparent to those of skill in the art.

[00183] In some embodiments, cardiac troponin, e.g., cTnI, is measured together, e.g., in the same sample, or in samples from the same individual taken at or near the same time, with a marker selected from the group consisting of creatine kinase (CK) and its myocardial fraction CK myocardial band (MB), aspartate aminotransferase, lactate dehydrogenase (LDH), α-hydroxybutyrate dehaydrogenase, myoglobin, glutamate oxaloacetate transaminase, glycogen phosphorylase BB, unbound free fatty acids, heart fatty acid binding protein (H-FABP), ischemia-modified albumin, myosin light chain 1, and myosin light chain 2. In some embodiments cardiac troponin, e.g., cTnI, is measured together with CK-MB, e.g., in the same sample, or in samples from the same individual taken at or near the same time.

[00184] In some embodiments, cardiac troponin, alone or in combination with other markers or clinical signs, measured as described herein, is used to determine reinfarction. In some embodiments, cardiac troponin, alone or in combination with other markers or clinical signs, measured as described herein, is used to determine characteristics of an infarct, e.g., size, or duration since infarct. In the latter case, fragments of troponin produced by proteolyis in

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the blood may be compared to total troponin; the greater the proportion of fragments, the more time has elapsed since infarct.

2. Conditions other than AMI

[00185] The methods of the invention also include methods of diagnosis, prognosis, and treatment based on concentration of cardiac troponin in a sample that are useful in conditions other than AMI.

[00186] Many conditions include potential or actual cardiac damage, and the ability to measure cardiac troponin at the levels described herein allow for early detection of such damage and early intervention. Knowledge of the concentration of cardiac troponin as measured by the methods and compositions of the invention is useful in diagnosis, prognosis, and determination of treatment for such conditions. Conditions include percutaneous coronary interventions, cardiac surgery, heart failure, acute rheumatic fever, amyloidosis, cardiac trauma (including contusion, ablation, pacing, firing, cardioversion, catheterization and cardiac surgery), reperfusion injury, cardiotoxycity from cancer therapy, congestive heart failure, end-stage renal failure, glycogen storage disease type II (Pompe's disease), heart transplantation, haeomoglobinopathy with transfusion haemosiderosis, hypertension, including gestational hypertension, hypotension, often with arrhythmias, hypothyroidism, myocarditis, pericarditis, post-operative non-cardiac surgery, pulmonary embolism, and sepsis.

[00187] In these embodiments, the troponin levels may be determined concomitantly with the levels of marker(s) that are specific for the non-cardiac disease, or other symptoms or clinical signs of the disease; the marker(s) concentration and/or information regarding other symptoms or clinical signs is combined with information regarding cardiac troponin concentrations, determined as described herein, to determine a diagnosis, prognosis, and/or method of treatment. For example, embodiments of the invention may employ, in addition to determination of cardiac troponin concentration, determination of the concentration of one or more of the polypeptides referenced above, or other protein markers useful in diagnosis, prognosis, or differentiation of disease. In some embodiments, a panel of markers for the disease is provided, where the panel includes cardiac troponin concentration, as described herein, and at least on other marker for the disease. The panel may include, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or more or individual markers, which including one or more cardiac troponins, e.g., total cTnI. The analysis of a single marker or subsets of marker can be carried out by one skilled in the art to optimize clinical sensitivity or specificity in various clinical settings. These include, but are not limited to ambulatory, urgent care, critical care, intensive care, monitoring unit, inpatient, outpatient, physician office, medical clinic, and health screening settings. Furthermore, one skilled in the art can use a single marker or a subset of markers in combination with an adjustment of the diagnostic threshold in each of the aforementioned settings to optimize clinical sensitivity and specificity.

[00188] a. Cardiac toxicity The compositions and methods of the invention are especially useful in determining and monitoring cardiac toxicity that results from a treatment, e.g., cardiac toxicity of drug treatment. Thus, for example, the invention provides a method of assessing cardiac toxicity of a treatment by measuring cardiac troponin in an individual by i) determining a concentration of cardiac troponin in a sample or determining the concentrations of cardiac troponin in a series of samples from the individual, where at least one of the samples is taken from the individual during or after a time when the individual is receiving the treatment, where the concentration or concentrations is determined by a cardiac troponin assay with a limit of detection for the cardiac troponin in said sample of less than about 50, 40, 30, 10, 5, 4,3,2 or 1 pg/ml, e.g., less than about 20 pg/ml; and ii) assessing the degree of cardiac toxicity of the treatment based on said concentration or concentrations. In some embodiments, the treatment is a drug treatment. In some embodiments, the treatment is a non-drug treatment. The

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method of determining the concentration of cardiac troponin includes any suitable method with the requisite sensitivity, e.g., the methods described herein. In some embodiments, the methods utilize a method of determining a concentration of cardiac troponin in the sample where the method comprises detecting single molecules of troponin, or complexes or fragments thereof.

[00189] Especially useful are methods of determination of cardiac toxicity utilizing the cross-reacting antibodies described herein, i.e., antibodies that react with troponin from at least two species, such as humans and another species such as rat, dog, mouse, or monkey. Such antibodies may be used in animal studies of drug toxicity, where the individual for which toxicity is assessed is, e.g., a mammal, such as a rat, mouse, dog, monkey, or other animal used in such studies. Toxicity in various species may be directly compared when the antibody used in the assay is the same antibody, thus reducing variability.

[00190] It will be appreciated that the compositions and methods of the invention may be used in conjunction with specific drugs whose side effects include cardiotoxicity in order to monitor the cardiac toxicity. Thus, the invention provides methods of monitoring cardiac toxicity in an individual who is receiving a drug that is known to cause cardiac toxicity by determining the concentration of cardiac troponin in one or more samples obtained from the individual, where the concentration or concentrations is determined by a cardiac troponin assay with a limit of detection for the cardiac troponin in said sample or samples of less than about 50, 40, 30, 10, 5, 4,3,2 or 1 pg/ml, e.g., less than about 20 pg/ml; and ii) assessing the degree of cardiac toxicity of the drug treatment based on said concentration or concentrations. In some embodiments the method further includes a step iii) determining whether or not to continue the drug treatment based on the assessment of step ii). Drugs whose side effects include cardiac toxicity are well-known in the art.

C. Business Methods

[00191] The present invention relates to systems and methods (including business methods) for establishing markers of cardiac troponin that can be used for diagnosing, prognosing, or determining a method of treatment of a biological state or a condition in an organism, preparing diagnostics based on such markers, and commercializing/marketing diagnostics and services utilizing such diagnostics. The biological state may be acute myocardial infarct, or cardiac damage due to drug toxictiy, or non-AMI states as described herein.

[00192] In one embodiment, the business methods herein comprise: establishing one or more cardiac troponin markers using a method comprising: establishing a range of concentrations for said marker or markers in biological samples obtained from a first population by measuring the concentrations of the marker or markers in the biological samples by detecting single molecules of the marker or markers at a level of detection of less than about 50, 20, 10, 5, or 1 pg/ml; and commercializing the one or more markers established in the above step, e.g., in a diagnostic product. The diagnostic product herein can include one or more antibodies that specifically binds to the cardiac troponin marker and a fluorescent moietythat is capable of emitting an average of at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

[00193] In one embodiment, the business methods herein comprise: establishing a range of normal values for a cardiac troponin marker using a system comprising: establishing a range of concentrations for said cardiac troponin marker in biological samples obtained from a first population by measuring the concentrations of the marker the biological samples by detecting single molecules of the marker at a level of detection less thatn about 50, 20, 10, 5, or 1 pg/ml; and providing a diagnostic service to determine if an organism has or does not have a state or condition

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of interest, e.g., AMI, cardiac toxicity due to drug treatment, or a non-AMI condition. A diagnostic service herein may be provided by a CLIA approved laboratory that is licensed under the business or the business itself. The diagnostic services herein can be provided directly to a health care provider, a health care insurer, or a patient. Thus the business methods herein can make revenue from selling e.g., diagnostic services or diagnostic products.

[00194] The business methods herein also contemplate providing diagnostic services to, for example, health care providers, insurers, patients, etc. The business herein can provide diagnostic services by either contracting out with a service lab or setting up a service lab (under Clinical Laboratory Improvement Amendment (CLIA) or other regulatory approval). Such service lab can then carry out the methods disclosed herein to identify if a cardiac troponin marker is within a sample.

VII. Compositions

[00195] The invention provides compositions useful in the detection and quantitation of cardiac troponin. Compositions include binding partners to cardiac troponin that are labeled with suitable labels for detection by the methods of the invention, pairs of binding partners in which one or both of the binding partners are labeled with suitable labels for detection by the methods of the invention, solid supports to which capture binding partners are attached, in some embodiments also with detection binding partners.

[00196] Exemplary embodiments include a composition for the detection of cardiac troponin that includes a binding partner to the cardiac troponin attached to a fluorescent moiety, where the fluorescent moiety is capable of emitting an average of at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the binding partner includes an antibody to the cardiac troponin. In some embodiments, the antibody is a polyclonal antibody. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the antibody is a cross-reacting antibody, e.g., an antibody that cross-reacts with cardiac troponin from at least two species, e.g., at least two species selected from the group consisting of human, monkey, dog, and mouse. In some embodiments the antibody cross-reacts with cardiac troponins from all of human, monkey, dog, and mouse. In some embodiments, the cardiac troponin is selected from the group consisting of cTnI and cTnT. In some embodiments, the cardiac troponin is cTnI. In some embodiments, cardiac troponin is cTnT. The antibody may specific to a specific region of the troponin molecule, e.g., specific to a region comprising amino acids 27-41 of cardiac troponin I. The fluorescent moiety may contain one or more molecules that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. group. The label composition may include a fluorescent moiety that includes one or more dye molecules selected from the group consisting of AlexaFluor 488, 532, 647, 700, or 750. The label composition may include a fluorescent moiety that includes one or more dye molecules selected from the group consisting of AlexaFluor 488, 532, 700, or 750. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 488. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 555. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 610. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 647. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 680. The label composition may

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include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 700. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 750.e

[00197] In some embodiments, the invention provides a composition that includes a set of standards for the determination of a concentration of a cardiac troponin, wherein at least one of the standards is at a concentration of cardiac troponin less than about 20, 15, 10, 5, 4, 3, 2, or 1 pg/ml. In some embodiments, the invention provides a composition that includes a set of standards for the determination of a concentration of a cardiac troponin, wherein at least one of the standards is at a concentration of cardiac troponin less than about 20 pg/ml. In some embodiments, the invention provides a composition that includes a set of standards for the determination of a concentration of a cardiac troponin, wherein at least one of the standards is at a concentration of cardiac troponin less than about 10 pg/ml. In some embodiments, the invention provides a composition that includes a set of standards for the determination of a concentration of a cardiac troponin, wherein at least one of the standards is at a concentration of cardiac troponin less than about 5 pg/ml. In some embodiments, the invention provides a composition that includes a set of standards for the determination of a concentration of a cardiac troponin, wherein at least one of the standards is at a concentration of cardiac troponin less than about 1 pg/ml.

[00198] Other compositions of the invention are as described herein.

VIII. Kits

[00199] The invention further provides kits. Kits of the invention include one or more compositions useful for the sensitive detection of cardiac troponin, as described herein, in suitable packaging. In some embodiments kits of the invention provide labels, e.g., binding partner such as an antibody that is specific for cardiac troponin, where the binding partner is attached to a fluorescent moiety. In some embodiments kits of the invention provide binding partner pairs, e.g., antibody pairs, that are specific for cardiac troponin, where at least one of the binding partners is a label for a cardiac troponin, as described herein. In some embodiments, the binding partners, e.g., antibodies, are provided in separate containers. In some embodiments, the binding partners, e.g., antibodies, are provided in the same container. In some embodiments, one of the binding partners, e.g., antibody, is immobilized on a solid support, e.g., a microtiter plate or a paramagnetic bead. In some of these embodiments, the other binding partner, e.g., antibody, is labeled with a fluorescent moiety as described herein.

[00200] Binding partners, e.g., antibodies, solid supports, and fluorescent labels for components of the kits may be any suitable such components as described herein.

[00201] The kits may additionally include reagents useful in the methods of the invention, e.g., buffers and other reagents used in binding reactions, washes, buffers or other reagents for preconditioning the instrument on which assays will be run, and elution buffers or other reagents for running samples through the instrument.

[00202] Kits may include one or more standards, e.g., standards for use in the assays of the invention, such as standards of highly purified, e.g., recombinant, human cTnI or human cTnT, or various fragments, complexes, and the like, thereof. Kits may further include instructions.

EXAMPLES

[00203] The following examples are offered by way of illustration and not by way of limiting the remaining disclosure.

[00204] Unless otherwise specified, processing samples in the Examples were analyzed in a single molecule detector (SMD) as described herein, with the following parameters: Laser: continuous wave gallium arsenite diode laser of wavelength 639 nm (Blue Sky Research, Milpitas, CA), focused to a spot size of approximately 2 microns (interrogation space of .004 pL as defined herein); flow rate = 5 microliter/min through a fused silica capillary of

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100 micron square ID and 300 micron square OD; non-confocal arrangement of lenses (see, e.g., Fig. 1A); focussing lens of 0.8 numerical aperture (Olympus); silicon avalanche photodiode detector (Perkin Elmer, Waltham, MA).

Example 1. Sandwich assays for biomarkers: cardiac Troponin I (cTnI)

[00205] The assay: The purpose of this assay was to detect the presence of cardiac Troponin I (cTNI) in human serum. The assay format was a two-step sandwich immunoassay based on a mouse monoclonal capture antibody and a goat polyconal detection antibody. Ten microliters of sample were required. The working range of the assay is 0 – 900 pg/ml with a typical analytical limit of detection of 1 – 3 pg/ml. The assay required about four hours of bench time to complete.

[00206] Materials: the following materials were used in the procedure described below: Assay plate: Nunc Maxisorp, product 464718, 384 well, clear, passively coated with monoclonal antibody, BiosPacific A34440228P Lot # A0316 (5μg/ml in 0.05 M sodium carbonate pH 9.6, overnight at room temperature); blocked with 5% sucrose, 1% BSA in PBS, and stored at 4oC. For the standard curve, Human cardiac Troponin I (BiosPacific Cat # J34000352) was used. The diluent for the standard concentrations was human serum that was immonodepleted of endogenous cTNI, aliquoted and stored at - 20oC. Dilution of the standards was done in a 96 well, conical, polypropylene, (Nunc product # 249944). The following buffers and solutions were used: (a) assay buffer: BBS with 1% BSA and 0.1% TritonX-100; (b) passive blocking solution in assay buffer containing 2mg/ml mouse IgG , (Equitech Bio); 2mg/ml goat IgG, (Equitech Bio); and 2mg/ml MAK33 poly, Roche# 11939661; (c) detection Antibody (Ab): Goat Polyclonal antibody affinity purified to Peptide 3, (BiosPacific G129C), which was label with a fluorescent dye AlexaFluor 647, and stored at 4oC; detection antibody diluent: 50% assay buffer, 50% passive blocking solution; wash buffer: borate buffer saline Triton Buffer (BBST) (1.0 M borate, 15.0 M sodium chloride, 10% Triton X-100, pH 8.3); elution buffer: BBS with 4M urea, 0.02% Triton X-100 and 0.001% BSA.

[00207] Preparation of AlexaFluor 647 labeled antibodies: the detection antibody G-129-C was conjugated to AlexaFluor 647 by first dissolving 100ug of G-129-C in 400uL of the coupling buffer (0.1M NaHCO3). The antibody solution was then concentrated to 50ul by transferring the solution into YM-30 filter and subjecting the solution and filter to centrifugation. The YM-30 filter and antibody was then washed three times by adding 400ul of the coupling buffer. The antibody was recovered by adding 50□l to the filter, inverting the filter, and centrifuging for 1 minute at 5,000 x g. The resulting antibody solution was 1-2ug/ul. AlexaFluor 647 NHS ester was reconstituted by adding 20ul DMSO to one vial of AlexaFluor 647, this solution was stored at -20oC for up to one month. 3ul of AlexaFluor 647 stock solution was added to the antibody solution, which was then mixed and incubated in the dark for one hour. After the one hour, 7.5ul 1M tris was added to the antibody AlexaFluor 647 solution and mixed. The solution was ultrafiltered with YM-30 to remove low molecular weight components. The volume of the retentate, which contained the antibody conjugated to AlexaFluor 647, was adjusted to 200-400□l by adding PBS. 3ul 10% NaN3 was added to the solution, the resulting solution was transferred to an Ultrafree 0.22 centrifugal unit and spun for 2 minutes at 12,000 x g. The filtrate containing the conjugated antibody was collected and used in the assays.

[00208] Procedure: cTnI standard and sample preparation and analysis:

[00209] The standard curve was prepared as follows: working standards were prepared (0 – 900 pg/ml) by serial dilutions of the stock of cTnI into standard diluent or to achieve a range of cTnI concentrations of between 1.2 pg/ml – 4.3 μg/ml.

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[00210] 10 µl passive blocking solution and 10 µl of standard or of sample were added to each well. Standards were run in quadruplicate. The plate was sealed with Axyseal sealing film, centrifuged for 1min at 3000 RPM, and incubated for 2 hours at 25oC with shaking. The plate was washed five times, and centrifuged until rotor reached 3000 RPM in an inverted position over a paper towel. A 1nM working dilution of detection antibody was prepared, and 20 µl detection antibody were added to each well. The plate was sealed and centrifuged, and the assay incubated for 1hour at 25oC with shaking. 30 µl elution buffer were added per well, the plate was sealed and the assay incubated for ½ hour at 25oC. The plate was either stored for up to 48 hours at 4oC prior to analysis, or the sample was analyzed immediately.

[00211] For analysis, 20 µl per well were acquired at 40 µl/minute, and 5 µl were analyzed at 5 µl/minute. The data were analyzed based on a threshold of 4 sigma. Raw signal versus concentration of the standards was plotted. A linear fit was performed for the low concentration range, and a non- linear fit was performed for the full standard curve. The limit of detection (LoD) was calculated as LOD = (3 x standard deviation of zeros) / slope of linear fit. The concentrations of the samples were determined from the equation (linear or non-linear) appropriate for the sample signal.

[00212] An aliquot was pumped into the analyzer. Individually-labeled antibodies were measured during capillary flow by setting the interrogation volume such that the emission of only 1 fluorescent label was detected in a defined space following laser excitation. With each signal representing a digital event, this configuration enables extremely high analytical sensitivities. Total fluorescent signal is determined as a sum of the individual digital events. Each molecule counted is a positive data point with hundreds to thousands of DMC events/sample. The limit of detection the cTnI assay of the invention was determined by the mean +3 SD method.

[00213] Results: Data for a typical cTnI standard curve measured in quadruplicate using the assay protocol is shown in Table 2.

Table 2

Standard Curve for cTnI

cTnI (pg/ml)	Signal	Standard Deviation	% CV
0	233	25	10.8
1.5625	346	31	8.9
3.125	463	35	7.5
6.25	695	39	5.6
12.5	1137	61	5.3
25	1988	139	7.0
50	3654	174	4.8
100	5493	350	6.4
200	8264	267	3.2
400	9702	149	1.5
800	9976	50	0.5

[00214] The sensitivity of the analyzer system was tested in 15 runs and was found routinely to detect sub femtomol/l (fM) levels of calibrator, as shown by the data in Table 3. The precision was 10% at 4 and 12 pg/ml cTnI.

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Table 3

Instrument Sensitivity

Calibrator (fM)	Signal counts	CV
0	11	
12	302	9
60	1341	8
300	4784	7

[00215] Linearized standard curve for the range concentrations of cTnI are shown in **Figure 5.**

[00216] The analytical limit of detection (LoD) was determined across 15 sequential assays. The LoD was the mean of the 0 std + 3 SD (n=4) intra-assay determinations. The average LoD was 1.7 pg/ml (range 0.4-2.8 pg/ml).

[00217] The recovery of the sample was determined by analyzing samples of serum that had been immunodepleted of cTnI and spiked with known amounts of cTnI. Table 4 shows the data for sample recovery by the system analyzed over 3 days.

Table 4

Sample Recovery

Spike (pg/ml)	Recovery (mean)	Standard Deviation	% CV
5	5.7	0.9	16
15	13.7	0.2	2
45	43	0.6	2
135	151	6.2	4

[00218] The linearity of the assay was determined in pooled human serum that was spiked with cTnI and diluted with standard diluent. The results in 56 show the dilutions and % of the signal expected for the corresponding dilution.

Table 5

Assay Linearity

Serum Dilution	% of expected
1:2	79
1:4	87
1:8	96

[00219] These data show that the analyzer system of the invention allows for performing highly sensitive laser-induced immunoassay for sub-femtomolar concentrations of cTnI.

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Example 2: Sandwich Bead-based Assays for TnI

[00220] The assays described above use the same microtiter plate format where the plastic surface is used to immobilize target molecules. The single particle analyzer system also is compatible with assays done in solution using microparticles or beads to achieve separation of bound from unbound entities.

[00221] Materials: MyOne Streptavidin C1 microparticles (MPs) are obtained from Dynal (650.01-03, 10 mg/ml stock). Buffers use in the assay include: 10X borate buffer saline Triton Buffer (BBST) (1.0 M borate, 15.0 M sodium chloride, 10% Triton X-100, pH 8.3); assay buffer (2 mg/ml normal goat IgG, 2 mg/ml normal mouse IgG, and 0.2 mg/ml MAB-33-IgG-Polymer in 0.1 M Tris (pH 8.1), 0.025 M EDTA, 0.15 M NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.1% NaN3, stored at 4C); .and elution buffer (BBS with 4 M urea, 0.02% Triton X-100, and 0.001% BSA, stored at 2-8C). Antibodies used in the sandwich bead-based assay include: Bio-Ab (A34650228P (BiosPacific) with 1-2 biotins per IgG) and Det-Ab (G-129-C (BiosPacific) conjugated to A647, 2-4 fluors per IgG). The standard is recombinant human cardiac troponin I (BiosPacific, cat # J34120352). The calibrator diluent is 30 mg/ml BSA in TBS wEDTA.

[00222] Microparticles Coating: 100 ul of the MPs stock is placed in an eppendorf tube. The MPs are washed three times with 100 ul of BBST wash buffer by applying a magnet, removing the supernatant, removing the magnet, and resuspending in wash buffer. After the washes the MPs are resuspended in 100 ul of assay buffer and 15 ug of Bio-Ab are added. The mixture is then incubated for an hour at room temperature with constant mixing. The MPs are washed five times with 1 ml wash buffer as described above. After the washes the MPs are resuspended in 15 ml of assay buffer (or 100 ul to store at 4 0C).

[00223] Preparation of Standard and Samples: The standard is diluted with calibrator diluent to prepare proper standard curve (usually 200 pg/ml down to 0.1 pg/ml). Frozen serum and plasma samples need to be centrifuged 10 minutes at room temperature at 13K rpm. Clarified serum/plasma is removed carefully to avoid taking any possible pellets or floaters and put into fresh tubes. 50 ul of each standard or sample is pippetted into appropriate wells.

[00224] Capture Target: 150 ul of MPs (after resuspension to 15 ml in assay buffer + 400 mM NaCl) are added to each well. The mixture is incubated on JitterBug, 5 at room temperature for 1 hr.

[00225] Washes and Detection: The plate is placed on a magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 250 ul of wash buffer are added after removing the plate from the magnet. The plate is then placed on the magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 20 ul Det-Ab are added per well (Det-Ab to 500 ng/ml is diluted in assay buffer + 400 mM NaCl)). The mixture is incubated on JitterBug, 5 at room temperature for 30 min.

[00226] Washes and Elution: The plate is placed on a magnet and washed three times with wash buffer. The supernatant is removed after ensuring that all MPs are captured by the magnet and 250 ul of wash buffer are added. After the washes the samples are transferred into a new 96-well plate. The new plate is then placed on the magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 250 ul of wash buffer are then added after removing the plate from the magnet. The plate is then placed on the magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 20 ul of elution buffer are then added and the mixture is incubated on JitterBug, 5 at room temperature for 30 min.

[00227] Filter out MPs and transfer to 384-well plate: The standard and samples are transferred into a 384-well filter plate placed on top of a 384-well assay plate. The plate is then centrifuged at room temperature at 3000 rpm

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with a plate rotor. The filter plate is removed and the appropriate calibrators are added. The plate is covered and is ready to be run on SMD.

[00228] SMD: An aliquot is pumped into the analyzer. Individually-labeled antibodies are measured during capillary flow by setting the interrogation volume such that the emission of only 1 fluorescent molecule is detected in a defined space following laser excitation. With each signal representing a digital event, this configuration enables extremely high analytical sensitivities. Total fluorescent signal is determined as a sum of the individual digital events. Each molecule counted is a positive data point with hundreds to thousands of DMC events/sample. The limit of detection the cTnI assay of the invention is determined by the mean +3 SD method.

Example 3. Concentration range for cTnI in a population of normal non-diseased subjects.

[00229] A reference range or normal range for cTnI concentrations in human serum was established using serum samples from 88 apparently healthy subjects (non-diseased). A sandwich immunoassay as described in Example 1 was performed and the number of signals or events as described above were counted using the single particle analyzer system of the invention. The concentration of serum troponin I was determined by correlating the signals detected by the analyzer with the standard curve as described above. All assays were perfumed in quadruplicate.

[00230] In accordance with recommendations by the current European and American Cardiology Societies (ESC/ACC) troponin assays should quantify accurately the 99th percentile of the normal range with an assay imprecision (CV) of less than 10% in order to distinguish reliably between patients with ACS and patients without ischemic heart disease, and risk stratification for adverse cardiac events. The assay showed that the biological threshold (cutoff concentration) for TnI is at a TnI concentration of 7pg/ml, which is established at the 99th percentile with a corresponding CV of 10% (Figure 5). At the 10% CV level the precision profile points at a TnI concentration of 4 and 12 pg/ml.

[00231] In addition, the assay correlates well with the Troponin-I standard measurements provided by the National Institute of Standards and Technology (Figure 6).

[00232] The assay of the invention is sufficiently sensitive and precise to fulfill the requirements of the ESC/ACC, and it is the most sensitive assay for cardiac troponin I when compared to assays such as those described by Koerbin et al.(Ann Clin Biochem, 42:19-23 (2005). The assay of the invention has a 10-20 fold greater sensitivity than that currently available assays, which has determined the biological threshold range to be 111-333 pg/ml cTnI.

Example 4. Detection of early release of TnI into the circulation of patients with acute myocardial infarction (AMI)

[00233] Study 1: 47 samples were obtained serially from 18 patients that presented with chest pain in the emergency department (ED). These patients all had non-ST elevated ECG were, and were diagnosed with AMI. The concentration of cTnI in the initial samples from all 18 patients was determined according to a commercial assay at the time of admission to the emergency room to be <350 pg/ml (10% cutpoint), and 12 were <100 pg/ml (99th%) percentile. These samples were tested at later times using the same commercial assay, and were determined to test positive for cTnI. The same serum samples were also assayed for TnI according to the assay of the invention as described in Examples 1 and 3, and the results compared to the results obtained using the commercial assay.

[00234] Blood was drawn for the first time at the time the patient presented with chest pain (sample 1), and subsequently at intervals between 4-8 hours (samples 2 at 12 hours; sample 3 at 16 hours; sample 4 at 24 hours;

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sample 5 at 30 hours; sample 6 at 36 hours; sample 7 at 42 hours; and sample 8 at 48 hours). The serum was analyzed by the methods of the invention and by a current commercial method, and the results obtained are shown in Figure 7. The analyzer of the invention detected TnI at the time the patient presented with chest pain (sample 1), while the commercial assay first detected cTnI at a much later time (sample 6 at 36 hours). The concentration of TnI in sample 3 exceeded the biological threshold level that was established using the analyzer of the invention (7 pg/ml, see Figure 5), and indicated that sample 3 is positive for TnI to suggest the incidence of a cardiac event. The biological threshold for the commercial assay lies between 111 and 333 pg/ml of TnI. Accordingly, sample 3 would not have been considered to indicate a possible cardiac event.

[00235] In addition, the methods and compositions of the present invention allow for much earlier diagnosis and possible intervention based on cardiac troponin levels, as evidenced by results for the first sample taken from the patients. In the 3 cases that had initial commercial assay cTnI values of between 100 and 350 ng/ml, all were positive for cTnI by the analytical methods of the invention (i.e., cTnI over 7 pg/ml). In the 12 cases that had initial commercial cTnI values of less than 100 pg/ml, 5 were determined to be positive for a cardiovascular event according to the assay of the invention (i.e., cTnI over 7 pg/ml). The prospective use of the assay of the invention would have detected 53% more AMI cases than the current commercial assay when the admission sample was tested.

[00236] Study 2: 50 additional serum samples, which tested negative according to the commercial assay, were tested using the analyzer and assay of the invention. The results are shown in Figure 8. Of the 50 samples, 36 were within the 99th% and determined to be within the normal range established by the assay of the invention. However, the remaining 14 samples that were determined to be within the commercial "normal" or non-diseased range, tested above the biological threshold established by the invention.

[00237] Therefore, the high sensitivity cTnI assay of the invention allows for the detection of myocardial damage in patients when cTnI serum levels are below threshold values by commercially available technology. The use of the highly sensitive and precise cTnI assay of the invention enables detection of AMI earlier than with existing cTnI assays, and thereby provides the opportunity for appropriate diagnosis and early medical intervention to improve the outcome.

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WHAT IS CLAIMED IS:

1. A method for determining the presence or absence of a single molecule of troponin or a fragment or complex thereof in a sample, comprising

i) labeling said molecule, fragment, or complex, if present, with a label; and

ii) detecting the presence or absence of said label, wherein detection of the presence of said label indicates the presence of said single molecule, fragment, or complex of troponin in said sample.

2. The method of claim 1 wherein the troponin is a cardiac isoform of troponin.

3. The method of claim 2 wherein the troponin is selected from the group consisting of cardiac troponin I (cTnI) and cardiac troponin C (cTnC).

4. The method of claim 3 wherein the troponin is cTnI.

5. The method of claim 1, wherein said detecting is capable of detecting said single molecule of troponin at a limit of detection of less than about 50 pg/ml

6. The method of claim 1 wherein said method is capable of detecting said troponin at a level of detection of less than about 20 pg/ml.

7. The method of claim 1, wherein said label comprises a fluorescent moiety.

8. The method of claim 1, wherein said fluorescent moiety capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

9. The method of claim 1 wherein said fluorescent moiety comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated group.

10. The method of claim 1 wherein said fluorescent moiety comprises a dye selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700.

11. The method of claim 1 wherein said fluorescent moiety comprises AlexaFluor 647.

12. The method of claim 1 wherein said label further comprises a binding partner for said troponin molecule, fragment, or complex.

13. The method of claim 12 wherein said binding partner comprises an antibody specific to said troponin molecule, fragment, or complex.

14. The method of claim 13 wherein said antibody is specific to a specific region of the troponin molecule.

15. The method of claim 14 wherein said antibody is specific to a region comprising amino acids 27-41 of cardiac troponin I.

16. The method of claim 13 wherein said antibody comprises a polyclonal antibody.

17. The method of claim 13 wherein said antibody is a monoclonal antibody.

18. The method of claim 1 further comprising capturing said troponin or troponin complex on a solid support.

19. The method of claim 18 wherein the solid support is selected from the group consisting of a microtiter plate and paramagnetic beads.

20. The method of claim 18 wherein said solid support comprises a capture partner specific for said troponin or troponin complex that is attached to said solid support.

21. The method of claim 20 wherein said attachment of said capture partner to said solid support is noncovalent.

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22. The method of claim 20 wherein said attachment of said capture partner to said solid support is covalent.

23. The method of claim 22 wherein said covalent attachment of said capture partner is such that said capture partner is attached to said solid support in a specific orientation.

24. The method of claim 23 wherein said specific orientation serves to maximize specific binding of said troponin or troponin complex to said capture partner.

25. The method of claim 20 wherein said capture partner comprises an antibody.

26. The method of claim 25 wherein said antibody is a monoclonal antibody.

27. The method of claim 25 wherein said antibody is specific to amino acids 87-91 of cardiac troponin I.

28. the method of claim 25 wherein said antibody is specific to amino acids 41-49 of cardiac troponin I.

29. The method of claim 1 wherein said sample is a blood, serum, or plasma sample

30. The method of claim 29 wherein said sample is a serum sample.

31. The method of claim 1 wherein said label comprises a fluorescent moiety, and wherein step ii) comprises passing said label through an single molecule detector.

32. The method of claim 31 wherein said single molecule detector comprises

a) an electromagnetic radiation source for stimulating said fluorescent moiety;

b) a capillary flow cell for passing said fluorescent moiety;

c) a source of motive force for moving said fluorescent moiety in said capillary flow cell;

d) an interrogation space defined within said capillary flow cell for receiving electromagnetic radiation emitted from said electromagnetic source;

e) an electromagnetic radiation detector operably connected to said interrogation space for measuring an electromagnetic characteristic of said stimulated fluorescent moiety; and

f) a microscope objective lens situated between said interrogation space and said detector, wherein the lens has a numerical aperture of 0.6 or greater.

33. A method for determining a diagnosis, prognosis, or method of treatment in an individual comprising

i) determining a concentration of cardiac troponin in a sample or determining the concentrations of cardiac troponin in a series of samples from said individual, wherein said concentration is determined by a cardiac troponin assay with a limit of detection for said cardiac troponin in said sample of less than about 50 pg/ml; and

ii) determining a diagnosis, prognosis, or method of treatment in said individual, based on said concentration in said sample, or on said concentrations in said series of samples.

34. The method of claim 33 wherein step ii) comprises an analysis selected from the group consisting of comparing said concentration or series of concentrations to a normal value for said concentration, comparing said concentration or series of concentrations to a predetermined threshold level, comparing said concentration or series of concentrations to a baseline value, and determining a rate of change of concentration for said series of concentrations.

35. The method of claim 34 wherein step ii) comprises comparing said concentration of troponin in said sample with a predetermined threshold concentration, and determining a diagnosis, prognosis, or method of treatment if the sample concentration is greater than the threshold level.

36. The method of claim 35 wherein said threshold concentration is determined by determining the 99th percentile concentration of troponin in a group of normal individuals, and setting said threshold concentration at said 99th percentile concentration.

37. The method of claim 33 wherein at least one sample is taken during or after a cardiac stress test.

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38. The method of claim 33 wherein said cardiac troponin is selected from the group consisting of cardiac troponin I and cardiac troponin T.

39. The method of claim 33 wherein the cardiac troponin is cardiac troponin I.

40. The method of claim 38 wherein said concentration of cardiac troponin is a concentration of total cardiac troponin.

41. The method of claim 40 wherein said concentration of cardiac troponin is a concentration of a cardiac troponin complex, cardiac troponin fragment, phosphorylated cardiac troponin, oxidized cardiac troponin, or a combination thereof.

42. The method of claim 41 wherein said concentration of cardiac troponin is compared to total cardiac troponin.

43. The method of claim 33 wherein said diagnosis, prognosis, or method of treatment is a diagnosis, prognosis, or method of treatment of myocardial infarct.

44. The method of claim 43 wherein said diagnosis, prognosis, or method of treatment comprises risk stratification for level of risk of myocardial infarct.

45. The method of claim 43, wherein said concentration or series of concentrations is determined at or near the time the individual presents to a health professional with one or more symptoms indicative of myocardial ischemia or infarct or the possibility thereof.

46. The method of claim 45 wherein said one or more symptoms is selected from the group consisting of chest pain, chest pressure, arm pain, abnormal EKG, abnormal enzyme levels, and shortness of breath.

47. The method of claim 33 wherein said concentration is determined by a method that comprises detecting single molecules of troponin, or complexes or fragments thereof.

48. The method of claim 47 comprising labeling said troponin or troponin complex with a label that comprises a fluorescent moiety.

49. The method of claim 33 wherein said fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules..

50. The method of claim 33 wherein said fluorescent moiety comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. group..

51. The method of claim 33 wherein said fluorescent moiety comprises a dye selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700.

52. The method of claim 51 wherein said fluorescent moiety comprises AlexaFluor 647.

53. The method of claim 33 wherein said label further comprises a binding partner for said troponin.

54. The method of claim 53 wherein said binding partner comprises an antibody specific to said troponin.

55. The method of claim 54 wherein said antibody comprises a polyclonal antibody.

56. The method of claim 33 further comprising capturing said troponin or troponin complex on a solid support.

57. The method of claim 56 wherein the solid support is selected from the group consisting of a microtiter plate and paramagnetic beads.

58. The method of claim 56 wherein said solid support comprises a capture partner specific for said troponin or troponin complex that is attached to said solid support.

59. The method of claim 33 wherein step i) further comprises assessing another indicator for said individual., and step ii) comprises determining a diagnosis, prognosis, or method of treatment in said individual, based on said

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concentration of troponin and said assessment of said other indicator of said non-troponin marker in said sample, or on said concentrations in said series of samples.

60. The method of claim 59 wherein said other indicator is a clinical indicator of myocardial ischemia or infarct.

61. The method of claim 60 wherein said other indicator is the concentration of one or more non-troponin markers in said sample or said series of samples.

62. The method of claim 59, wherein said one or more markers are markers of cardiac ischemia, or markers of inflammation and of plaque instability.

63. The method of claim 62, wherein said one or more markers of cardiac ischemia are selected from the group consisting of creatine kinase (CK) and its myocardial fraction CK myocardial band (MB), aspartate aminotransferase, lactate dehydrogenase (LDH), α-hydroxybutyrate dehaydrogenase, myoglobin, glutamate oxaloacetate transaminase, glycogen phosphorylase BB, unbound free fatty acids, heart fatty acid binding protein (H-FABP), ischemia-modified albumin, myosin light chain 1, myosin light chain 2.

64. The method of claim 63, wherein said one or more markers comprise one or more specific markers of myocardial injury.

65. The method of claim 33 wherein said diagnosis, prognosis, or method of treatment is a diagnosis, prognosis, or method of treatment of a condition that is not myocardial infarct.

66. The method of claim 65 wherein said condition is cardiac toxicity.

67. The method of claim 66 wherein said cardiac toxicity is associated with the administration of a drug to the individual.

68. The method of claim 65 wherein the condition is selected from the group consisting of acute rheumatic fever, amyloidosis, cardiac trauma (including contusion, ablation, pacing, firing, cardioversion, catheterization and cardiac surgery), reperfusion injury, congestive heart failure, end-stage renal failure, glycogen storage disease type II (Pompe's disease), heart transplantation, haeomoglobinopathy with transfusion haemosiderosis, hypertension, including gestational hypertension, hypotension, often with arrhythmias, hypothyroidism, myocarditis, pericarditis, post-operative non-cardiac surgery, pulmonary embolism, and sepsis.

69. A composition for the detection of a troponin isoform comprising a binding partner to the troponin isoform attached to a fluorescent moiety, wherein said fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

70. The composition of claim 69 wherein said binding partner comprises an antibody to said troponin isoform.

71. The composition of claim 70 wherein said antibody is a polyclonal antibody.

72. The composition of claim 70 wherein said antibody is a monoclonal antibody.

73. The composition of claim 69 wherein said troponin isoform is a cardiac isoform.

74. The composition of claim 73 wherein said cardiac isoform is selected from the group consisting of cTnI and cTnT.

75. The composition of claim 74 wherein said cardiac isoform is cTnI.

76. The composition of claim 75 wherein said antibody is specific to a specific region of the troponin molecule.

77. The composition of claim 76 wherein said antibody is specific to a region comprising amino acids 27-41 of cardiac troponin I.

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78. The composition of claim 69 wherein said fluorescent moiety comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. group.

79. The composition of claim 69 wherein said fluorescent moiety comprises a dye selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700.

80. The composition of claim 79 wherein said fluorescent moiety comprises AlexaFluor 647.

81. A composition comprising a set of standards for the determination of a concentration of a cardiac troponin, wherein at least one of the standards is at a concentration of cardiac troponin less than about 10 pg/ml.

82. A kit comprising a composition comprising an antibody to cardiac troponin attached to a fluorescent dye moiety, wherein said moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules, wherein said composition is packaged in suitable packaging.

83. The kit of claim 82 wherein the cardiac troponin is cardiac troponin I or cardiac troponin T.

84. The kit of claim 82 wherein the cardiac troponin is cardiac troponin I.

85. The kit of claim 82 further comprising instructions.

86. The kit of claim 82 further comprising a composition comprising a capture antibody for said cardiac troponin I attached to a solid support.

87. The kit of claim 86 wherein said solid support comprises a microtiter plate or paramagnetic microparticles.

88. The kit of claim 82 further comprising a component selected from the group consisting of wash buffer, assay buffer, elution buffer, and calibrator diluent.

89. The kit of claim 82 further comprising a standard for the cardiac troponin.

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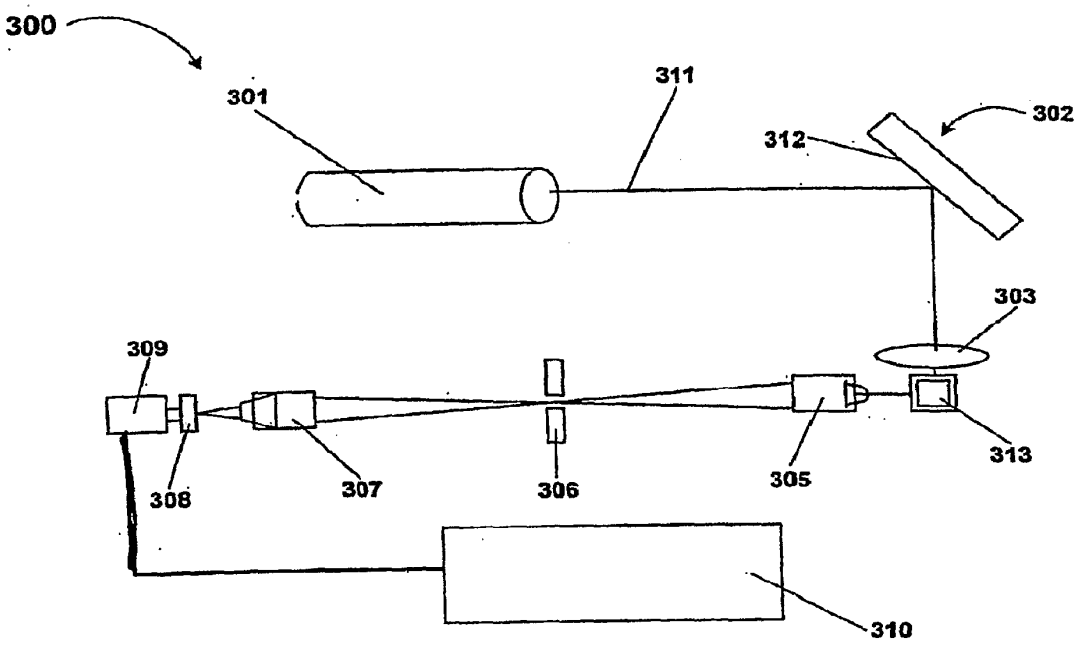


Figure 1A

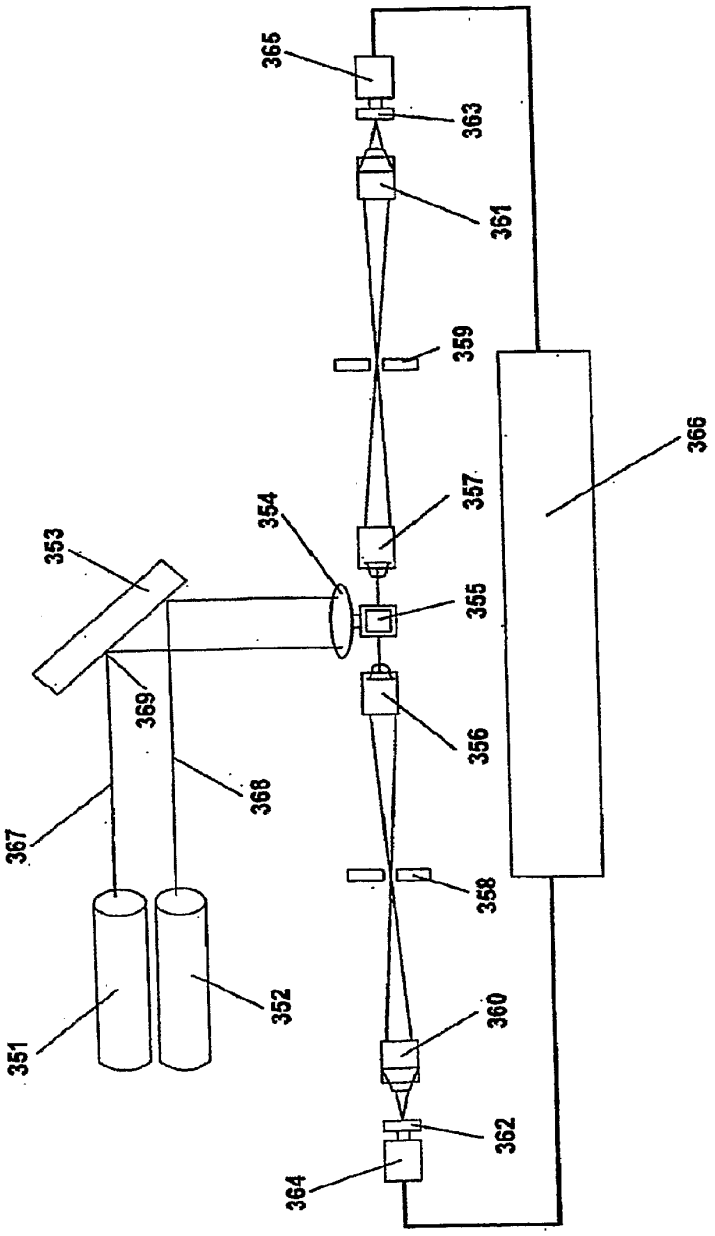

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Figure 1B

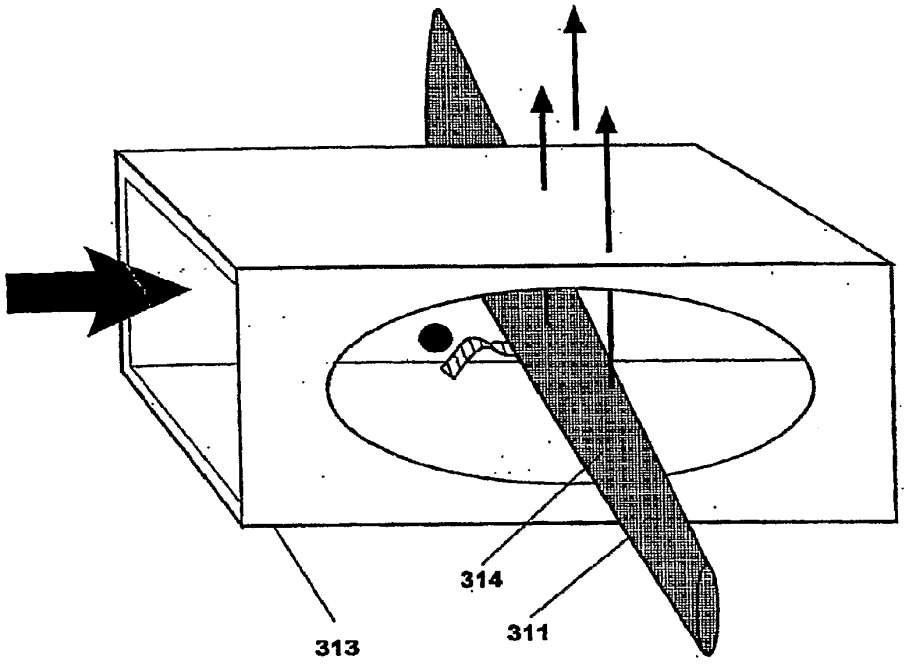


Figure 2A

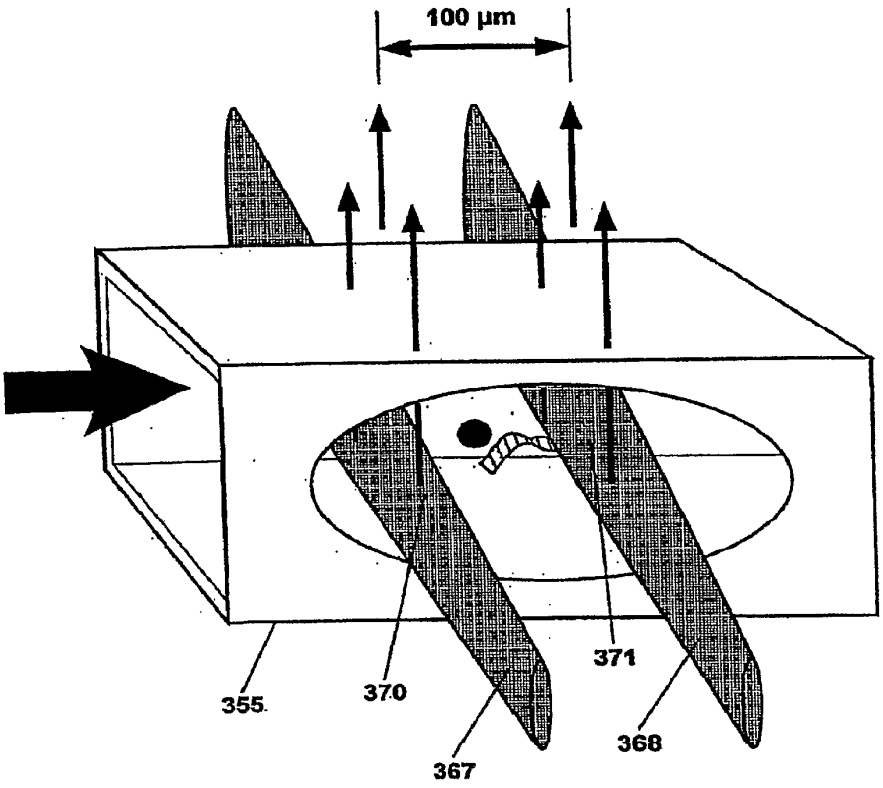


Figure 2B

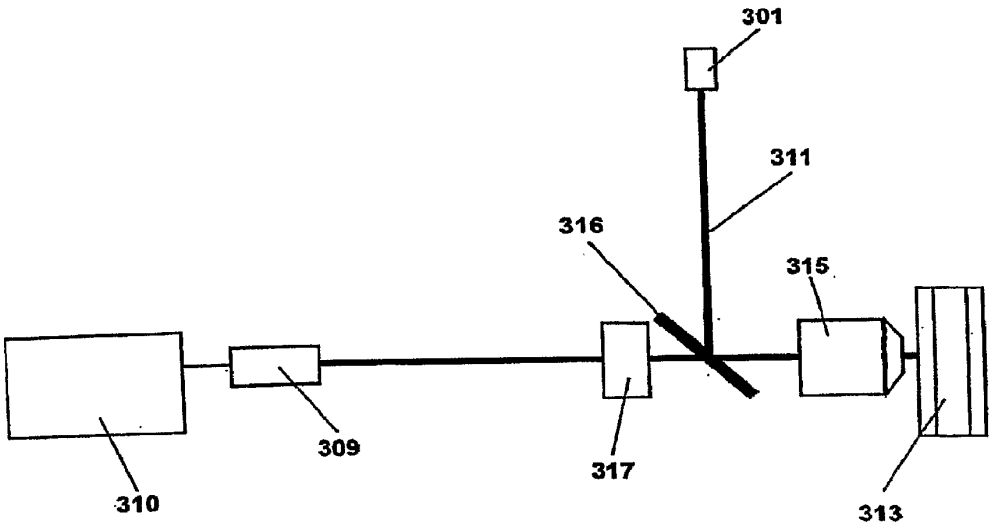


Figure 3A

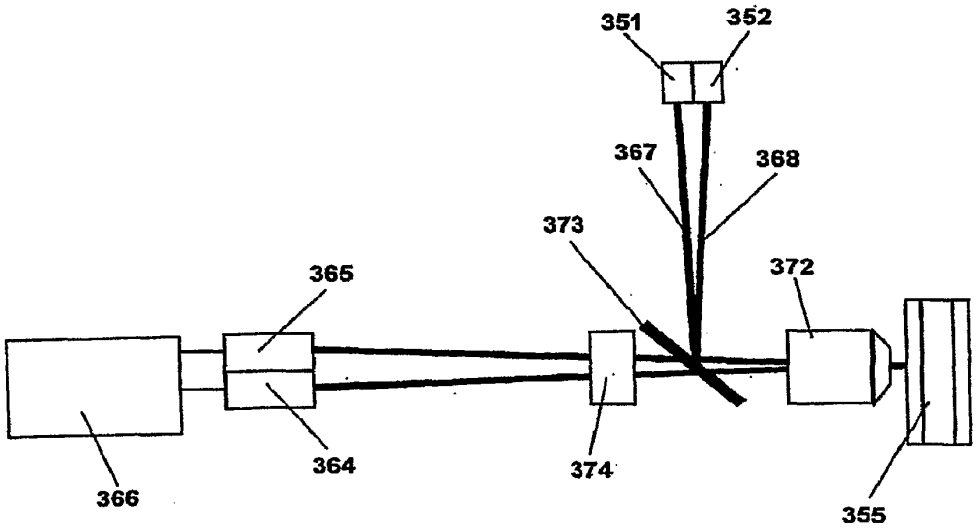


Figure 3B

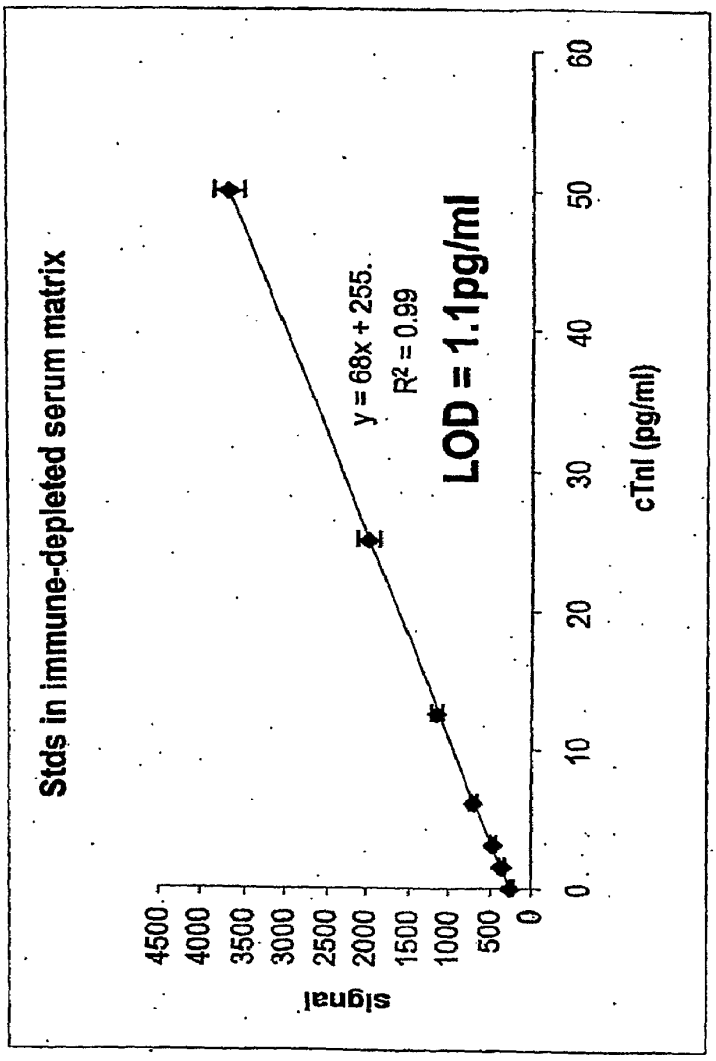

Stds in immune-depleted serum matrix
y = 68x + 255.
R² = 0.99
LOD = 1.1pg/ml
signal
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
cTnI (pg/ml)

Figure 4

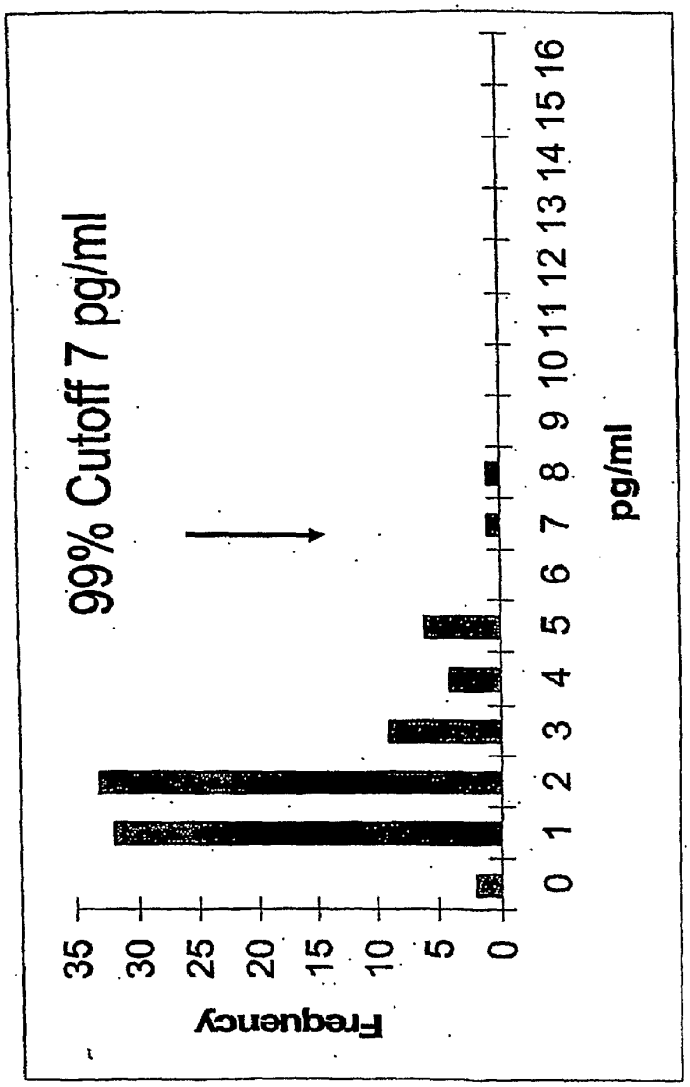

99% Cutoff 7 pg/ml
Frequency
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
pg/ml

Figure 5

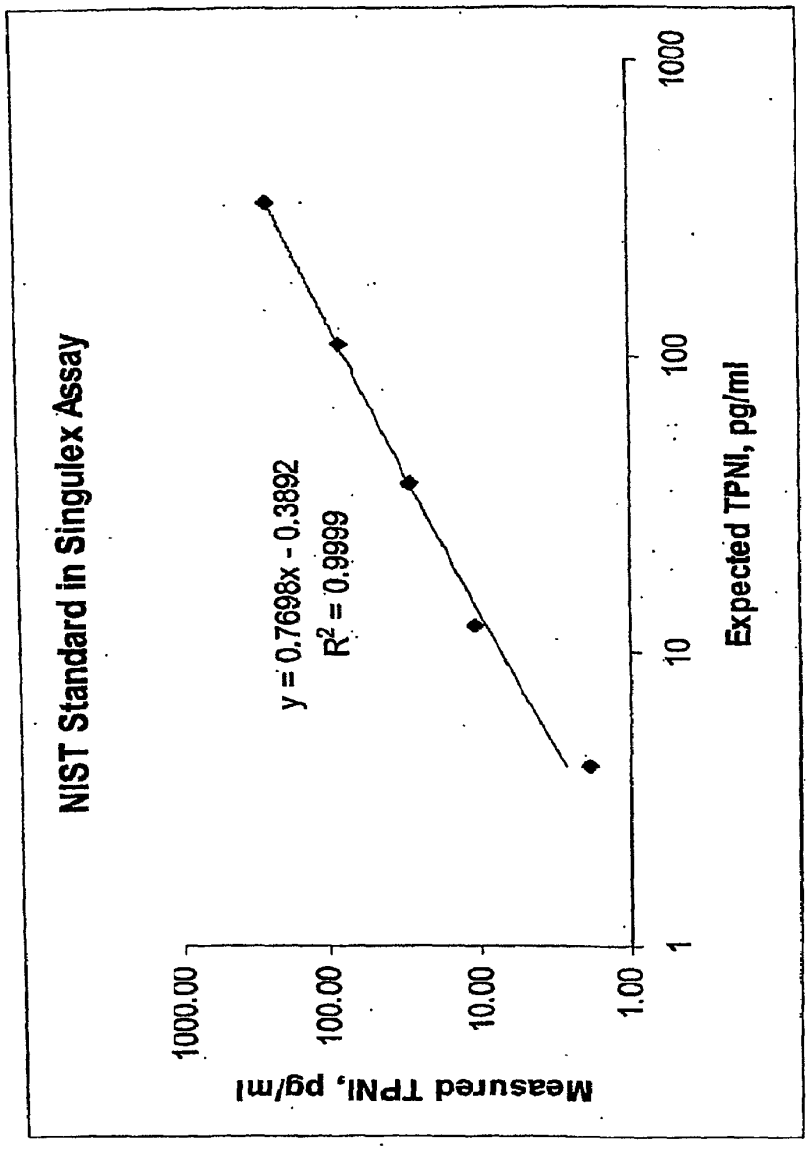

NIST Standard in Singulex Assay
y = 0.7698x - 0.3892
R2 = 0.9999
Measured TPNI, pg/ml
1000.00
100.00
10.00
1.00
Expected TPNI, pg/ml
1000
100
10
1

Figure 6

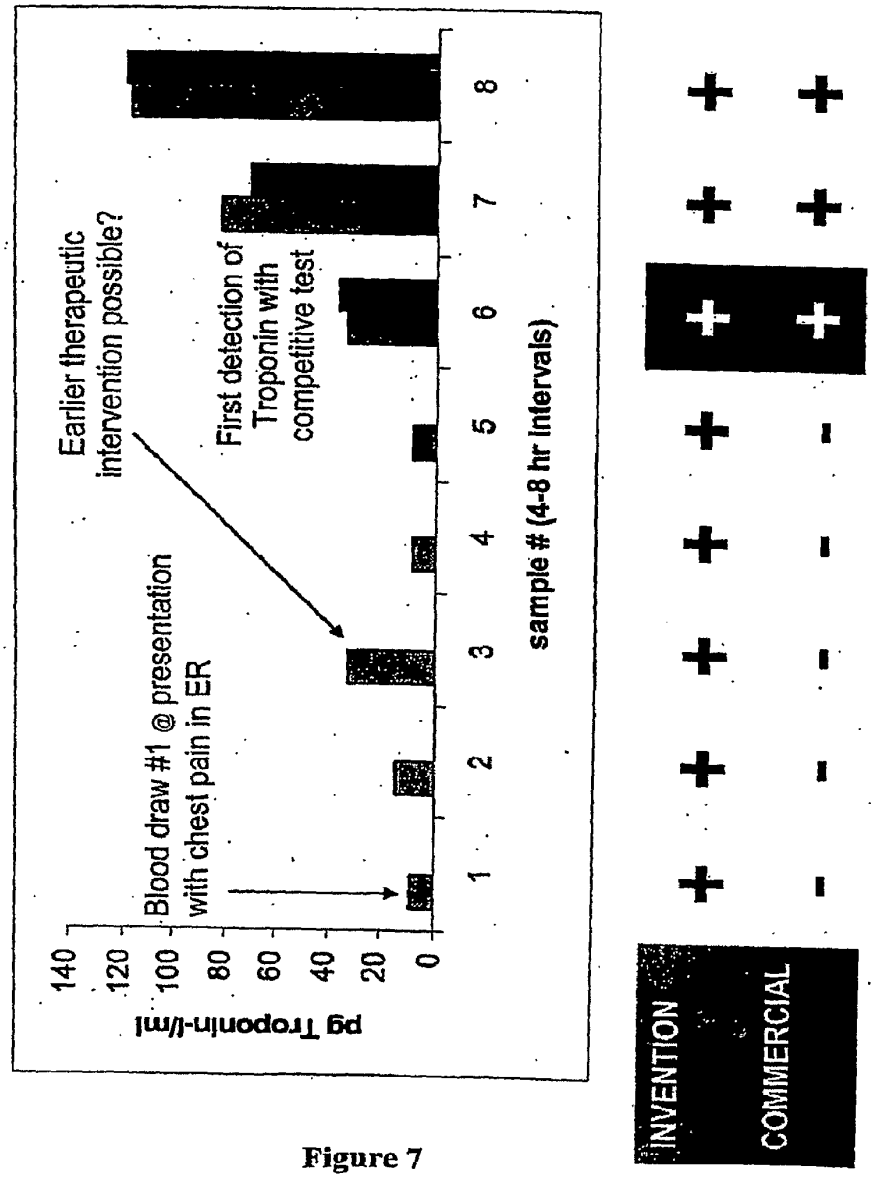

pg Troponin-I/ml
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
sample # (4-8 hr intervals)
Blood draw #1 @ presentation with chest pain in ER
Earlier therapeutic intervention possible?
First detection of Troponin with competitive test
INVENTION
COMMERCIAL

Figure 7

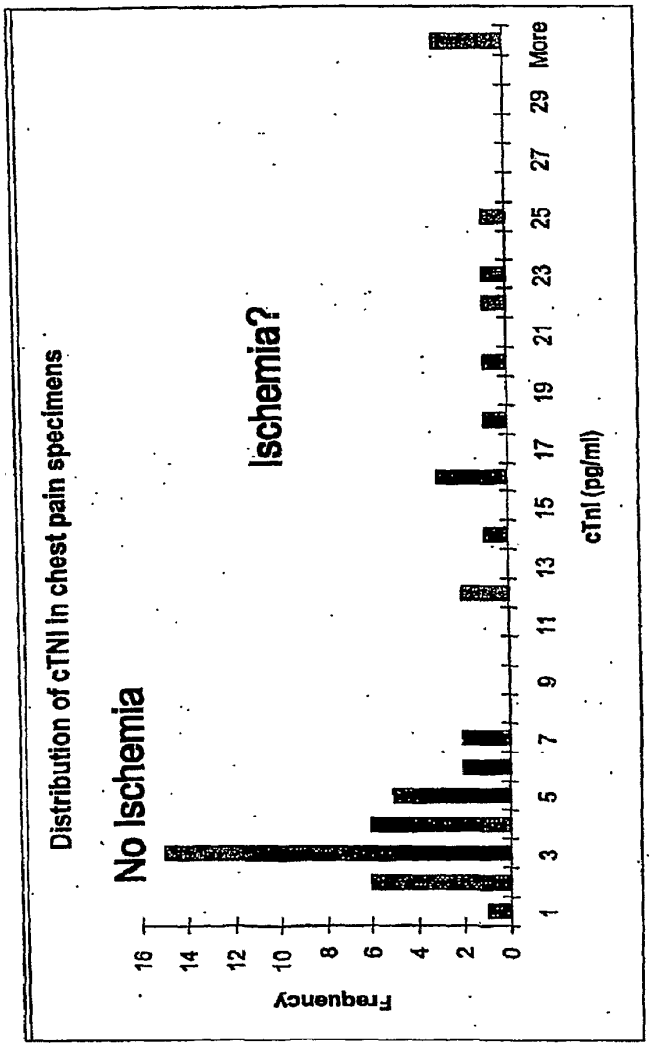

Distribution of cTNI in chest pain specimens
No Ischemia
Ischemia?
Frequency
0
2
4
6
8
10
12
14
16
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
More
cTnI (pg/ml)

Figure 8

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60/861,498	28 November 2006 (28.11.2006)	US
60/872,986	4 December 2006 (04.12.2006)	US

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(54) Title: METHODS AND COMPOSITIONS FOR HIGHLY SENSITIVE ANALYSIS OF MARKERS AND DETECTION OF MOLECULES

(57) Abstract: Disclosed are methods, kits, and compositions for the highly sensitive detection of molecules. The methods, kits, and compositions are useful in determining concentrations of molecules in samples to levels of 1 femtomolar, 1 attomolar, or lower. The methods, kits, and compositions also allow the determination of concentration over a wide range, e.g., 7-log range, without need for sample dilution.

METHODS AND COMPOSITIONS FOR HIGHLY SENSITIVE ANALYSIS OF MARKERS AND DETECTION OF MOLECULES

CROSS-REFERENCE

[0001] This application is a continuation-in-part application of Serial No. 11/048,660, filed January 28, 2005, which is incorporated herein by reference in its entirety and to which application we claim priority under 35 USC § 120. Application Serial No. 11/048,660 claims the benefit of U.S. Provisional Application No. 60/613,881, filed September 28, 2004, U.S. Provisional Application No. 60/624,785, filed October 27, 2004, and U.S. Provisional Application No. 60/636,158, filed December 13, 2004.

[0002] This application also claims priority under 35 USC § 119 to U.S. Provisional Application No. 60/789,304, filed April 4, 2006, U.S. Provisional Application No. 60/793,664, filed April 19, 2006, U.S. Provisional Application No. 60/808,662, filed May 26, 2006, U.S. Provisional Application No. 60/861,498, filed November 28, 2006, and U.S. Provisional Application No. 60/872,986, filed December 4, 2006, all of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

[0003] Advances in biomedical research, medical diagnosis, prognosis, monitoring and treatment selection, bioterrorism detection, and other fields involving the analysis of multiple samples of low volume and concentration of analytes have led to development of sample analysis systems capable of sensitively detecting particles in a sample at ever-decreasing concentrations. U.S. Patent Nos. 4,793,705 and 5,209,834 describe previous systems in which extremely sensitive detection has been achieved. The present invention provides further development in this field.

SUMMARY OF THE INVENTION

[0004] In one aspect the invention involves an apparatus.

[0005] In some embodiments of the apparatus, the invention involves an analyzer system kit for detecting a single protein molecule in a sample, the kit comprising an analyzer and at least one label comprising a fluorescent moiety and a binding partner for the protein molecule, and where the analyzer contains: a) an electromagnetic radiation source for stimulating the fluorescent moiety; b) a capillary flow cell for passing the label; c) a source of motive force for moving the label in the capillary flow cell; d) an interrogation space defined within the capillary flow cell for receiving electromagnetic radiation emitted from the electromagnetic source; and e) an electromagnetic radiation detector operably connected to the interrogation space for measuring an electromagnetic characteristic of the stimulated fluorescent moiety; where the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments of the apparatus of the invention, the analyzer system comprises not more than one interrogation space. In some embodiments of the apparatus of the invention, the electromagnetic radiation source is a laser, and where the laser has a power output of at least about 3, 5, 10, or 20 mW. In some embodiments of the apparatus of the invention, the fluorescent moiety involves a fluorescent molecule. In some embodiments, the fluorescent molecule is a dye molecule. In some embodiments, the dye molecule include at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the

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fluorescent moiety is a quantum dot. In some embodiments of the apparatus of the invention, the electromagnetic radiation source is a continuous wave electromagnetic radiation source. In some embodiments, the continuous wave electromagnetic radiation source is a light-emitting diode or a continuous wave laser. In some embodiments of the apparatus of the invention, the motive force is pressure. In some embodiments of the apparatus of the invention, the detector is an avalanche photodiode detector.

[0006] In some embodiments of the apparatus of the invention, the analyzer system further include a confocal optical arrangement for deflecting a laser beam onto the interrogation space and for imaging the stimulated dye molecule, where the confocal optical arrangement comprises an objective lens having a numerical aperture of at least about 0.8. In some embodiments of the apparatus of the invention, the analyzer system further includes a sampling system capable of automatically sampling a plurality of samples and providing a fluid communication between a sample container and the interrogation space. In some embodiments of the apparatus of the invention, the analyzer system further includes a sample recovery system in fluid communication with the interrogation space, where the recovery system is capable of recovering substantially all of the sample.

[0007] In some embodiments the invention includes an analyzer for determining the concentration of species in a sample, where the analyzer is capable of determining the concentration over a dynamic range of concentrations of at least about 10^5. In some embodiments, the dynamic range is from about 1 femtomolar to about 100 picomolar.

[0008] In another aspect the invention includes methods.

[0009] In some embodiments the invention include a method for determining the presence or absence of a single molecule of a protein in a biological sample, including labeling the molecule with a label and detecting the presence or absence of the label in a single molecule detector, where the label includes a fluorescent moiety that is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments the single molecule detector comprises not more than one interrogation space. In some embodiments of the methods of the invention, the limit of detection of the single molecule in the sample is less than about 10, 1, 0.1, 0.01, or 0.001 femtomolar. In some embodiments, the limit of detection is less than about 1 femtomolar. In some embodiments of the methods of the invention, detecting the presence or absence of the label in a single molecule detector involves detecting electromagnetic radiation emitted by the fluorescent moiety. In some embodiments of the methods of the invention, the methods further include exposing the fluorescent moiety to electromagnetic radiation. In some embodiments, the electromagnetic radiation is provided by a laser. In some embodiments, the laser stimulates the moiety at a power output of less than about 20 mW. In some embodiments, the laser stimulates the moiety for a duration of less than about 1000, 250, 100, 50, 25 or 10 microseconds. In some embodiments of the methods of the invention, the label further includes a binding partner specific for binding the molecule. In some embodiments, the binding partner is an antibody. In some embodiments of the methods of the invention, the fluorescent moiety comprises a fluorescent dye molecule. In some embodiments, the dye molecule includes at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the dye molecule is an AlexFluor molecule selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the dye molecule is an AlexaFluor647 dye molecule. In some embodiments, the fluorescent moiety comprises a plurality of AlexaFluor 647 molecules. In some embodiments, the plurality of AlexaFluor 647 molecules comprises about 2-4 AlexaFluor 647 molecules. In some embodiments, the fluorescent moiety is a quantum dot. In some embodiments of the methods of the invention, the methods further

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include measuring the concentration of the protein in the sample. In some embodiments of the methods of the invention, the detecting the presence or absence of the label includes: (i) passing a portion of the sample through an interrogation space; and (ii) subjecting the interrogation space to exposure to electromagnetic radiation, the electromagnetic radiation being sufficient to stimulate the fluorescent moiety to emit photons, if the label is present; and (iii) detecting photons emitted during the exposure of step (ii). In some embodiments of the methods of the invention, the methods further includes determining a background photon level in the interrogation space, where the background level represents the average photon emission of the interrogation space when it is subjected to electromagnetic radiation in the same manner as in step (ii), but without label in the interrogation space. In some embodiments of the methods of the invention, the method further includes comparing the amount of photons detected in step (iii) to a threshold photon level, where the threshold photon level is a function of the background photon level, where an amount of photons detected in step (iii) greater that the threshold level indicates the presence of the label, and an amount of photons detected in step (iii) equal to or less than the threshold level indicates the absence of the label.

[0010] In some embodiments the invention involve a method for determining the presence or absence of a biological state in a subject, including: a) performing an immunoassay on a sample from the subject, where the immunoassay comprises binding a plurality of labels for a marker to a plurality of molecules of the marker in the sample, where the label is specific to the marker and where one label binds to one molecule of marker, and where the label comprises an antibody attached to a fluorescent moiety that is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules; b) detecting the labels where the detecting comprises detecting single labels with a single molecule detector; and c) determining a concentration for the marker in the sample based on the number of labels detected in step b). In some embodiments of the methods of the invention, the method further include comparing the concentration obtained in step b) to a concentration or range of concentrations for the marker that are known to be indicative of the presence or absence of the biological state. In some embodiments of the methods of the invention, the subject is a human. In some embodiments, the sample is blood, serum, plasma, urine or exhaled breath condensate.

[0011] In another aspect the invention provides compositions.

[0012] In some embodiments the invention provides a label for detecting a biological molecule comprising a binding partner for the biological molecule that is attached to a fluorescent moiety, where the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules, where the moiety comprises about 2 to 4 fluorescent entities. In some embodiments of the composition of the invention, the biological molecule is a protein or a small molecule. In some embodiments, the biological molecule is a protein. In some embodiments, the fluorescent entities comprise fluorescent dye molecules. In some embodiments, the fluorescent dye molecules include at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the dye molecules are AlexFluor molecules that can be AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the dye molecules are AlexaFluor647 dye molecules. In some embodiments of the methods of the invention, the dye molecules comprise a first type and a second type of dye molecules. In some embodiments, the first type and second type of dye molecules have different emission

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spectra. In some embodiments, the ratio of the number of first type to second type of dye molecule is 4:1, 3:1, 2: 1, 1:1, 1:2, 1:3 or 1:4. In some embodiments of the methods of the invention, the binding partner is an antibody.

[0013] In one aspect the invention provides methods.

[0014] In some embodiments, the invention provides a method of establishing a marker for a biological state by i) establishing a range of concentrations for said marker in biological samples obtained from a first population, where said establishing comprises measuring the concentrations of said marker in said biological samples by detecting single molecules of said marker. In some embodiments, said first population is a population that does not exhibit said biological state. In some embodiments, the method further comprises establishing a range of range of levels for said marker in biological samples obtained from a second population, wherein the members of said second population exhibit the biological state, and wherein said establishing comprises measuring the levels of said marker in said biological samples by detecting single molecules of said marker. In some embodiments, the first and second populations are different.In some embodiments, at least one member of said second population is a member of said first population, or at least one member of said first population is a member of said second population. In some embodiments, substantially all the members of the second population are members of the first population who have developed the biological state. In some embodiments, the detecting of single molecules of said marker is performed using a method with a limit of detection for said marker of less than about 1000, 100, 50, 20, 10, 5, 1, or 0.5 femtomolar of said marker in said samples. In some embodiments, the biological state is a phenotypic states; a condition affecting the organism; a state of development; age; health; pathology; disease; disease process; disease staging; infection; toxicity; or response to chemical, environmental, or drug factors (such as drug response phenotyping, drug toxicity phenotyping, or drug effectiveness phenotyping). In some embodiments, the biological state is a pathology. In some embodiments, the marker is a polypeptide or small molecule. In some embodiments, the state is a disease state, e.g., cancer, cardiovascular disease, inflammatory disease, autoimmune disease, neurological disease, infectious disease or pregnancy related disorders. In some embodiments, the state is a disease stage state. In some embodiments, the disease stage state is a cancer disease stage state. In some embodiments, the state is a treatment response state. In some embodiments, the treatment is a drug treatment. In some embodiments, the response is selected from the group consisting of a therapeutic effect and a side effect. In some embodiments, the effect is a therapeutic effect. In some embodiments, the response is a side effect, e.g., an adverse effect. In some embodiments, the detecting comprises labeling said marker with a label comprising a fluorescent moiety capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, said fluorescent moiety comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. group. In some embodiments, said fluorescent moiety comprises a dye selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, said fluorescent moiety comprises AlexaFluor 647. In some embodiments, said label further comprises a binding partner for said marker. In some embodiments, said binding partner comprises an antibody specific for said marker. In some embodiments, said antibody comprises a polyclonal antibody. In some embodiments, said antibody is a monoclonal antibody. In some embodiments, the method further comprises establishing a threshold level for said marker based on said first and second ranges, wherein the presence of said marker in a biological sample from an individual at a level above said threshold level indicates a substantially increased probability of the presence of the biological state in said individual.

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[0015] In some embodiments, the invention provides a method for detecting the presence or absence of a biological state of an organism, comprising i) measuring the concentration of a marker in a biological sample from said organism, wherein said marker is a marker established by a method comprising establishing a range of concentrations for said marker in biological samples obtained from a first population, where said establishing comprises measuring the concentrations of said marker in said biological samples by detecting single molecules of said marker; and ii) determining the presence of absence of said biological state based on said concentration of said marker in said organism.

[0016] In a further embodiment, the invention provides a method for detecting the presence or absence of a biological state in an organism, comprising i) measuring the concentrations of a marker in a plurality of biological samples from said organism, wherein said marker is a marker established by by a method comprising establishing a range of concentrations for said marker in biological samples obtained from a first population, where said establishing comprises measuring the concentrations of said marker in said biological samples by detecting single molecules of said marker; and ii) determining the presence of absence of said biological state based on said concentrations of said marker in said plurality of samples. In some embodiments, said samples are of different types. In some embodiments, said determining is based on a comparison of the concentrations of said marker in said different types of samples. In some embodiments, the samples are of the same type, and the samples are taken at intervals. In some embodiments, the biological sample is blood, plasma, or serum.

[0017] In some embodiments, the invention provides a business method comprising: identifying one or more markers using the system of claim 1 that is associated with a biological state of an organism; and commercializing said one or more markers in a diagnostic to detect the presence or absence of said biological state in said organism. In some embodiments, the business method of claim 36 wherein said one or more markers are polypeptides or small molecules. In some embodiments, one or more markers are new chemical entities. In some embodiments, said business method further comprises performing a diagnostic service for fee. In some embodiments, said business method further comprises selling a diagnostic product based on said one or more biomarkers.

[0018] In some embodiments, the invention provides a business method comprising: identifying one or more markers using a method comprising establishing a range of concentrations for said marker in biological samples obtained from a first population, where said establishing comprises measuring the concentrations of said marker in said biological samples by detecting single molecules of said marker that is associated with a biological state of an organism; and providing a diagnostic service of determining if said organism has or does not have said biological state. In some embodiments, said diagnostic service is provided by a CLIA approved laboratory. In some embodiments, said diagnostic service is provided to a health care provider, insurer or patient.

[0019] In one aspect, the invention provides compositions.

[0020] In some embodiments, the invention provides a label for the detection of a marker, wherein the label comprises a binding partner for the marker and a fluorescent moiety, wherein the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the fluorescent moiety comprises a fluorescent molecule. In some embodiments, the fluorescent moiety comprises a plurality of fluorescent molecules. In some embodiments, the label comprises about 2-10, 2-8, 2-6, 2-4, 3-10, 3-8, 3-6 fluorescent molecules. In some embodiments, the label comprises about 2-4 fluorescent molecules. In some embodiments, the fluorescent dye molecules comprise at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a

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conjugated substance. In some embodiments, the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the binding partner comprises an antibody. In some embodiments, said antibody is a monoclonal antibody. In some embodiments, said antibody is a polyclonal antibody. In some embodiments, said antibody is specific for a marker of inflammation. In some embodiments, said antibody is specific for a marker selected from the group consisting of chemokines, growth factors, proteases and inhibitors, cell adhesion molecules, stem cell factors, signal transduction and apoptosis molecules, neurotrophic factors, and developmental proteins. In some embodiments, the antibody is specific for a growth factor. In some embodiments, the antibody is specific for a cytokine. In some embodiments, the cytokine is selected from the group consisting of IL-12p70, IL-10, IL-1 alpha, IL-3, IL-12 p40, IL-1ra, IL-12, IL-6, IL-4, IL-18, IL-10, IL-5, eotaxin, IL-16, MIG, IL-8, IL-17, IL-7, IL-15, IL-13, IL-2R (soluble), IL-2, LIF/HILDA, IL-1 beta, Fas/CD95/Apo-1, or MCP-1. In some emboediments, the fluorescent moiety comprises a fluorescent molecule. In some embodiments, the fluorescent moiety comprises a plurality of fluorescent molecules. In some embodiments, the label comprises about 2-10, 2-8, 2-6, 2-4, 3-10, 3-8, or 3-6 fluorescent molecules. In some embodiments, the label comprises about 2-4 fluorescent molecules. In some embodiments, the fluorescent dye molecules comprise at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the fluorescent molecules are AlexaFluor 647 molecules.

INCORPORATION BY REFERENCE

[0021] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0023] Figures 1A and 1B. Schematic diagram of the arrangement of the components of a single particle analyzer. Figure 3A shows an analyzer that includes one electromagnetic source and one electromagnetic detector; Figure 3B shows an analyzer that includes two electromagnetic sources and one electromagnetic detectors.

[0024] Figures 2A and 2B. Schematic diagrams of a capillary flow cell for a single particle analyzer. Figure 4A shows the flow cell of an analyzer that includes one electromagnetic source; and Figure 4B shows the flow cell of an analyzer that includes two electromagnetic sources.

[0025] Figures 3A and 3B. Schematic diagrams showing the conventional (A) and confocal (B) positioning of laser and detector optics of a single particle analyzer. Figure 5A shows the arrangement for an analyzer that has one electromagnetic source and one electromagnetic detector; Figure 5B shows the arrangement for an analyzer that has two electromagnetic sources and two electromagnetic detectors.

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[0026] Figure 4. Linearized standard curve for the range concentrations of cTnI.

[0027] Figure 5. Biological threshold (cutoff concentration) for cTnI is at a cTnI concentration of 7pg/ml, as established at the 99th percentile with a corresponding CV of 10%.

[0028] Figure 6. Correlation of assay results of cTnI determined using the analyzer system of the invention with standard measurements provided by the National Institute of Standards and Technology (R2 = 0.9999).

[0029] Figure 7. Detection of cTnI in serial serum samples from patients who presented at the emergency room with chest pain. The measurements made with the analyzer system of the invention were compared to measurements made with a commercially available assay.

[0030] Figure 8. Distribution of normal biological concentrations of cTnI and concentrations of cTnI in serum samples from patients presenting with chest pain

[0031] Figure 9. Competition curve for LTE4. The LOD was determined to be 1.5 pg/ml LTE4.

[0032] Figure 10. Graph showing the standard curve for concentrations of Akt1. The LOD was calculated to be 25 pg/ml Akt1.

[0033] Figure 11. Graph showing the standard curve for concentrations of TGFβ. The LOD was calculated to be 350 pg/ml Akt1.

[0034] Figure 12 A schematic representation of a kit that includes an analyzer system for detecting a single protein molecule in a sample and least one label that inlcudes a fluorescent moiety and a binding partner for the protein molecule, where the analyzer includes an electromagnetic radiation source for stimulating the fluorescent moiety; a capillary flow cell for passing the label; a source of motive force for moving the label in the capillary flow cell; an interrogation space defined within the capillary flow cell for receiving electromagnetic radiation emitted from the electromagnetic source; and an electromagnetic radiation detector operably connected to the interrogation space for measuring an electromagnetic characteristic of the stimulated fluorescent moiety, where the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules

[0035] Fig. 13: Standard curve of TREM-1 measured in a sandwich molecule immunoassay developed for the single particle analyzer system. The linear range of the assay is 100-1500 fM.

[0036] Fig. 14 A-F. Detection of IL-6 and IL-8. A-B) Standard curve for IL-6. A) IL-6 standards, diluted according to a commercially available kit (R & D Systems, Minneapolis, MN) gave a linear response between 0.1 and 10 pg/ml. B) IL-6 standard curve below 1 pg/ml. C) Distribution of IL-6 and IL-8 identified in blood bank donor EDTA specimens. D) Range of detection at low concentrations of any analyte can be extended to higher concentrations by switching the detection of the analyzer from counting molecules (digital signal) to detecting the sum of photons (analog signal) that are generated at the higher concentrations of analyte. The single particle analyzer has an expanded linear dynamic range of 6 logs. E) Six-log range of detection based on switching from digital to analog detection. F) Non-linearized standard curve showing range of low concentrations of IL-6 (0.1 fg/ml- 10 fg/ml) determined by counting photons emitted by individual particles (digital signal), and higher range of concentrations of IL-6 (10 fg/ml – 1 pg/ml).

Fig. 15. Comparison of assays of the invention with conventional assays.

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DETAILED DESCRIPTION OF THE INVENTION

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I. Introduction

[0037] The invention provides instruments, kits, compositions, and methods for the highly sensitive detection of single molecules, and for the determination of the concentration of the molecules in a sample. The sensitivity and precision of the instruments, compositions, methods, and kits of the invention may be achieved in some embodiments by a combination of factors selected from, but not limited to, electromagnetic sources of appropriate wavelength and power output, appropriate interrogation space size, high numerical aperture lenses, detectors capable of detecting single photons, and data analysis systems for counting single molecules. The instruments of the invention are referred to as "single molecule detectors" or "single particle detectors," and are also encompassed by the terms "single molecule analyzers" and "single particle analyzers." The sensitivity and precision of the kits and methods of the invention are achieved in some embodiments by the use of the instruments of the invention together with by a combination of factors selected from, but not limited to, labels for molecules that exhibit characteristics that allow the molecules to be detected at the level of the single molecule, and methods assaying the label in the instruments described herein. "

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[0038] The instruments, kits, and methods of the invention are especially useful in the sensitive and precise detection of single protein molecules or small molecules, and for the determination of the concentration of said molecules in a sample.

[0039] Thus the invention provides, in some embodiments, instruments and kits for the sensitive detection and determination of concentration of molecules by detection of single molecules, labels for such detection and determination, and methods using such instruments and labels in the analysis of samples. In particular, the sensitivity and precision of the instruments, kits, and methods of the invention make possible the detection and determination of concentration of molecules, e.g., markers for biological states, at extremely low concentrations, e.g., concentrations below about 100, 10, 1, 0.1, 0.01, or 0.001 femtomolar. In further embodiments, the instruments and kits of the invention are capable of determining a concentration of a species, e.g., molecule, in a sample over a large dynamic range of concentrations without the need for dilution or other treatment of samples, e.g., over a concentration range of more than 10^5-fold, 10^6-fold, or 10^7-fold

[0040] The high sensitivity of the instruments, kits, and methods of the invention allows the establishment of uses for markers, e.g., biological markers, that have not previously been possible because of a lack of sensitivity of detection, as well as the establishment of new markers. There are numerous markers currently available which, while potentially of use in determining a biological state, are not currently of practical use because their lower ranges are unknown. In some cases, abnormally high levels of the marker are detectable by current methodologies, but normal ranges have not been established. In some cases, upper normal ranges of the marker are detectable, but not lower normal ranges, or levels below normal. In some cases, for example, markers specific to tumors, or markers of infection, any level of the marker indicates the potential presence of the biological state, and enhancing sensitivity of detection is an advantage for early diagnosis. In some cases, the rate of change, or lack of change, in the concentration of the marker over multiple timepoints provides the most useful information, but present methods of analysis do not permit timepoint sampling in the early stages of a condition, when it is typically at its most treatable. In some cases, the marker may be detected at clinically useful levels only through the use of cumbersome methods that are not practical or useful in a clinical setting, such as methods that require complex sample treatment and time-consuming analysis. In addition, there are potential markers of biological states that exist in sufficiently low concentrations that their presence remains extremely difficult or impossible to detect by current methods.

[0041] The analytical methods and compositions of the present invention provide levels of sensitivity, precision, and robustness that allow the detection of markers for biological states at concentrations at which the markers have been previously undetectable, thus allowing the "repurposing" of such markers from confirmatory markers, or markers useful only in limited research settings, to diagnostic, prognostic, treatment-directing, or other types of markers useful in clinical settings and/or in large-scale clinical settings such as clinical trials. Such methods allow the determination of normal and abnormal ranges for such markers

[0042] The markers thus repurposed can be used for, e.g., detection of normal state (normal ranges), detection of responder/non-responder (e.g., to a treatment, such as administration of a drug); early disease or pathological occurrence detection (e.g., detection of cancer in its earliest stages, early detection of cardiac ischemia); disease staging (e.g., cancer); disease monitoring (e.g., diabetes monitoring, monitoring for recurrence of cancer after treatment); study of disease mechanism; and study of treatment toxicity, such as toxicity of drug treatments.

[0043] The invention thus provides methods and compositions for the sensitive detection of markers, and further methods of establishing values for normal and abnormal levels of the markers. In further embodiments, the invention provides methods of diagnosis, prognosis, and/or treatment selection based on values established for the

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markers. The invention also provides compositions for use in such methods, e.g., detection reagents for the ultrasensitive detection of markers.

II. Molecules for Sensitive Detection By the Methods and Compositions of the Invention

[0044] The instruments, kits and methods of the invention can be used for the sensitive detection and determination of concentration of a number of different types of single molecules. In particular, the instruments, kits, and methods are useful in the sensitive detection and determination of concentration of markers of biological states. "Detection of a single molecule," as that term is used herein, refers to both direct and indirect detection. For example, a single molecule may be labeled with a fluorescent label, and the molecule-label complex detected in the instruments described herein. Alternatively, a single molecule may be labled with a fluorescent label, then the fluorescent label is detached from the single molecule, and the label detected in the instruments described herein. The term detection of a single molecule encompasses both forms of detection.

A. General

[0045] Examples of molecules which can be detected using the analyzer and related methods of the present invention include: biopolymers such as proteins, nucleic acids, carbohydrates, and small molecules, both organic and inorganic. In particular, the instruments, kits, and methods described herein are useful in the detection of single molecules of proteins and small molecules in biological samples, and the determination of concentration of such molecules in the sample.

[0046] The terms "protein," "polypeptide," "peptide," and "oligopeptide," are used interchangeably herein and include any composition that includes two or more amino acids joined together by a peptide bond. It may be appreciated that polypeptides can contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids. Also, polypeptides can include one or more amino acids, including the terminal amino acids, which are modified by any means known in the art (whether naturally or non-naturally). Examples of polypeptide modifications include e.g., by glycosylation, or other-post-translational modification. Modifications which may be present in polypeptides of the present invention, include, but are not limited to, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a polynucleotide or polynucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

[0047] The molecules detected by the present instruments, kits, and methods may be free or may be part of a complex, e.g., an antibody-antigen complex, or more generally a protein-protein complex, e.g., complexes of troponin.

B. Markers of Biological States

[0048] In some embodiments, the invention provides compositions and methods for the sensitive detection of biological markers, and for the use of such markers in diagnosis, prognosis, and/or determination of methods of treatment.

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[0049] Markers of the present invention may be, for example, any composition and/or molecule or a complex of compositions and/or molecules that is associated with a biological state of an organism (e.g., a condition such as a disease or a non-disease state). A marker can be, for example, a small molecule, a polypeptide, a nucleic acid, such as DNA and RNA, a lipid, such as a phospholipid or a micelle, a cellular component such as a mitochondrion or chloroplast, etc. Markers contemplated by the present invention can be previously known or unknown. For example, in some embodiments, the methods herein may identify novel polypeptides that can be used as markers for a biological state of interest or condition of interest, while in other embodiments, known polypeptides are identified as markers for a biological state of interest or condition. Using the systems of the invention it is possible that one can observe those markers, e.g., polypeptides with high potential use in determining the biological state of an organism, but that are only present at low concentrations, such as those "leaked" from diseased tissue. Other high potentially useful markers or polypeptides may be those that are related to the disease, for instance, those that are generated in the tumor-host environment. Any suitable marker that provides information regarding a biological state may be used in the methods and compositions of the invention. A "marker," as that term is used herein, encompasses any molecule that may be detected in a sample from an organism and whose detection or quantitation provides information about the biological state of the organism.

[0050] Biological states include but are not limited to phenotypic states; conditions affecting an organism; states of development; age; health; pathology; disease detection, process, or staging; infection; toxicity; or response to chemical, environmental, or drug factors (such as drug response phenotyping, drug toxicity phenotyping, or drug effectiveness phenotyping).

[0051] The term "organism" as used herein refers to any living being comprised of a least one cell. An organism can be as simple as a one cell organism or as complex as a mammal. An organism of the present invention is preferably a mammal. Such mammal can be, for example, a human or an animal such as a primate (e.g., a monkey, chimpanzee, etc.), a domesticated animal (e.g., a dog, cat, horse, etc.), farm animal (e.g., goat, sheep, pig, cattle, etc.), or laboratory animal (e.g., mouse, rat, etc.). Preferably, an organism is a human.

[0052] In some embodiments, the methods and compositions of the invention are directed to classes of markers, e.g., cytokines, growth factors, oncology markers, markers of inflammation, endocrine markers, autoimmune markers, thyroid markers, cardiovascular markers, markers of diabetes, markers of infectious disease, neurological markers, respiratory markers, gastrointestinal markers, musculoskeletal markers, dermatological disorders, and metabolic markers.

[0053] Table 1, below, provides examples of these classes of markers that have been measured by the methods and compositions of the invention, and provides the concentration of the markers as detected by the methods and compositions of the invention and number of particles that are counted by the single particle analyzer system of the invention for the particular marker.

Table 1

CLASSES OF MARKERS AND EXEMPLARY MARKERS IN THE CLASSES

Cytokines	Molar Conc.	Molecules
IL-12 p70	2.02×10^{-14}	$6.09 \times 10^{+5}$
IL-10	5.36×10^{-14}	$1.61 \times 10^{+6}$
IL-1 alpha	5.56×10^{-14}	$1.67 \times 10^{+6}$

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IL-3	5.85×10^{-14}	$1.76 \times 10^{+6}$
IL-12 p40	6.07×10^{-14}	$1.83 \times 10^{+6}$
IL-1ra	6.12×10^{-14}	$1.84 \times 10^{+6}$
IL-12	8.08×10^{-14}	$2.44 \times 10^{+6}$
IL-6	9.53×10^{-14}	$2.87 \times 10^{+6}$
IL-4	1.15×10^{-13}	$3.47 \times 10^{+6}$
IL-18	1.80×10^{-13}	$5.43 \times 10^{+6}$
IP-10	1.88×10^{-13}	$1.13 \times 10^{+7}$
IL-5	1.99×10^{-13}	$5.98 \times 10^{+6}$
Eotaxin	2.06×10^{-13}	$1.24 \times 10^{+7}$
IL-16	3.77×10^{-13}	$1.14 \times 10^{+7}$
MIG	3.83×10^{-13}	$1.15 \times 10^{+7}$
IL-8	4.56×10^{-13}	$1.37 \times 10^{+7}$
IL-17	5.18×10^{-13}	$1.56 \times 10^{+7}$
IL-7	5.97×10^{-13}	$1.80 \times 10^{+7}$
IL-15	6.13×10^{-13}	$1.84 \times 10^{+7}$
IL-13	8.46×10^{-13}	$2.55 \times 10^{+7}$
IL-2R (soluble)	8.89×10^{-13}	$2.68 \times 10^{+7}$
IL-2	8.94×10^{-13}	$2.69 \times 10^{+7}$
LIF/HILDA	9.09×10^{-13}	$5.47 \times 10^{+7}$
IL-1 beta	1.17×10^{-12}	$3.51 \times 10^{+7}$
Fas/CD95/Apo-1	1.53×10^{-12}	$9.24 \times 10^{+7}$
MCP-1	2.30×10^{-12}	$6.92 \times 10^{+7}$
Oncology	**Molar Conc.**	**Molecules**
EGF	4.75×10^{-14}	$2.86 \times 10^{+6}$
TNF-alpha	6.64×10^{-14}	$8.00 \times 10^{+6}$
PSA (3rd generation)	1.15×10^{-13}	$6.92 \times 10^{+6}$
VEGF	2.31×10^{-13}	$6.97 \times 10^{+6}$
TGF-beta1	2.42×10^{-13}	$3.65 \times 10^{+7}$
FGFb	2.81×10^{-13}	$1.69 \times 10^{+7}$
TRAIL	5.93×10^{-13}	$3.57 \times 10^{+7}$
TNF-RI (p55)	2.17×10^{-12}	$2.62 \times 10^{+8}$
Inflammation	**Molar Conc.**	**Molecules**
ICAM-1 (soluble)	8.67×10^{-15}	$5.22 \times 10^{+4}$
RANTES	6.16×10^{-14}	$3.71 \times 10^{+6}$
MIP-2	9.92×10^{-14}	$2.99 \times 10^{+6}$
MIP-1 beta	1.98×10^{-13}	$5.97 \times 10^{+6}$
MIP-1 alpha	2.01×10^{-13}	$6.05 \times 10^{+6}$
MMP-3	1.75×10^{-12}	$5.28 \times 10^{+7}$

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Endocrinology	Molar Conc.	Molecules
17 beta-Estradiol (E2)	4.69×10^{-14}	$2.82 \times 10^{+6}$
DHEA	4.44×10^{-13}	$2.67 \times 10^{+7}$
ACTH	1.32×10^{-12}	$7.96 \times 10^{+7}$
Gastrin	2.19×10^{-12}	$1.32 \times 10^{+8}$
Growth Hormone (hGH)	2.74×10^{-12}	$1.65 \times 10^{+8}$
Autoimmune	**Molar Conc.**	**Molecules**
GM-CSF	1.35×10^{-13}	$8.15 \times 10^{+6}$
C-Reactive Protein (CRP)	3.98×10^{-13}	$2.40 \times 10^{+7}$
G-CSF	1.76×10^{-12}	$1.06 \times 10^{+8}$
Thyroid	**Molar Conc.**	**Molecules**
Cyclic AMP	9.02×10^{-15}	$5.43 \times 10^{+5}$
Calcitonin	3.25×10^{-14}	$1.95 \times 10^{+6}$
Parathyroid Hormone (PTH)	1.56×10^{-13}	$9.37 \times 10^{+6}$
Cardiovascular	**Molar Conc.**	**Molecules**
B-Natriuretic Pepide	2.86×10^{-13}	$1.72 \times 10^{+7}$
NT-proBNP	2.86×10^{-12}	$8.60 \times 10^{+7}$
C-Reactive Protein, HS	3.98×10^{-13}	$2.40 \times 10^{+7}$
Beta-Thromboglobulin (BTG)	*5.59×10^{-13}*	$3.36 \times 10^{+7}$
Diabetes	**Molar Conc.**	**Molecules**
C-Peptide	2.41×10^{-15}	$1.45 \times 10^{+5}$
Leptin	1.89×10^{-13}	$1.14 \times 10^{+7}$
Infectious Dis.	**Molar Conc.**	**Molecules**
IFN-gamma	2.08×10^{-13}	$1.25 \times 10^{+7}$
IFN-alpha	4.55×10^{-13}	$2.74 \times 10^{+7}$
Metabolism	**Molar Conc.**	**Molecules**
Bio-Intact PTH (1-84)	1.59×10^{-12}	$1.44 \times 10^{+8}$
PTH	1.05×10^{-13}	$9.51 \times 10^{+6}$

[0054] Cytokines For both research and diagnostics, cytokines are useful as markers of a number of conditions, diseases, pathologies, and the like, and the compositions and methods of the invention include labels for detection and quantitation of cytokines and methods using such labels to determine normal and abnormal levels of cytokines, as well as methods of diagnosis, prognosis, and/or determination of treatment based on such levels.

[0055] There are currently over 100 cytokines/chemokines whose coordinate or discordant regulation is of clinical interest. In order to correlate a specific disease process with changes in cytokine levels, the ideal approach requires analyzing a sample for a given cytokine, or multiple cytokines, with high sensitivity. Exemplary cytokines that are presently used in marker panels and that may be used in methods and compositions of the invention include, but are not limited to, BDNF, CREB pS133, CREB Total, DR-5, EGF,ENA-78, Eotaxin, Fatty Acid Binding Protein, FGF-basic, granulocyte colony-stimulating factor (G-CSF), GCP-2 , Granulocyte-macrophage Colony-stimulating Factor GM-CSF (GM-CSF), growth-related oncogene - keratinocytes (GRO-KC), HGF, ICAM-1, IFN-alpha, IFN-gamma, the interleukins IL-10, IL-11, IL-12, IL-12 p40, IL-12 p40/p70, IL-12 p70, IL-13, IL-15, IL-16, IL-17, IL-18, IL-

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1alpha, IL-1beta, IL-1ra, IL-1ra/IL-1F3, IL-2 , IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, interferon-inducible protein (10 IP-10), JE/MCP-1, keratinocytes (KC), KC/GROa, LIF, Lymphotacin, M-CSF, monocyte chemoattractant protein-1 (MCP-1), MCP-1(MCAF), MCP-3, MCP-5, MDC, MIG, macrophage inflammatory (MIP-1 alpha), MIP-1 beta, MIP-1 gamma, MIP-2, MIP-3 beta, OSM, PDGF-BB, regulated upon activation, normal T cell. expressed and secreted (RANTES), Rb (pT821), Rb (total), Rb pSpT249/252, Tau (pS214), Tau (pS396), Tau (total), Tissue Factor, tumor necrosis factor-alpha (TNF-alpha), TNF-beta, TNF-RI, TNF-RII, VCAM-1, and VEGF. In some embodiments, the cytokine is IL-12p70, IL-10, IL-1 alpha, IL-3, IL-12 p40, IL-1ra, IL-12, IL-6, IL-4, IL-18, IL-10, IL-5, eotaxin, IL-16, MIG, IL-8, IL-17, IL-7, IL-15, IL-13, IL-2R (soluble), IL-2, LIF/HILDA, IL-1 beta, Fas/CD95/Apo-1, or MCP-1.

[0056] Growth factors Growth factors include EGF Ligands such as Amphiregulin, LRIG3, Betacellulin, Neuregulin-1/NRG1, EGF, Neuregulin-3/NRG3, Epigen, TGF-alpha, Epiregulin, TMEFF1/Tomoregulin-1, HB-EGF, TMEFF2, LRIG1; EGF R/ErbB Receptor Family such as EGF R, ErbB3, ErbB2, ErbB4; FGF Family such as FGF LigandsFGF acidic, FGF-12, FGF basic, FGF-13, FGF-3, FGF-16, FGF-4, FGF-17, FGF-5, FGF-19, FGF-6, FGF-20, FGF-8, FGF-21, FGF-9, FGF-22, FGF-10, FGF-23, FGF-11, KGF/FGF-7, FGF Receptors FGF R1-4, FGF R3, FGF R1, FGF R4, FGF R2, FGF R5, FGF Regulators FGF-BP; the Hedgehog Family Desert Hedgehog, Sonic Hedgehog, Indian Hedgehog; Hedgehog Related Molecules & Regulators BOC, GLI-3, CDO, GSK-3 alpha/beta, DISP1, GSK-3 alpha, Gas1, GSK-3 beta, GLI-1, Hip, GLI-2; the IGF FamilyIGF LigandsIGF-I, IGF-II, IGF-I Receptor (CD221)IGF-I R , and IGF Binding Protein (IGFBP) Family ALS, IGFBP-5, CTGF/CCN2, IGFBP-6, Cyr61/CCN1, IGFBP-L1, Endocan, IGFBP-rp1/IGFBP-7, IGFBP-1, IGFBP-rP10, IGFBP-2, NOV/CCN3, IGFBP-3, WISP-1/CCN4, IGFBP-4; Receptor Tyrosine Kinases Axl, FGF R4, C1q R1/CD93, FGF R5, DDR1, Flt-3, DDR2, HGF R, Dtk, IGF-I R, EGF, R IGF-II R, Eph, INSRR, EphA1, Insulin R/CD220, EphA2, M-CSF R, EphA3, Mer, EphA4, MSP R/Ron, EphA5, MuSK, EphA6, PDGF R alpha, EphA7, PDGF R beta, EphA8, Ret, EphB1, RTK-like Orphan Receptor 1/ROR1, EphB2, RTK-like Orphan Receptor 2/ROR2, EphB3, SCF R/c-kit, EphB4, Tie-1, EphB6, Tie-2, ErbB2, TrkA, ErbB3, TrkB, ErbB4, TrkC, FGF, R1-4 VEGF R, FGF R1, VEGF R1/Flt-1, FGF R2, VEGF R2/KDR/Flk-1, FGF R3, VEGF R3/Flt-4; Proteoglycans & Regulators Proteoglycans Aggrecan, Mimecan, Agrin, NG2/MCSP, Biglycan, Osteoadherin, Decorin, Podocan, DSPG3, delta-Sarcoglycan, Endocan, Syndecan-1/CD138, Endoglycan, Syndecan-2, Endorepellin/Perlecan, Syndecan-3, Glypican 2, Syndecan-4, Glypican 3, Testican 1/SPOCK1, Glypican 5, Testican 2/SPOCK2, Glypican 6, Testican 3/SPOCK3, Lumican, Versican, Proteoglycan Regulators

Arylsulfatase A/ARSA, Glucosamine (N-acetyl)-6-Sulfatase/GNS, Exostosin-like 2/EXTL2, HS6ST2, Exostosin-like 3/EXTL3, Iduronate 2-Sulfatase/IDS, GalNAc4S-6ST; SCF, Flt-3 Ligand & M-CSF Flt-3, M-CSF R, Flt-3 Ligand, SCF, M-CSF, SCF R/c-kit; TGF-beta Superfamily (same as listed for inflammatory markers); VEGF/PDGF Family Neuropilin-1, PlGF, Neuropilin-2, PlGF-2, PDGF, VEGF, PDGF R alpha, VEGF-B, PDGF R beta, VEGF-C, PDGF-A, VEGF-D, PDGF-AB, VEGF R, PDGF-B, VEGF R1/Flt-1, PDGF-C, VEGF R2/KDR/Flk-1, PDGF-D, VEGF R3/Flt-4; Wnt-related Molecules Dickkopf Proteins & Wnt InhibitorsDkk-1, Dkk-4, Dkk-2, Soggy-1, Dkk-3, WIF-1 Frizzled & Related Proteins Frizzled-1, Frizzled-8, Frizzled-2, Frizzled-9, Frizzled-3, sFRP-1, Frizzled-4, sFRP-2, Frizzled-5, sFRP-3, Frizzled-6, sFRP-4, Frizzled-7, MFRP Wnt Ligands Wnt-1, Wnt-8a, Wnt-2b, Wnt-8b, Wnt-3a, Wnt-9a, Wnt-4, Wnt-9b, Wnt-5a, Wnt-10a, Wnt-5b, Wnt-10b, Wnt-7a, Wnt-11, Wnt-7b ; Other Wnt-related Molecules APC, Kremen-2, Axin-1, LRP-1, beta-Catenin, LRP-6, Dishevelled-1, Norrin, Dishevelled-3, PKC beta 1, Glypican 3, Pygopus-1, Glypican 5, Pygopus-2, GSK-3 alpha/beta, R-Spondin 1, GSK-3 alpha, R-Spondin 2, GSK-3 beta, R-Spondin 3, ICAT, RTK-like Orphan Receptor 1/ROR1, Kremen-1, RTK-like Orphan Receptor 2/ROR, and Other Growth Factors CTGF/CCN2, beta-NGF, Cyr61/CCN1, Norrin, DANCE, NOV/CCN3,

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EG-VEGF/PK1, Osteocrin, Hepassocin, PD-ECGF, HGF, Progranulin, LECT2, Thrombopoietin, LEDGF, WISP-1/CCN4.

[0057] Markers of Inflammation Markers of inflammation include ICAM-1, RANTES, MIP-2, MIP-1-beta, MIP-1-alpha, and MMP-3. Further markers of inflammation include Adhesion molecules such as the integrins α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, αVβ1, α4β7, α6β4, αDβ2, αLβ2, αMβ2, αVβ3, αVβ5, αVβ6, αVβ8, αXβ2, αIIbβ3, □IELbβ7, beta-2 integrin, beta-3 integrin, beta-2 integrin, beta-4 integrin, beta-5 integrin, beta-6 integrin, beta-7 integrin, beta-8 integrin, alpha-1 integrin, alpha-2 integrin, alpha-3 integrin, alpha-4 integrin, alpha-5 integrin, alpha-6 integrin, alpha-7 integrin, alpha-8 integrin, alpha-9 integrin, alpha-D integrin, alpha-L integrin, alpha-M integrin, alpha-V integrin, alpha-X integrin, alpha-IIb integrin, alphaIELb integrin,; Integrin-associated Molecules such as Beta IG-H3, Melusin, CD47, MEPE, CD151, Osteopontin, IBSP/Sialoprotein II, RAGE, IGSF8; Selectins such as E-Selectin, P-Selectin, L-Selectin,; Ligands such as CD34, GlyCAM-1, MadCAM-1, PSGL-1, vitronectic, vitronectin receptor, fibronectin, vitronectin, collagen, laminin. ICAM-1, ICAM-3, BL-CAM, LFA-2, VCAM-1, NCAM, PECAM. Further markers of inflammation include Cytokines such as IFN-α, IFN-β, IFN-ε, –κ, -τ, and –ζ, IFN-ω, IFN-γ, IL29, IL28A and IL28B, IL-1, IL-1□ and .□, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, TCCR/WSX-1. Further markers of inflammation include cytokine receptors such as Common beta chain, IL-3 R alpha, IL-3 R beta, GM-CSF R, IL-5 R alpha, Common gamma Chain/IL-2 R gamma, IL-2 R alpha, IL-9 R, IL-2 R beta, IL-4 R, IL-21 R, IL-15 R alpha, IL-7 R alpha/CD127, IL-1ra/IL-1F3, IL-1 R8, IL-1 RI, IL-1 R9, IL-1 RII, IL-18 R alpha/IL-1 R5, IL-1 R3/IL-1 R AcP, IL-18 R beta/IL-1 R7, IL-1 R4/ST2 SIGIRR, IL-1 R6/IL-1 R rp2, IL-11 R alpha, IL-31 RA, CNTF R alpha, Leptin R, G-CSF R, LIF R alpha, IL-6 R, OSM R beta, IFN-alpha/beta R1, IFN-alpha/beta R2, IFN-gamma R1, IFN-gamma R2, IL-10 R alpha, IL-10 R beta, IL-20 R alpha, IL-20 R beta, IL-22 R, IL-17 R, IL-17 RD, IL-17 RC, IL-17B R, IL-13 R alpha 2, IL-23 R, IL-12 R beta 1, IL-12 R beta 2, TCCR/WSX-1, IL-13 R alpha 1. Further markers of inflammation include Chemokines such as CCL-1, CCL -2, CCL -3, CCL -4, CCL -5, CCL -6, CCL -7, CCL -8, CCL -9, CCL -10, CCL -11, CCL -12, CCL -13, CCL -14, CCL -15, CCL -16, CCL -17, CCL -18, CCL -19, CCL -20, CCL -21, CCL -22, CCL -23, CCL -24, CCL -25, CCL -26, CCL -27, CCL -28, MCK-2, MIP-2, CINC-1, CINC-2, KC, CINC-3, LIX, GRO, Thymus Chemokine-1, CXCL-1, CXCL -2, CXCL -3, CXCL -4, CXCL -5, CXCL -6, CXCL -7, CXCL -8, CXCL -9, CXCL -10, CXCL -11, CXCL -12, CXCL -13, CXCL -14, CXCL -15, CXCL -16, CXCL -17, XCL1, XCL2, Chemerin. Further markers of inflammation include Chemokine receptors such as CCR-1, CCR -2, CCR -3, CCR -4, CCR -5, CCR -6, CCR -7, CCR -8, CCR -9, CCR-10, CXCR3, CXCR6, CXCR4, CXCR1, CXCR5, CXCR2, Chem R23. Further markers of inflammation include Tumor necrosis factors (TNFs), such as TNF.alpha., 4-1BB Ligand/TNFSF9, LIGHT/TNFSF14, APRIL/TNFSF13, Lymphotoxin, BAFF/TNFSF13B, Lymphotoxin beta/TNFSF3, CD27 Ligand/TNFSF7, OX40 Ligand/TNFSF4, CD30 Ligand/TNFSF8, TL1A/TNFSF15, CD40 Ligand/TNFSF5, TNF-alpha/TNFSF1A, EDA, TNF-beta/TNFSF1B, EDA-A2, TRAIL/TNFSF10, Fas Ligand/TNFSF6, TRANCE/TNFSF11, GITR Ligand/TNFSF18 ,TWEAK/TNFSF12. Further markers of inflammation include TNF Superfamily Receptors such as 4-1BB/TNFRSF9, NGF R/TNFRSF16, BAFF R/TNFRSF13C, Osteoprotegerin/TNFRSF11B, BCMA/TNFRSF17, OX40/TNFRSF4, CD27/TNFRSF7, RANK/TNFRSF11A, CD30/TNFRSF8, RELT/TNFRSF19L, CD40/TNFRSF5, TACI/TNFRSF13B, DcR3/TNFRSF6B, TNF RI/TNFRSF1A, DcTRAIL R1/TNFRSF23, TNF RII/TNFRSF1B, DcTRAIL R2/TNFRSF22, TRAIL R1/TNFRSF10A, DR3/TNFRSF25, TRAIL R2/TNFRSF10B, DR6/TNFRSF21, TRAIL R3/TNFRSF10C, EDAR, TRAIL R4/TNFRSF10D, Fas/TNFRSF6, TROY/TNFRSF19, GITR/TNFRSF18, TWEAK R/TNFRSF12, HVEM/TNFRSF14, XEDAR. Further markers of inflammation include TNF Superfamily

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Regulators such as FADD, TRAF-2, RIP1, TRAF-3, TRADD, TRAF-4, TRAF-1, TRAF-6. Further markers of inflammation include Acute-phase reactants and acute phase proteins. Further markers of inflammation include TGF-beta superfamily ligands such as Activins, Activin A, Activin B, Activin AB, Activin C, BMPs (Bone Morphogenetic Proteins), BMP-2, BMP-7, BMP-3, BMP-8, BMP-3b/GDF-10, BMP-9, BMP-4, BMP-10, BMP-5, BMP-15/GDF-9B, BMP-6, Decapentaplegic, Growth/Differentiation Factors (GDFs), GDF-1, GDF-8, GDF-3, GDF-9 GDF-5, GDF-11, GDF-6, GDF-15, GDF-7, GDNF Family Ligands, Artemin, Neurturin, GDNF, Persephin, TGF-beta, TGF-beta , TGF-beta 3, TGF-beta 1, TGF-beta 5, LAP (TGF-beta 1), Latent TGF-beta bp1, Latent TGF-beta 1, Latent TGF-beta bp2, TGF-beta 1.2, Latent TGF-beta bp4, TGF-beta 2, Lefty, MIS/AMH, Lefty-1, Nodal, Lefty-A , Activin RIA/ALK-2, GFR alpha-1/GDNF R alpha-1, Activin RIB/ALK-4, GFR alpha-2/GDNF R alpha-2, Activin RIIA, GFR alpha-3/GDNF R alpha-3, Activin RIIB, GFR alpha-4/GDNF R alpha-4, ALK-1, MIS RII, ALK-7, Ret, BMPR-IA/ALK-3, TGF-beta RI/ALK-5 ,BMPR-IB/ALK-6, TGF-beta RII, BMPR-II, TGF-beta RIIb, Endoglin/CD105, TGF-beta RIII. Further markers of inflammation include TGF-beta superfamily Modulators such as Amnionless, NCAM-1/CD56, BAMBI/NMA, Noggin, BMP-1/PCP, NOMO, Caronte, PRDC, Cerberus 1, SKI, Chordin, Smad1, Chordin-Like 1, Smad2, Chordin-Like 2, Smad3, COCO, Smad4, CRIM1, Smad5, Cripto, Smad7, Crossveinless-2, Smad8, Cryptic, SOST, DAN, Latent TGF-beta bp1, Decorin, Latent TGF-beta bp2, FLRG, Latent TGF-beta bp4, Follistatin, TMEFF1/Tomoregulin-1, Follistatin-like 1, TMEFF2, GASP-1/WFIKKNRP, TSG, GASP-2/WFIKKN, TSK, Gremlin, Vasorin. Further markers of inflammation include EGF Ligands such as Amphiregulin, LRIG3, Betacellulin, Neuregulin-1/NRG1, EGF, Neuregulin-3/NRG3, Epigen, TGF-alpha, Epiregulin, TMEFF1/Tomoregulin-1, HB-EGF, TMEFF2, LRIG1. Further markers of inflammation include EGF R/ErbB Receptor Family, such as EGF R, ErbB3, ErbB2, ErbB4. Further markers of inflammation include Fibrinogen. Further markers of inflammation include SAA. Further markers of inflammation include glial markers, such as alpha.1-antitrypsin, C-reactive protein (CRP), .alpha.2-macroglobulin, glial fibrillary acidic protein (GFAP), Mac-1, F4/80. Further markers of inflammation include myeloperoxidase. Further markers of inflammation include Complement markers such as C3d, C1q, C5, C4d, C4bp, and C5a-C9. Further markers of inflammation include Major histocompatibility complex (MHC) glycoproteins, such as HLA-DR and HLA-A, D,C. Further markers of inflammation include Microglial markers, such as CR3 receptor, MHC I, MHC II, CD 31, CD11a, CD11b, CD11c, CD68, CD45RO, CD45RD, CD18, CD59, CR4, CD45, CD64, and CD44. Further markers of inflammation include alpha.2 macroglobulin receptor, Fibroblast growth factor, Fc gamma RI, Fc gamma RII, CD8, LCA (CD45), CD18 (), CD59, Apo J, clusterin, type 2 plasminogen activator inhibitor,. CD44, Macrophage colony stimulating factor receptor, MRP14, 27E10, 4-hydroxynonenal-protein conjugates , I.kappa.B, NF.kappa.B, cPLA.sub.2, COX-2, Matrix metalloproteinases, Membrane lipid peroxidation, and ATPase activity. HSPC228, EMP1, CDC42, TLE3, SPRY2, p40BBP, HSPC060 or NAB2, or a down-regulation of HSPA1A, HSPA1B, MAPRE2 and OAS1 expression, TACE/ADAM17, alpha-1-Acid Glycoprotein, Angiopoietin-1, MIF, Angiopoietin-2, CD14, beta-Defensin 2, MMP-2, ECF-L/CHI3L3, MMP-7, EGF, MMP-9, EMAP-II, MSP, EN-RAGE, Nitric Oxide, Endothelin-1, Osteoactivin/GPNMB , FPR1, PDGF, FPRL1, Pentraxin 3/TSG-14, FPRL2, Gas6, PLUNC, GM-CSF, RAGE, S100A10, S100A8, S100A9, HIF-1 alpha, Substance P, TFPI, TGF-beta 1, TIMP-1, TIMP-2, TIMP-3, TIMP-4, TLR4, LBP, TREM-1, Leukotriene A4, Hydrolase TSG-6, Lipocalin-1, uPA, M-CSF, and VEGF

[0058] Oncology markers include EGF, TNF-alpha, PSA, VEGF, TGF-beta1, FGFb, TRAIL, and TNF-RI (p55)

[0059] Markers of endocrine function include 17 beta-estradiol (E2), DHEA, ACTH, gastrin, and growth hormone (hGH).

[0060] Marker of autimmunity include GM-CSF, C-Reactive Protein, and G-CSF.

[0061] Markers of thyroid function include cyclicAMP, calcitonin, and parathyroid hormone.

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[0062] Cardiovascular markers include cardiac troponin I, cardiac troponin T, B-natriuretic peptide, NT-proBNP, C-ractive Protein HS, and betatrhromboglobulin.

[0063] Makers of diabetes inlcude C-peptide and leptin.

[0064] Markers of infectious disease include IFN-gamma and IFN-alpha.

[0065] Markers of metabolism include Bio-intact PTH (1-84) and PTH.

[0066] Markers of biological states Markers may indicate the presence of a particular phenotypic state of interest. Examples of phenotypic states include, phenotypes resulting from an altered environment, drug treatment, genetic manipulations or mutations, injury, change in diet, aging, or any other characteristic(s) of a single organism or a class or subclass of organisms.

[0067] In some embodiments, a phenotypic state of interest is a clinically diagnosed disease state. Such disease states include, for example, cancer, cardiovascular disease, inflammatory disease, autoimmune disease, neurological disease, infectious disease and pregnancy related disorders. Alternatively, states of health can be detected using markers.

[0068] Cancer phenotypes are included in some aspects of the invention. Examples of cancer herein include, but are not limited to: breast cancer, skin cancer, bone cancer, prostate cancer, liver cancer, lung cancer, brain cancer, cancer of the larynx, gallbladder, pancreas, rectum, parathyroid, thyroid, adrenal, neural tissue, head and neck, colon, stomach, bronchi, kidneys, basal cell carcinoma, squamous cell carcinoma of both ulcerating and papillary type, metastatic skin carcinoma, osteo sarcoma, Ewing's sarcoma, veticulum cell sarcoma, myeloma, giant cell tumor, small-cell lung tumor, non-small cell lung carcinoma gallstones, islet cell tumor, primary brain tumor, acute and chronic lymphocytic and granulocytic tumors, hairy-cell tumor, adenoma, hyperplasia, medullary carcinoma, pheochromocytoma, mucosal neuronms, intestinal ganglloneuromas, hyperplastic corneal nerve tumor, marfanoid habitus tumor, Wilm's tumor, seminoma, ovarian tumor, leiomyomater tumor, cervical dysplasia and in situ carcinoma, neuroblastoma, retinoblastoma, soft tissue sarcoma, malignant carcinoid, topical skin lesion, mycosis fungoide, rhabdomyosarcoma, Kaposi's sarcoma, osteogenic and other sarcoma, malignant hypercalcemia, renal cell tumor, polycythermia vera, adenocarcinoma, glioblastoma multiforma, leukemias, lymphomas, malignant melanomas, epidermoid carcinomas, and other carcinomas and sarcomas.

[0069] Cardivascular disease may be included in other applications of the invention. Examples of cardiovascular disease include, but are not limited to, congestive heart failure, high blood pressure, arrhythmias, atherosclerosis, cholesterol, Wolff-Parkinson-White Syndrome, long QT syndrome, angina pectoris, tachycardia, bradycardia, atrial fibrillation, ventricular fibrillation, congestive heart failure, myocardial ischemia, myocardial infarction, cardiac tamponade, myocarditis, pericarditis, arrhythmogenic right ventricular dysplasia, hypertrophic cardiomyopathy, Williams syndrome, heart valve diseases, endocarditis, bacterial, pulmonary atresia, aortic valve stenosis, Raynaud's disease, Raynaud's disease, cholesterol embolism, Wallenberg syndrome, Hippel-Lindau disease, and telangiectasis.

[0070] Inflammatory disease and autoimmune disease may be included in other embodiments of the invention. Examples of inflammatory disease and autoimmune disease include, but are not limited to, rheumatoid arthritis, non-specific arthritis, inflammatory disease of the larynx, inflammatory bowel disorder, psoriasis, hypothyroidism (e.g., Hashimoto thyroidism), colitis, Type 1 diabetes, pelvic inflammatory disease, inflammatory disease of the central nervous system, temporal arteritis, polymyalgia rheumatica, ankylosing spondylitis, polyarteritis nodosa, Reiter's syndrome, scleroderma, systemis lupus and erythematosus.

[0071] The methods and compositions of the invention can also provide laboratory information about markers of infectious disease including markers of Adenovirus, Bordella pertussis, Chlamydia pneumoiea, Chlamydia trachomais, Cholera Toxin, Cholera Toxin β, Campylobacter jejuni, Cytomegalovirus, Diptheria Toxin, Epstein-

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Barr NA, Epstein-Barr EA, Epstein-Barr VCA, Helicobacter Pylori, Hepatitis B virus (HBV) Core, Hepatitis B virus (HBV) Envelope, Hepatitis B virus (HBV) Survace (Ay), Hepatitis C virus (HCV) Core, Hepatitis C virus (HCV) NS3, Hepatitis C virus (HCV) NS4, Hepatitis C virus (IICV) NS5, Hepititis A, Hepititis D, Hepatitis E virus (HEV) orf2 3KD, Hepatitis E virus (HEV) orf2 6KD, Hepatitis E virus (HEV) orf3 3KD, Human immunodeficiency virus (HIV)-1 p24, Human immunodeficiency virus (HIV)-1 gp41, Human immunodeficiency virus (HIV)-1 gp120, Human papilloma virus (HPV), Herpes simplex virus HSV-1/2, Herpes simplex virus HSV-1 gD, Herpes simplex virus HSV-2 gG, Human T-cell leukemia virus (HTLV)-1/2, Influenza A, Influenza A H3N2, Influenza B, Leishmanina donovani, Lyme disease, Mumps, M. pneumoniae, M. teberculosis, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Polio Virus, Respiratory syncytial virus (RSV), Rubella, Rubeola, Streptolysin O, Tetanus Toxin, T. pallidum 15kd, T. pallidum p47, T. cruzi, Toxoplasma, and Varicella Zoater.

III. Labels

[0072] In some embodiments, the invention provides methods and compositions that include labels for the highly sensitive detection and quantitation of molecules, e.g., of markers.

[0073] One skilled in the art will recognize that many strategies can be used for labeling target molecules to enable their detection or discrimination in a mixture of particles. The labels may be attached by any known means, including methods that utilize non-specific or specific interactions of label and target. Labels may provide a detectable signal or affect the mobility of the particle in an electric field. In addition, labeling can be accomplished directly or through binding partners.

[0074] In some embodiments, the label comprises a binding partner to the molecule of interest, where the binding partner is attached to a fluorescent moiety. The compositions and methods of the invention may utilize highly fluorescent moieties, e.g., a moiety capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. Moieties suitable for the compositions and methods of the invention are described in more detail below.

[0075] In some embodiments, the invention provides a label for detecting a biological molecule comprising a binding partner for said biological molecule that is attached to a fluorescent moiety, wherein said fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the moiety comprises a plurality of fluorescent entities, e.g., about 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, or 3-5, 3-6, 3-7, 3-8, 3-9, or 3-10 fluorescent entities. In some embodiments, the moiety comprises about 2 to 4 fluorescent entities. In some embodiments, biological molecule is a protein or a small molecule, In some embodiments. the biological molecule is a protein. The fluorescent entities may be fluorescent dye molecules. In some embodiments, the fluorescent dye molecules comprise at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the dye molecules are AlexFluor molecules selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the dye molecules are AlexFluor molecules selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the dye molecules are AlexaFluor647 dye molecules. In some embodiments, the dye molecules comprise a first type and a second type of dye molecules,

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e.g., two different AlexaFluor molecules, e.g., where the first type and second type of dye molecules have different emission spectra. The ratio of the number of first type to second type of dye molecule may be, e.g., 4:1, 3:1, 2: 1, 1:1, 1:2, 1:3 or 1:4. The binding partner may be, e.g., an antibody.

[0076] In some embodiments, the invention provides a label for the detection of a marker, wherein the label comprises a binding partner for the marker and a fluorescent moiety, wherein the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the fluorescent moiety comprises a fluorescent molecule. In some embodiments, the fluorescent moiety comprises a plurality of fluorescent molecules, e.g., about 2-10, 2-8, 2-6, 2-4, 3-10, 3-8, 3-6 fluorescent molecules. In some embodiments, the label comprises about 2-4 fluorescent molecules. In some embodiments, the fluorescent dye molecules comprise at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the binding partner comprises an antibody. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, antibody is a polyclonal antibody.

[0077] The antibody may be specific to any suitable marker. In some embodiments, the antibody is specific to a marker that is selected from the group consisting of cytokines, growth factors, oncology markers, markers of inflammation, endocrine markers, autoimmune markers, thyroid markers, cardiovascular markers, markers of diabetes, markers of infectious disease, neurological markers, respiratory markers, gastrointestinal markers, musculoskeletal markers, dermatological disorders, and metabolic markers.

[0078] In some embodiments, the antibody is specific to a marker that is a cytokine. In some embodiments, the cytokine is selected from the group consisting of BDNF, CREB pS133, CREB Total, DR-5, EGF,ENA-78, Eotaxin, Fatty Acid Binding Protein, FGF-basic, granulocyte colony-stimulating factor (G-CSF), GCP-2 , Granulocyte-macrophage Colony-stimulating Factor GM-CSF (GM-CSF), growth-related oncogene - keratinocytes (GRO-KC), HGF, ICAM-1, IFN-alpha, IFN-gamma, the interleukins IL-10, IL-11, IL-12, IL-12 p40, IL-12 p40/p70, IL-12 p70, IL-13, IL-15, IL-16, IL-17, IL-18, IL-1alpha, IL-1beta, IL-1ra, IL-1ra/IL-1F3, IL-2 , IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, interferon-inducible protein (10 IP-10), JE/MCP-1, keratinocytes (KC), KC/GROa, LIF, Lymphotacin, M-CSF, monocyte chemoattractant protein-1 (MCP-1), MCP-1(MCAF), MCP-3, MCP-5, MDC, MIG, macrophage inflammatory (MIP-1 alpha), MIP-1 beta, MIP-1 gamma, MIP-2, MIP-3 beta, OSM, PDGF-BB, regulated upon activation, normal T cell. expressed and secreted (RANTES), Rb (pT821), Rb (total), Rb pSpT249/252, Tau (pS214), Tau (pS396), Tau (total), Tissue Factor, tumor necrosis factor-alpha (TNF-alpha), TNF-beta, TNF-RI, TNF-RII, VCAM-1, and VEGF.

[0079] In some embodiments, the cytokine is selected from the group consisting of IL-12p70, IL-10, IL-1 alpha, IL-3, IL-12 p40, IL-1ra, IL-12, IL-6, IL-4, IL-18, IL-10, IL-5, Eotaxin, IL-16, MIG, IL-8, IL-17, IL-7, IL-15, IL-13, IL-2R (soluble), IL-2, LIF/HILDA, IL-1 beta, Fas/CD95/Apo-1 and MCP-1.

[0080] In some embodiments, the antibody is specifc to a marker that is a growth factor. In some embodiments, the antibody is specifc to a marker that is a growth factor that is TGF-beta. In some embodiments, the growth factor is GF Ligands such as Amphiregulin, LRIG3, Betacellulin, Neuregulin-1/NRG1, EGF, Neuregulin-3/NRG3, Epigen, TGF-alpha, Epiregulin, TMEFF1/Tomoregulin-1, HB-EGF, TMEFF2, LRIG1; EGF R/ErbB Receptor Family such

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as EGF R, ErbB3, ErbB2, ErbB4; FGF Family such as FGF LigandsFGF acidic, FGF-12, FGF basic, FGF-13, FGF-3, FGF-16, FGF-4, FGF-17, FGF-5, FGF-19, FGF-6, FGF-20, FGF-8, FGF-21, FGF-9, FGF-22, FGF-10, FGF-23, FGF-11, KGF/FGF-7, FGF Receptors FGF R1-4, FGF R3, FGF R1, FGF R4, FGF R2, FGF R5, FGF Regulators FGF-BP; the Hedgehog Family Desert Hedgehog, Sonic Hedgehog, Indian Hedgehog; Hedgehog Related Molecules & Regulators BOC, GLI-3, CDO, GSK-3 alpha/beta, DISP1, GSK-3 alpha, Gas1, GSK-3 beta, GLI-1, Hip, GLI-2; the IGF FamilyIGF LigandsIGF-I, IGF-II, IGF-I Receptor (CD221)IGF-I R , and IGF Binding Protein (IGFBP) Family ALS, IGFBP-5, CTGF/CCN2, IGFBP-6, Cyr61/CCN1, IGFBP-L1, Endocan, IGFBP-rp1/IGFBP-7, IGFBP-1, IGFBP-rP10, IGFBP-2, NOV/CCN3, IGFBP-3, WISP-1/CCN4, IGFBP-4; Receptor Tyrosine Kinases Axl, FGF R4, C1q R1/CD93, FGF R5, DDR1, Flt-3, DDR2, HGF R, Dtk, IGF-I R, EGF, R IGF-II R, Eph, INSRR, EphA1, Insulin R/CD220, EphA2, M-CSF R, EphA3, Mer, EphA4, MSP R/Ron, EphA5, MuSK, EphA6, PDGF R alpha, EphA7, PDGF R beta, EphA8, Ret, EphB1, RTK-like Orphan Receptor 1/ROR1, EphB2, RTK-like Orphan Receptor 2/ROR2, EphB3, SCF R/c-kit, EphB4, Tie-1, EphB6, Tie-2, ErbB2, TrkA, ErbB3, TrkB, ErbB4, TrkC, FGF, R1-4 VEGF R, FGF R1, VEGF R1/Flt-1, FGF R2, VEGF R2/KDR/Flk-1, FGF R3, VEGF R3/Flt-4; Proteoglycans & Regulators Proteoglycans Aggrecan, Mimecan, Agrin, NG2/MCSP, Biglycan, Osteoadherin, Decorin, Podocan, DSPG3, delta-Sarcoglycan, Endocan, Syndecan-1/CD138, Endoglycan, Syndecan-2, Endorepellin/Perlecan, Syndecan-3, Glypican 2, Syndecan-4, Glypican 3, Testican 1/SPOCK1, Glypican 5, Testican 2/SPOCK2, Glypican 6, Testican 3/SPOCK3, Lumican, Versican, Proteoglycan Regulators

[0081] Arylsulfatase A/ARSA, Glucosamine (N-acetyl)-6-Sulfatase/GNS, Exostosin-like 2/EXTL2, HS6ST2, Exostosin-like 3/EXTL3, Iduronate 2-Sulfatase/IDS, GalNAc4S-6ST; SCF, Flt-3 Ligand & M-CSF Flt-3, M-CSF R, Flt-3 Ligand, SCF, M-CSF, SCF R/c-kit; TGF-beta Superfamily (same as listed for inflammatory markers); VEGF/PDGF Family Neuropilin-1, PlGF, Neuropilin-2, PlGF-2, PDGF, VEGF, PDGF R alpha, VEGF-B, PDGF R beta, VEGF-C, PDGF-A, VEGF-D, PDGF-AB, VEGF R, PDGF-B, VEGF R1/Flt-1, PDGF-C, VEGF R2/KDR/Flk-1, PDGF-D, VEGF R3/Flt-4; Wnt-related Molecules Dickkopf Proteins & Wnt InhibitorsDkk-1, Dkk-4, Dkk-2, Soggy-1, Dkk-3, WIF-1 Frizzled & Related Proteins Frizzled-1, Frizzled-8, Frizzled-2, Frizzled-9, Frizzled-3, sFRP-1, Frizzled-4, sFRP-2, Frizzled-5, sFRP-3, Frizzled-6, sFRP-4, Frizzled-7, MFRP Wnt Ligands Wnt-1, Wnt-8a, Wnt-2b, Wnt-8b, Wnt-3a, Wnt-9a, Wnt-4, Wnt-9b, Wnt-5a, Wnt-10a, Wnt-5b, Wnt-10b, Wnt-7a, Wnt-11, Wnt-7b ; Other Wnt-related Molecules APC, Kremen-2, Axin-1, LRP-1, beta-Catenin, LRP-6, Dishevelled-1, Norrin, Dishevelled-3, PKC beta 1, Glypican 3, Pygopus-1, Glypican 5, Pygopus-2, GSK-3 alpha/beta, R-Spondin 1, GSK-3 alpha, R-Spondin 2, GSK-3 beta, R-Spondin 3, ICAT, RTK-like Orphan Receptor 1/ROR1, Kremen-1, RTK-like Orphan Receptor 2/ROR, and Other Growth Factors CTGF/CCN2, beta-NGF, Cyr61/CCN1, Norrin, DANCE, NOV/CCN3, EG-VEGF/PK1, Osteocrin, Hepassocin, PD-ECGF, HGF, Progranulin, LECT2, Thrombopoietin, LEDGF, or WISP-1/CCN4.

[0082] In some embodiments, the antibody is specific to a marker that is a marker for cancer (oncology marker). In some embodiments, the antibody is specific to a marker that is a marker for cancer that is EGF. In some embodiments, the antibody is specific to a marker that is a marker for cancer that is TNF-alpha. In some embodiments, the antibody is specific to a marker that is a marker for cancer that is PSA. In some embodiments, the antibody is specific to a marker that is a marker for cancer that is VEGF. In some embodiments, the antibody is specific to a marker that is a marker for cancer that is TGF-beta. In some embodiments, the antibody is specific to a marker that is a marker for cancer that is FGFb. In some embodiments, the antibody is specific to a marker that is a marker for cancer that isTRAIL. In some embodiments, the antibody is specific to a marker that is a marker for cancer that is TNF-RI (p55).

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[0083] In further embodiments, the antibody is specific to a marker for cancer that is alpha-Fetoprotein. In some embodiments, the antibody is specific to a marker for cancer that is ER beta/NR3A2. In some embodiments, the antibody is specific to a marker for cancer that is ErbB2. In some embodiments, the antibody is specific to a marker for cancer that is Kallikrein 3/PSA. In some embodiments, the antibody is specific to a marker for cancer that is ER alpha/NR3A1. In some embodiments, the antibody is specific to a marker for cancer that is Progesterone R/NR3C3. In some embodiments, the antibody is specific to a marker for cancer that is A33. In some embodiments, the antibody is specific to a marker for cancer that is MIA. In some embodiments, the antibody is specific to a marker for cancer that is Aurora A. In some embodiments, the antibody is specific to a marker for cancer that is MMP-2. In some embodiments, the antibody is specific to a marker for cancer that is Bcl-2. In some embodiments, the antibody is specific to a marker for cancer that is MMP-3. In some embodiments, the antibody is specific to a marker for cancer that is Cadherin-13. In some embodiments, the antibody is specific to a marker for cancer that is MMP-9. In some embodiments, the antibody is specific to a marker for cancer that is E-Cadherin. In some embodiments, the antibody is specific to a marker for cancer that is NEK2. In some embodiments, the antibody is specific to a marker for cancer that is Carbonic Anhydrase IX. In some embodiments, the antibody is specific to a marker for cancer that is Nestin. In some embodiments, the antibody is specific to a marker for cancer that is beta-Catenin. In some embodiments, the antibody is specific to a marker for cancer that is NG2/MCSP. In some embodiments, the antibody is specific to a marker for cancer that is Cathepsin D. In some embodiments, the antibody is specific to a marker for cancer that is Osteopontin. In some embodiments, the antibody is specific to a marker for cancer that is CD44. In some embodiments, the antibody is specific to a marker for cancer that is p21/CIP1/CDKN1A. In some embodiments, the antibody is specific to a marker for cancer that is CEACAM-6. In some embodiments, the antibody is specific to a marker for cancer that is p27/Kip1. In some embodiments, the antibody is specific to a marker for cancer that is Cornulin. In some embodiments, the antibody is specific to a marker for cancer that is p53. In some embodiments, the antibody is specific to a marker for cancer that is DPPA4. In some embodiments, the antibody is specific to a marker for cancer that is Prolactin. In some embodiments, the antibody is specific to a marker for cancer that is ECM-1. In some embodiments, the antibody is specific to a marker for cancer that is PSP94. In some embodiments, the antibody is specific to a marker for cancer that is EGF. In some embodiments, the antibody is specific to a marker for cancer that is S100B. In some embodiments, the antibody is specific to a marker for cancer that is EGF R. In some embodiments, the antibody is specific to a marker for cancer that is S100P. In some embodiments, the antibody is specific to a marker for cancer that is EMMPRIN/CD147. In some embodiments, the antibody is specific to a marker for cancer that is SCF R/c-kit. In some embodiments, the antibody is specific to a marker for cancer that is Fibroblast Activation Protein alpha/FAP. In some embodiments, the antibody is specific to a marker for cancer that is Serpin E1/PAI-1. In some embodiments, the antibody is specific to a marker for cancer that is FGF acidic. In some embodiments, the antibody is specific to a marker for cancer that is Serum Amyloid A4. In some embodiments, the antibody is specific to a marker for cancer that is FGF basic. In some embodiments, the antibody is specific to a marker for cancer that is Survivin. In some embodiments, the antibody is specific to a marker for cancer that is Galectin-3. In some embodiments, the antibody is specific to a marker for cancer that is TEM8. In some embodiments, the antibody is specific to a marker for cancer that is Glypican 3. In some embodiments, the antibody is specific to a marker for cancer that is TIMP-1. In some embodiments, the antibody is specific to a marker for cancer that is HIN-1/Secretoglobulin 3A1. In some embodiments, the antibody is specific to a marker for cancer that is TIMP-2. In some embodiments, the antibody is specific to a marker for cancer that is IGF-I. In some embodiments, the antibody is specific to a marker for cancer that is TIMP-3. In some embodiments, the antibody is specific to a

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marker for cancer that is IGFBP-3. In some embodiments, the antibody is specific to a marker for cancer that is TIMP-4. In some embodiments, the antibody is specific to a marker for cancer that is IL-6. In some embodiments, the antibody is specific to a marker for cancer that is TNF-alpha/TNFSF1A. In some embodiments, the antibody is specific to a marker for cancer that is Kallikrein 6/Neurosin. In some embodiments, the antibody is specific to a marker for cancer that is TRAF-4. In some embodiments, the antibody is specific to a marker for cancer that is M-CSF. In some embodiments, the antibody is specific to a marker for cancer that is uPA. In some embodiments, the antibody is specific to a marker for cancer that is Matriptase/ST14. In some embodiments, the antibody is specific to a marker for cancer that is uPAR. In some embodiments, the antibody is specific to a marker for cancer that is Mesothelin. In some embodiments, the antibody is specific to a marker for cancer that is VCAM-1. In some embodiments, the antibody is specific to a marker for cancer that is Methionine Aminopeptidase. In some embodiments, the antibody is specific to a marker for cancer that is VEGF. In some embodiments, the antibody is specific to a marker for cancer that is Methionine Aminopeptidase 2.

[0084] In some embodiments, the antibody is specific to a marker that is a marker for inflammation. In some embodiments, the antibody is specific to a marker that is a marker for inflammation that is ICAM-1. In some embodiments, the antibody is specific to a marker that is a marker for inflammation that is RANTES. In some embodiments, the antibody is specific to a marker that is a marker for inflammation that is MIP-2. In some embodiments, the antibody is specific to a marker that is a marker for inflammation that is MIP-1 beta. In some embodiments, the antibody is specific to a marker that is a marker for inflammation that is MIP-1 alpha. In some embodiments, the antibody is specific to a marker that is a marker for inflammation that is MMP-3.

[0085] In some embodiments, the antibody is specific to a marker that is a marker for endocrine function. In some embodiments, the antibody is specific to a marker that is a marker for endocrine function that is 17 beta-estradiol (E2). In some embodiments, the antibody is specific to a marker that is a marker for endocrine function that is DHEA. In some embodiments, the antibody is specific to a marker that is a marker for endocrine function that is ACTH. In some embodiments, the antibody is specific to a marker that is a marker for endocrine function that is gastrin. In some embodiments, the antibody is specific to a marker that is a marker for endocrine function that is growth hormone.

[0086] In some embodiments, the antibody is specific to a marker that is a marker for autoimmune disease. In some embodiments, the antibody is specific to a marker that is a marker for autoimmune disease that is GM-CSF. In some embodiments, the antibody is specific to a marker that is a marker for autoimmune disease that is C-reactive protein (CRP). In some embodiments, the antibody is specific to a marker that is a marker for autoimmune disease that is G-CSF.

[0087] In some embodiments, the antibody is specific to a marker for thyroid function. In some embodiments, the antibody is specific to a marker for thyroid function that is cyclic AMP. In some embodiments, the antibody is specific to a marker for thyroid function. In some embodiments, the antibody is specific to a marker for thyroid function that is calcitonin. In some embodiments, the antibody is specific to a marker for thyroid function. In some embodiments, the antibody is specific to a marker for thyroid function that is parathyroid hormone.

[0088] In some embodiments, the antibody is specific to a marker for cardiovascular function. In some embodiments, the antibody is specific to a marker for cardiovascular function that is B-natruiretic peptide. In some embodiments, the antibody is specific to a marker for cardiovascular function that isNT-proBNP. In some embodiments, the antibody is specific to a marker for cardiovascular function that is C-reactive protein, HS. In some embodiments, the antibody is specific to a marker for cardiovascular function that is beta-thromboglobulin. In some embodiments, the antibody is specific to a marker for cardiovascular function that is a cardiac troponin. In

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some embodiments, the antibody is specific to a marker for cardiovascular function that is cardiac troponin I. In some embodiments, the antibody is specific to a marker for cardiovascular function that is cardiac troponin T.

[0089] In some embodiments, the antibody is specific to a marker for diabetes. In some embodiments, the antibody is specific to a marker for diabetes that is C-peptide. In some embodiments, the antibody is specific to a marker for diabetes that is leptin.

[0090] In some embodiments, the antibody is specific to a marker for infectious disease. In some embodiments, the antibody is specific to a marker for infectious disease that is IFN gamma. In some embodiments, the antibody is specific to a marker for infectious disease that is IFN alpha. In some embodiments, the antibody is specific to a marker for infectious disease that is TREM-1.

[0091] In some embodiments, the antibody is specific to a marker for metabolism. In some embodiments, the antibody is specific to a marker for metabolism that is bio-intact PTH (1-84). In some embodiments, the antibody is specific to a marker for metabolism that is PTH.

[0092] In some embodiments, the antibody is specific to a marker that is IL-1 beta. In some embodiments, the antibody is specific to a marker that is TNF-alpha. In some embodiments, the antibody is specific to a marker that is IL-6. In some embodiments, the antibody is specific to a marker that is TnI (cardiac troponin I). In some embodiments, the antibody is specific to a marker that is is IL-8.

[0093] In some embodiments, the antibody is specific to a marker that is Abeta 40. In some embodiments, the antibody is specific to a marker that is Abeta 42. In some embodiments, the antibody is specific to a marker that is cAMP. In some embodiments, the antibody is specific to a marker that is FAS Ligand. In some embodiments, the antibody is specific to a marker that is FGF-basic. In some embodiments, the antibody is specific to a marker that is GM-CSF. In some embodiments, the antibody is specific to a marker that is IFN-alpha. In some embodiments, the antibody is specific to a marker that is IFN-gamma. In some embodiments, the antibody is specific to a marker that is IL-1a. In some embodiments, the antibody is specific to a marker that is IL-2. In some embodiments, the antibody is specific to a marker that is IL-4. In some embodiments, the antibody is specific to a marker that is IL-5. In some embodiments, the antibody is specific to a marker that is IL-7. In some embodiments, the antibody is specific to a marker that is IL-12. In some embodiments, the antibody is specific to a marker that is In some embodiments, the antibody is specific to a marker that is IL-13. In some embodiments, the antibody is specific to a marker that is IL-17. In some embodiments, the antibody is specific to a marker that is MCP-1. In some embodiments, the antibody is specific to a marker that is MIP-1a. In some embodiments, the antibody is specific to a marker that is RANTES. In some embodiments, the antibody is specific to a marker that is VEGF.

[0094] In some embodiments, the antibody is specific to a marker that is ACE. In some embodiments, the antibody is specific to a marker that is activin A. In some embodiments, the antibody is specific to a marker that is adiponectin. In some embodiments, the antibody is specific to a marker that is adipsin. In some embodiments, the antibody is specific to a marker that is AgRP. In some embodiments, the antibody is specific to a marker that is AKT1. In some embodiments, the antibody is specific to a marker that is albumin. In some embodiments, the antibody is specific to a marker that is betacellulin. In some embodiments, the antibody is specific to a marker that is bombesin. In some embodiments, the antibody is specific to a marker that is CD14. In some embodiments, the antibody is specific to a marker that is CD-26. In some embodiments, the antibody is specific to a marker that is CD-38. In some embodiments, the antibody is specific to a marker that is CD-40L. In some embodiments, the antibody is specific to a marker that is CD-40s. In some embodiments, the antibody is specific to a marker that is CDK5. In some embodiments, the antibody is specific to a marker that is Complement C3. In some embodiments, the antibody is specific to a marker that is Complement C4. In some embodiments, the antibody is specific to a

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marker that is C-peptide. In some embodiments, the antibody is specific to a marker that is CRP. In some embodiments, the antibody is specific to a marker that is EGF. In some embodiments, the antibody is specific to a marker that is E-selectin. In some embodiments, the antibody is specific to a marker that is FAS. In some embodiments, the antibody is specific to a marker that is FASLG. In some embodiments, the antibody is specific to a marker that is Fetuin A. In some embodiments, the antibody is specific to a marker that is fibrinogen. In some embodiments, the antibody is specific to a marker that is ghrelin. In some embodiments, the antibody is specific to a marker that is glucagon. In some embodiments, the antibody is specific to a marker that is growth hormone. In some embodiments, the antibody is specific to a marker that is haptoglobulin. In some embodiments, the antibody is specific to a marker that is hepatocyte growth factor. In some embodiments, the antibody is specific to a marker that is HGF. In some embodiments, the antibody is specific to a marker that is ICAM1. In some embodiments, the antibody is specific to a marker that is IFNG. In some embodiments, the antibody is specific to a marker that is IGF1. In some embodiments, the antibody is specific to a marker that is IL-1RA. In some embodiments, the antibody is specific to a marker that is Il-6sr. In some embodiments, the antibody is specific to a marker that is IL-8. In some embodiments, the antibody is specific to a marker that is IL-10. In some embodiments, the antibody is specific to a marker that is IL-18. In some embodiments, the antibody is specific to a marker that is ILGFBP1. In some embodiments, the antibody is specific to a marker that is ILGFBP3. In some embodiments, the antibody is specific to a marker that is insulin-like growth factor 1. In some embodiments, the antibody is specific to a marker that is LEP. In some embodiments, the antibody is specific to a marker that is M-CSF. In some embodiments, the antibody is specific to a marker that is MMP2. In some embodiments, the antibody is specific to a marker that is MMP9. In some embodiments, the antibody is specific to a marker that is NGF. In some embodiments, the antibody is specific to a marker that is PAI-1. In some embodiments, the antibody is specific to a marker that is RAGE. In some embodiments, the antibody is specific to a marker that is RSP4. In some embodiments, the antibody is specific to a marker that is resistin. In some embodiments, the antibody is specific to a marker that is sex hormone binding globulin. In some embodiments, the antibody is specific to a marker that is SOCX3. In some embodiments, the antibody is specific to a marker that is TGF beta. In some embodiments, the antibody is specific to a marker that is thromboplastin. In some embodiments, the antibody is specific to a marker that is TNF R1. In some embodiments, the antibody is specific to a marker that is VCAM-1. In some embodiments, the antibody is specific to a marker that is VWF. In some embodiments, the antibody is specific to a marker that is TSH. In some embodiments, the antibody is specific to a marker that is EPITOME.

[0095] In some embodiments, the antibody is specific to a marker that is cardiac troponin I. In some embodiments, the antibody is specific to a marker that is TREM-1. In some embodiments, the antibody is specific to a marker that is IL-6. In some embodiments, the antibody is specific to a marker that is IL-8. In some embodiments, the antibody is specific to a marker that is Leukotriene T4. In some embodiments, the antibody is specific to a marker that is Akt1. In some embodiments, the antibody is specific to a marker that is TGF-beta. In some embodiments, the antibody is specific to a marker that is Fas ligand.

[0096] In some embodiments, the fluorescent moiety comprises a fluorescent molecule. In some embodiments, the fluorescent moiety comprises a plurality of fluorescent molecules, e.g., about 2-10, 2-8, 2-6, 2-4, 3-10, 3-8, or 3-6 fluorescent molecules. In some embodiments, the label comprises about 2-4 fluorescent molecules. In some embodiments, the fluorescent molecule comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. group. In some embodiments, the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some

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embodiments, the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the fluorescent molecules are AlexaFluor 647 molecules.

A. Binding partners

[0097] Any suitable binding partner with the requisite specificity for the form of molecule, e.g., marker, to be detected may be used. If the molecule, e.g., marker, has several different forms, various specificities of binding partners are possible. Suitable binding partners are known in the art and include antibodies, aptamers, lectins, and receptors. A useful and versatile type of binding partner is an antibody.

1. Antibodies

[0098] Thus, in some embodiments, the binding partner is an antibody specific for a molecule to be detected. **The term "antibody," as used herein,** is a broad term and is used in its ordinary sense, including, without limitation, to refer to naturally occurring antibodies as well as non-naturally occurring antibodies, including, for example, single chain antibodies, chimeric, bifunctional and humanized antibodies, as well as antigen-binding fragments thereof.. It will be appreciated that the choice of epitope or region of the molecule to which the antibody is raised will determine its specificity, e.g., for various forms of the molecule, if present, or for total (e.g., all, or substantially all of the molecule).

[0099] Methods for producing antibodies are well-established. One skilled in the art will recognize that many procedures are available for the production of antibodies, for example, as described in Antibodies, A Laboratory Manual, Ed Harlow and David Lane, Cold Spring Harbor Laboratory (1988), Cold Spring Harbor, N.Y. One skilled in the art will also appreciate that binding fragments or Fab fragments which mimic antibodies can also be prepared from genetic information by various procedures (Antibody Engineering: A Practical Approach (Borrebaeck, C., ed.), 1995, Oxford University Press, Oxford; J. Immunol. 149, 3914-3920 (1992)). Monoclonal and polyclonal antibodies to molecules, e.g., proteins, and markers also commercially available (R and D Systems, Minneapolis, Minnesota; HyTest, HyTest Ltd.,Turku Finland; Abcam Inc., Cambridge, MA, USA, Life Diagnostics, Inc., West Chester, PA, USA; Fitzgerald Industries International, Inc., Concord, MA 01742-3049 USA; BiosPacific, Emeryville, CA).

[00100] In some embodiments, the antibody is a polyclonal antibody. In other embodiments, the antibody is a monoclonal antibody.

[00101] Capture binding partners and detection binding partner pairs, e.g., capture and detection antibody pairs, may be used in embodiments of the invention. Thus, in some embodiments, a heterogeneous assay protocol is used in which, typically, two binding partners, e.g., two antibodies, are used. One binding partner is a capture partner, usually immobilized on a solid support, and the other binding partner is a detection binding partner, typically with a detectable label attached. Such antibody pairs are available from the sources described above, e.g., BiosPacific, Emeryville, CA. Antibody pairs can also be designed and prepared by methods well-known in the art. Compositions of the invention include antibody pairs wherein one member of the antibody pair is a label as described herein, and the other member is a capture antibody.

[00102] In some embodiments it is useful to use an antibody that cross-reacts with a variety of species, either as a capture antibody, a detection antibody, or both. Such embodiments include the measurement of drug toxicity by determining, e.g., release of cardiac troponin into the blood as a marker of cardiac damage. A cross-reacting antibody allows studies of toxicity to be done in one species, e.g. a non-human species, and direct transfer of the

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results to studies or clinical observations of another species, e.g., humans, using the same antibody or antibody pair in the reagents of the assays, thus decreasing variability between assays. Thus, in some embodiments, one or more of the antibodies for use as a binding partner to the marker, e.g., cardiac troponin, such as cardiac troponin I, may be a cross-reacting antibody. In some embodiments, the antibody cross-reacts with the marker, e.g. cardiac troponin, from at least two species selected from the group consisting of human, monkey, dog, and mouse. In some embodiments the antibody cross-reacts with the marker e.g. cardiac troponin, from all of the group consisting of human, monkey, dog, and mouse.

B. Fluorescent Moieties

[00103] In some embodiments of labels used in the invention, the binding partner, e.g., antibody, is attached to a fluorescent moiety. The fluorescence of the moiety will be sufficient to allow detection in a single molecule detector, such as the single molecule detectors described herein.

[00104] A "fluorescent moiety," as that term is used herein, includes one or more fluorescent entities whose total fluorescence is such that the moiety may be detected in the single molecule detectors described herein. Thus, a fluorescent moiety may comprise a single entity (e.g., a Quantum Dot or fluorescent molecule) or a plurality of entities (e.g., a plurality of fluorescent molecules). It will be appreciated that when "moiety," as that term is used herein, refers to a group of fluorescent entities, e.g., a plurality of fluorescent dye molecules, each individual entity may be attached to the binding partner separately or the entities may be attached together, as long as the entities as a group provide sufficient fluorescence to be detected.

[00105] Typically, the fluorescence of the moiety involves a combination of quantum efficiency and lack of photobleaching sufficient that the moiety is detectable above background levels in a single molecule detector, with the consistency necessary for the desired limit of detection, accuracy, and precision of the assay. For example, in some embodiments, the fluorescence of the fluorescent moiety is such that it allows detection and/or quantitation of a molecule, e.g., a marker, at a limit of detection of less than about 10, 5, 4, 3, 2, 1, 0.1, 0.01, 0.001, 0.00001, or 0.000001 pg/ml and with a coefficient of variation of less than about 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1% or less, e.g., about 10% or less, in the instruments described herein. In some embodiments, the fluorescence of the fluorescent moiety is such that it allows detection and/or quantitation of a molecule, e.g., a marker, at a limit of detection of less than about 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 pg/ml and with a coefficient of variation of less than about 10%, in the instruments described herein.

[00106] "Limit of detection," as that term is used herein, includes the lowest concentration at which one can identify a sample as containing a molecule of the substance of interest, e.g., the first non-zero value. It can be defined by the variability of zeros and the slope of the standard curve. For example, the limit of detection of an assay may be determined by running a standard curve, determining the standard curve zero value, and adding 2 standard deviations to that value. A concentration of the substance of interest that produces a signal equal to this value is the "lower limit of detection" concentration.

[00107] Furthermore, the moiety has properties that are consistent with its use in the assay of choice. In some embodiments, the assay is an immunoassay, where the fluorescent moiety is attached to an antibody; the moiety must have properties such that it does not aggregate with other antibodies or proteins, or experiences no more aggregation than is consistent with the required accuracy and precision of the assay. In some embodiments, fluorescent moieties that are preferred are fluorescent moieties, e.g., dye molecules that have a combination of 1)

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high absorption coefficient; 2) high quantum yield; 3) high photostability (low photobleaching); and 4) compatibility with labeling the molecule of interest (e.g., protein) so that it may be analyzed using the analyzers and systems of the invention (e.g., does not cause precipitation of the protein of interest, or precipitation of a protein to which the moiety has been attached).

[00108] Fluorescent moieties, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that are useful in some embodiments of the invention may be defined in terms of their photon emission characteristics when stimulated by EM radiation. For example, in some embodiments, the invention utilizes a fluorescent moiety, e.g., a moiety comprising a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 350, 400, 500, 600, 700, 800, 900, or 1000, photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules. It will be appreciated that the total energy may be achieved by many different combinations of power output of the laser and length of time of exposure of the dye moiety. E.g., a laser of a power output of 1 mW may be used for 3 ms, 3 mW for 1 ms, 6 mW for 0.5 ms, 12 mW for 0.25 ms, and so on.

[00109] In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 50 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 100 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 150 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety, e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 300 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye moiety e.g., a single fluorescent dye molecule or a plurality of fluorescent dye molecules, that is capable of emitting an average of at least about 500 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns

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in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

[00110] In some embodiments, the fluorescent moiety comprises an average of at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 fluorescent entities, e.g., fluorescent molecules. In some embodiments, the fluorescent moiety comprises an average of no more than about 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 fluorescent entities, e.g., fluorescent molecules. In some embodiments, the fluorescent moiety comprises an average of about 1 to 11, or about 2 to 10, or about 2 to 8, or about 2 to 6, or about 2 to 5, or about 2 to 4, or about 3 to 10, or about 3 to 8, or about 3 to 6, or about 3 to 5, or about 4 to 10, or about 4 to 8, or about 4 to 6, or about 2, 3, 4, 5, 6, or more than about 6 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 2 to 8 fluorescent moieties are attached. In some embodiments, an average of about 2 to 6 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 2 to 4 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 3 to 10 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 3 to 8 fluorescent entities. In some embodiments, the fluorescent moiety comprises an average of about 3 to 6 fluorescent entities. By "average" is meant that, in a given sample that is a representative sample of a group of labels of the invention, where the sample contains a plurality of the binding partner-fluorescent moiety units, the molar ratio of the particular fluorescent entity of which the fluorescent moiety is comprise, to the binding partner, as determined by standard analytical methods, corresponds to the number or range of numbers specified. For example, in embodiments in which the label comprises a binding partner that is an antibody and a fluorescent moiety that comprises a plurality of fluorescent dye molecules of a specific absorbance, a spectrophometric assay may be used in which a solution of the label is diluted to an appropriate level and the absorbance at 280 nm is taken to determine the molarity of the protein (antibody) and an absorbance at, e.g., 650 nm (for AlexaFluor 647) is taken to determine the molarity of the fluorescent dye molecule. The ratio of the latter molarity to the former represents the average number of fluorescent entities (dye molecules) in the fluorescent moiety attached to each antibody.

1. Dyes

[00111] In some embodiments, the invention utilizes fluorescent moieties that comprise fluorescent dye molecules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 50 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 75 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 100 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of emitting an average of at least about 150 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the invention utilizes a fluorescent dye molecule that is capable of

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emitting an average of at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the molecule, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the molecule, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules

[00112] A non-inclusive list of useful fluorescent entities for use in the fluorescent moieties of the invention is given in **Table 2**, below. In some embodiments, the fluorescent dye is selected from the group consisting of Alexa Flour 488, 532, 647, 700, 750, Fluorescein, B-phycoerythrin, allophycocyanin, PBXL-3, and Qdot 605. In some embodiments, the fluorescent dye is selected from the group consisting of Alexa Flour 488, 532, 700, 750, Fluorescein, B-phycoerythrin, allophycocyanin, PBXL-3, and Qdot 605.

TABLE 2

FLUORESCENT ENTITIES

Dye	E Ex (nm)	E (M)-1	Em (nm)	MMw
Bimane	380	5,700	458	282.31
Dapoxyl	373	22,000	551	362.83
Dimethylamino coumarin-4-acetic acid	375	22,000	470	344.32
Marina blue	365	19,000	460	367.26
8-Anilino naphthalene-1-sulfonic acid	372		480	
Cascade blue	376	23,000	420	607.42
Alexa Fluor 405	402	35,000	421	1028.26
Cascade blue	400	29,000	420	607.42
Cascade yellow	402	24,000	545	563.54
Pacific blue	410	46,000	455	339.21
PyMPO	415	26,000	570	582.41
Alexa 430	433	15,000	539	701.75
Atto-425	438		486	
NBD	465	22,000	535	391.34
Alexa 488	495	73,000	519	643.41
Fluorescein	494	79,000	518	376.32
Oregon Green 488	496	76,000	524	509.38
Atto 495	495		522	
Cy2	489	150,000	506	713.78
DY-480-XL	500	40,000	630	514.60
DY-485-XL	485	20,000	560	502.59
DY-490-XL	486	27,000	532	536.58
DY-500-XL	505	90,000	555	596.68
DY-520-XL	520	40,000	664	514.60
Alexa Fluor 532	531	81,000	554	723.77
BODIPY 530/550	534	77,000	554	513.31
6-HEX	535	98,000	556	680.07
6-JOE	522	75,000	550	602.34
Rhodamine 6G	525	108,000	555	555.59
Atto-520	520		542	
Cy3B	558	130,000	572	658.00
Alexa Fluor 610	612	138,000	628	
Alexa Fluor 633	632	159,000	647	ca. 1200
Alexa Fluor 647	650	250,000	668	ca. 1250
BODIPY 630/650	625	101,000	640	660.50
Cy5	649	250,000	670	791.99
Alexa Fluor 660	663	110,000	690	
Alexa Fluor 680	679	184,000	702	
Alexa Fluor 700	702	192,000	723	
Alexa Fluor 750	749	240,000	782	
B-phycoerythrin	546, 565	2,410,000	575	240,000
R-phycoerythrin	480, 546, 565	1,960,000	578	240,000
Allophycocyanin	650	700,000	660	700,000
PBXL-1	545		666	

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Dye	E Ex (nm)	E (M)-1	Em (nm)	MMw
PBXL-3	614		662	

Atto-tec dyes

Name	Ex (nm)	Em (nm)	QY	□ (ns)
Atto 425	436	486	0.9	3.5
Atto 495	495	522	0.45	2.4
Atto 520	520	542	0.9	3.6
Atto 560	561	585	0.92	3.4
Atto 590	598	634	0.8	3.7
Atto 610	605	630	0.7	3.3
Atto 655	665	690	0.3	1.9
Atto 680	680	702	0.3	1.8

Dyomics Fluors

label	Ex (nm)	Molar absorbance* [l·mol-1·cm-1]	Em (nm)	molecular weight# [g·mol-1]
DY-495/5	495	70,000	520	489.47
DY-495/6	495	70,000	520	489.47
DY-495X/5	495	70,000	520	525.95
DY-495X/6	495	70,000	520	525.95
DY-505/5	505	85,000	530	485.49
DY-505/6	505	85,000	530	485.49
DY-505X/5	505	85,000	530	523.97
DY-505X/6	505	85,000	530	523.97
DY-550	553	122,000	578	667.76
DY-555	555	100.000	580	636.18
DY-610	609	81.000	629	667.75
DY-615	621	200.000	641	578.73
DY-630	636	200.000	657	634.84
DY-631	637	185.000	658	736.88
DY-633	637	180.000	657	751.92
DY-635	647	175.000	671	658.86
DY-636	645	190.000	671	760.91
DY-650	653	170.000	674	686.92
DY-651	653	160.000	678	888.96
DYQ-660	660	117,000	-	668.86
DYQ-661	661	116,000	-	770.90
DY-675	674	110.000	699	706.91
DY-676	674	145.000	699	807.95
DY-680	690	125.000	709	634.84
DY-681	691	125.000	708	736.88
DY-700	702	96.000	723	668.86
DY-701	706	115.000	731	770.90
DY-730	734	185.000	750	660.88
DY-731	736	225.000	759	762.92
DY-750	747	240.000	776	712.96
DY-751	751	220.000	779	814.99
DY-776	771	147.000	801	834.98
DY-780-OH	770	70.000	810	757.34
DY-780-P	770	70.000	810	957.55
DY-781	783	98.000	800	762.92
DY-782	782	102.000	800	660.88
EVOblue-10	651	101.440	664	389.88
EVOblue-30	652	102.000	672	447.51

Quantum Dots :Qdot 525, 565, 585, 605, 655, 705, 800

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[00113] Suitable dyes for use in the invention include modified carbocyanine dyes. The modification of carbocyanine dyes includes the modification of an indolium ring of the carbocyanine dye to permit a reactive group or conjugated substance at the number 3 position. The modification of the indolium ring provides dye conjugates that are uniformly and substantially more fluorescent on proteins, nucleic acids and other biopolymers, than conjugates labeled with structurally similar carbocyanine dyes bound through the nitrogen atom at the number one position. In addition to having more intense fluorescence emission than structurally similar dyes at virtually identical wavelengths, and decreased artifacts in their absorption spectra upon conjugation to biopolymers, the modified carbocyanine dyes have greater photostability and higher absorbance (extinction coefficients) at the wavelengths of peak absorbance than the structurally similar dyes. Thus, the modified carbocyanine dyes result in greater sensitivity in assays that use the modified dyes and their conjugates. Preferred modified dyes include compounds that have at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. Other dye compounds include compounds that incorporate an azabenzazolium ring moiety and at least one sulfonate moiety. The modified carbocyanine dyes that can be used to detect individual molecules in various embodiments of the invention are described in U.S. Patent 6,977,305, which is herein incorporated by reference in its entirety. Thus, in some embodiments the labels of the invention utilize a fluorescent dye that includes a substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group.

[00114] In some embodiments, the label comprises a fluorescent moiety that includes one or more Alexa dyes (Molecular Probes, Eugene, OR). The Alexa dyes are disclosed in U.S. Patent 6,977,305; 6,974,874; 6,130,101; and 6,974,305 which are herein incorporated by reference in their entirety. Some embodiments of the invention utilize a dye chosen from the group consisting of AlexaFluor 647, AlexaFluor 488, AlexaFluor 532, AlexaFluor 555, AlexaFluor 610, AlexaFluor 680, AlexaFluor 700, and AlexaFluor 750. Some embodiments of the invention utilize a dye chosen from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 700 and AlexaFluor 750. Some embodiments of the invention utilize a dye chosen from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 555, AlexaFluor 610, AlexaFluor 680, AlexaFluor 700, and AlexaFluor 750. Some embodiments of the invention utilize the AlexaFluor 647 molecule, which has an absorption maximum between about 650 and 660 nm and an emission maximum between about 660 and 670 nm. The AlexaFluor 647 dye is used alone or in combination with other AlexaFluor dyes.

[00115] In addition, currently available organic fluors can be improved by rendering them less hydrophobic by adding hydrophilic groups such as polyethylene. Alternatively, currently sulfonated organic fluors such as the AlexaFluor 647 dye can be rendered less acidic by making them zwitterionic. Particles such as antibodies that are labeled with the modified fluors are less likely to bind non-specifically to surfaces and proteins in immunoassays, and thus enable assays that have greater sensitivity and lower backgrounds. Methods for modifying and improving the properties of fluorescent dyes for the purpose of increasing the sensitivity of a system that detects single particles are known in the art. Preferably, the modification improves the Stokes shift while maintaining a high quantum yield.

2 Quantum dots

[00116] In some embodiments, the fluorescent label moiety that is used to detect a molecule in a sample using the analyzer systems of the invention is a quantum dot. Quantum dots (QDs), also known as semiconductor nanocrystals or artificial atoms, are semiconductor crystals that contain anywhere between 100 to 1,000 electrons

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and range from 2-10 nm.. Some QDs can be between 10-20 nm in diameter. QDs have high quantum yields, which makes them particularly useful for optical applications. QDs are fluorophores that fluoresce by forming excitons, which can be thought of the excited state of traditional fluorophores, but have much longer lifetimes of up to 200 nanoseconds. This property provides QDs with low photobleaching. The energy level of QDs can be controlled by changing the size and shape of the QD, and the depth of the QDs' potential. One of the optical features of small excitonic QDs is coloration, which is determined by the size of the dot. The larger the dot, the redder, or more towards the red end of the spectrum the fluorescence. The smaller the dot, the bluer or more towards the blue end it is. The bandgap energy that determines the energy and hence the color of the fluoresced light is inversely proportional to the square of the size of the QD. Larger QDs have more energy levels which are more closely spaced, thus allowing the QD to absorb photons containing less energy, i.e. those closer to the red end of the spectrum. Because the emission frequency of a dots dependent on the bandgap, it is therefore possible to control the output wavelength of a dot with extreme precision. In some embodiments the protein that is detected with the single particle analyzer system is labeled with a QD. In some embodiments, the single particle analyzer is used to detect a protein labeled with one QD and using a filter to allow for the detection of different proteins at different wavelengths.

QDs have broad excitation and narrow emission properties which when used with color filtering require only a single electromagnetic source for multiplex analysis of multiple targets in a single sample to resolve individual signals. Thus, in some embodiments, the analyzer system comprises one continuous wave laser and particles that are each labeled with one QD. Colloidally prepared QDs are free floating and can be attached to a variety of molecules via metal coordinating functional groups. These groups include but are not limited to thiol, amine, nitrile, phosphine, phosphine oxide, phosphonic acid, carboxylic acids or other ligands. By bonding appropriate molecules to the surface, the quantum dots can be dispersed or dissolved in nearly any solvent or incorporated into a variety of inorganic and organic films. Quantum dots (QDs) can be coupled to streptavidin directly through a maleimide ester coupling reaction or to antibodies through a meleimide-thiol coupling reaction. This yields a material with a biomolecule covalently attached on the surface, which produces conjugates with high specific activity. In some embodiments, the protein that is detected with the single particle analyzer is labeled with one quantum dot. In some embodiments the quantum dot is between 10 and 20 nm in diameter. In other embodiments, the quantum dot is between 2 and 10 nm in diameter. Useful Quantum Dots include QD 605, QD 610, QD 655, and QD 705. A particularly preferred Quantum Dot is QD 605.

C. Binding Partner-Fluorescent Moiety Compositions

[00117] The labels of the invention generally contain a binding partner, e.g., antibody, bound to a fluorescent moiety to provide the requisite fluorescence for detection and quantitation in the instruments described herein. Any suitable combination of binding partner and fluorescent moiety for detection in the single molecule detectors described herein may be used as a label in the invention. In some embodiments, the invention provides a label for a marker of a biological state, where the label includes an antibody to the marker and a fluorescent moiety. The marker may be any of the markers described above. The antibody may be any antibody as described above. A fluorescent moiety may be attached such that the label is capable of emitting an average of at least about 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 350, 400, 500, 600, 700, 800, 900, or 1000, photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the label, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the fluorescent moiety may be a fluorescent moiety that

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is capable of emitting an average of at least about 50, 100, 150, or 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot of not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. The fluorescent moiety may be a fluorescent moiety that includes one or more dye molecules with a structure that includes a substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance group.. The label composition may include a fluorescent moiety that includes one or more dye molecules selected from the group consisting of AlexaFluor 488, 532, 647, 700, or 750. The label composition may include a fluorescent moiety that includes one or more dye molecules selected from the group consisting of AlexaFluor 488, 532, 700, or 750. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 488. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 555. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 610. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 647. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 680. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 700. The label composition may include a fluorescent moiety that includes one or more dye molecules that are AlexaFluor 750.

[00118] In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an AlexFluor molecule, e.g. an AlexaFluor molecule selected from the described groups, such as an AlexaFluor 647 molecule attached to a to an antibody specific for the marker. In some embodiments the composition includes an average of 1 to 11, or about 2 to 10, or about 2 to 8, or about 2 to 6, or about 2 to 5, or about 2 to 4, or about 3 to 10, or about 3 to 8, or about 3 to 6, or about 3 to 5, or about 4 to 10, or about 4 to 8, or about 4 to 6, or about 2, 3, 4, 5, 6, or more than about 6 AlexaFluor 647 molecules molecule attached to an antibody for the marker. In some embodiments the invention provides a composition for the detection a marker of a biological state that includes an average of 1 to 11, or about 2 to 10, or about 2 to 8, or about 2 to 6, or about 2 to 5, or about 2 to 4, or about 3 to 10, or about 3 to 8, or about 3 to 6, or about 3 to 5, or about 4 to 10, or about 4 to 8, or about 4 to 6, or about 2, 3, 4, 5, 6, or more than about 6 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker. In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an average of about 2 to 10 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker. In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an average of about 2 to 8 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker. In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an average of about 2 to 6 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker. In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an average of about 2 to 4 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker. In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an average of about 3 to 8 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker. In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an average of about 3 to 6 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker. In some embodiments the invention provides a composition for the detection of a marker of a biological state that includes an average of about 4 to 8 AlexaFluor 647 molecules molecule attached to an antibody specific to the marker.

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[00119] Attachment of the fluorescent moiety, or fluorescent entities that make up the fluorescent moiety, to the binding partner, e.g., antibody, may be by any suitable means; such methods are well-known in the art and exemplary methods are given in the Examples. In some embodiments, after attachment of the fluorescent moiety to the binding partner to form a label for use in the methods of the invention, and prior to the use of the label for labeling the protein of interest, it is useful to perform a filtration step. E.g., an antibody-dye label may be filtered prior to use, e.g., through a 0.2 micron filter, or any suitable filter for removing aggregates. Other reagents for use in the assays of the invention may also be filtered, e.g., e.g., through a 0.2 micron filter, or any suitable filter. Without being bound by theory, it is thought that such filtration removes a portion of the aggregates of the, e.g., antibody-dye labels. As such aggregates will bind as a unit to the protein of interest, but upon release in elution buffer are likely to disaggregate, false positives may result; i.e., several labels will be detected from an aggregate that has bound to only a single protein molecule of interest. Regardless of theory, filtration has been found to reduce false positives in the subsequent assay and to improve accuracy and precision.

[00120] It will be appreciated that immunoassays often employ a sandwhich format, in which binding partner pairs, e.g. antibodies, to the same molecule, e.g., marker are used. The invention also encompasses binding partner pairs, e.g., antibodies, wherein both antibodies are specific to the same molecule, e.g., the same marker, and wherein at least one member of the pair is a label as described herein. Thus, for any label that includes a binding-partner and a fluorescent moiety, the invention also encompasses a pair of binding partners wherein the first binding partner, e.g., antibody, is part of the label, and the second binding partner, e.g., antibody, is, typically, unlabeled and serves as a capture binding partner. In addition, binding partner pairs are frequently used in FRET assays. FRET asaays useful in the invention are disclosed in U.S. Patent Application No. 11/048,660, incorporated by reference herein in its entirety, and the present invention also encompasses binding partner pairs, each of which includes a FRET label.

IV. Highly Sensitive Analysis of Molecules

[00121] In one aspect, the invention provides a method for determining the presence or absence of a single molecule, e.g., a molecule of a marker of a biological state, in a sample, by i) labeling the molecule if present, with a label; and ii) detecting the presence or absence of the label, where the detection of the presence of the label indicates the presence of the single molecule in the sample. In some embodiments, the method is capable of detecting the molecule at a limit of detection of less than about 100, 80, 60, 50, 40, 30, 20, 15, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2. 0.1, 0.05, 0.01, 0.005, or 0.001 femtomolar. In some embodiments, the method is capable of detecting the molecule at a limit of detection of less than about 100 femtomolar. In some embodiments, the method is capable of detecting the molecule at a limit of detection of less than about 10 femtomolar. In some embodiments, the method is capable of detecting the molecule at a limit of detection of less than about 1 femtomolar. In some embodiments, the method is capable of detecting the molecule at a limit of detection of less than about 0.1 femtomolar. In some embodiments, the method is capable of detecting the molecule at a limit of detection of less than about 0.01 femtomolar. In some embodiments, the method is capable of detecting the molecule at a limit of detection of less than about 0.001 femtomolar. Detection limits may be determined by use of an appropriate standard, e.g., National Institute of Standards and Technology reference standard material.

[00122] The methods also provide methods of determining a concentration of a molecule, e.g., a marker of a biological state, in a sample by detecting single molecules of the molecule in the sample. The "detecting" of a single molecule includes detecting the molecule directly or indirectly. In the case of indirect detection, labels that corresponds to single molecules, e.g., a labels that have been attached to the single molecules, may be detected.

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[00123] In some embodiments, the invention provides a method for determining the presence or absence of a single molecule of a protein in a biological sample, comprising labeling said molecule with a label and detecting the presence or absence of said label in a single molecule detector, wherein said label comprises a fluorescent moiety that is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. The single molecule detector may, in some embodiments, comprise not more than one interrogation space. The limit of detection of the single molecule in the sample may be less than about 10, 1, 0.1, 0.01, or 0.001 femtomolar. In some embodiments, the limit of detection is less than about 1 femtomolar. The detecting may comprise detecting electromagnetic radiation emitted by said fluorescent moiety. The method may further comprise exposing said fluorescent moiety to electromagnetic radiation, e.g., electromagnetic radiation provided by a laser, such as a laser with a power output of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 mW. In some embodiments, the laser stimulates provides light to the interrogation space for a duration of about 10-1000 microseconds, or about 1000, 250, 100, 50, 25 or 10 microseconds. In some embodiments, the label further comprises a binding partner specific for binding said molecule, such as an antibody. In some embodiments, the fluorescent moiety comprises a fluorescent dye molecule, such as a dye molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the dye molecule is an AlexFluor molecule selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the dye molecule is an AlexaFluor647 dye molecule. In some embodiments, the fluorescent moiety comprises a plurality of AlexaFluor 647 molecules. In some embodiments, the plurality of AlexaFluor 647 molecules comprises about 2-4 AlexaFluor 647 molecules, or about 3-6 AlexaFluor 647 molecules. In some embodiments, the fluorescent moiety is a quantum dot. The method may further comprise measuring the concentration of said protein in the sample.

[00124] In some embodiments, detecting the presence or absence of said label comprises: (i) passing a portion of said sample through an interrogation space; (ii) subjecting said interrogation space to exposure to electromagnetic radiation, said electromagnetic radiation being sufficient to stimulate said fluorescent moiety to emit photons, if said label is present; and (iii) detecting photons emitted during said exposure of step (ii). The method may further comprise determining a background photon level in said interrogation space, wherein said background level represents the average photon emission of the interrogation space when it is subjected to electromagnetic radiation in the same manner as in step (ii), but without label in the interrogation space. The method may further comprise comparing the amount of photons detected in step (iii) to a threshold photon level, wherein said threshold photon level is a function of said background photon level, wherein an amount of photons detected in step (iii) greater that the threshold level indicates the presence of said label, and an amount of photons detected in step (iii) equal to or less than the threshold level indicates the absence of said label.

A. Sample

[00125] The sample may be any suitable sample. Typically, the sample is a biological sample, e.g., a biological fluid. Such fluids include, without limitation, bronchoalveolar lavage fluid (BAL), blood, serum, plasma, urine, nasal swab, cerebrospinal fluid, pleural fluid, synovial fluid, peritoneal fluid, amniotic fluid, gastric fluid, lymph fluid, interstitial fluid, tissue homogenate, cell extracts, saliva, sputum, stool, physiological secretions, tears, mucus, sweat, milk, semen, seminal fluid, vaginal secretions, fluid from ulcers and other surface eruptions, blisters, and

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abscesses, and extracts of tissues including biopsies of normal, malignant, and suspect tissues or any other constituents of the body which may contain the target particle of interest. Other similar specimens such as cell or tissue culture or culture broth are also of interest.

In some embodiments, the sample is a blood sample. In some embodiments the sample is a plasma sample. In some embodiments the sample is a serum sample. In some embodiments, the sample is a urine sample. In some embodiments, the sample is a nasal swab.

B. Sample preparation

[00126] In general, any method of sample preparation may be used that produces a label corresponding to a molecule of interest, e.g., a marker of a biological state, that is wished to be measured, where the label is detectable in the instruments described herein. As is known in the art, sample preparation in which a label is added to one or more molecules may be performed in a homogeneous or heterogeneous format. In some embodiments, the sample preparation is formed in a homogenous format. In analyzer system employing a homogenous format, unbound label is not removed from the sample. See, e.g., U.S. Patent Application No. 11/048,660. In some embodiments, the particle or particles of interest are labeled by addition of labeled antibody or antibodies that bind to the particle or particles of interest.

[00127] In some embodiments, a heterogeneous assay format is used, where, typically, a step is employed for removing unbound label. Such assay formats are well-known in the art. One particularly useful assay format is a sandwich assay, e.g., a sandwich immunoassay. In this format, the molecule of interest, e.g., marker of a biological state, is captured, e.g., on a solid support, using a capture binding partner. Unwanted molecules and other substances may then optionally be washed away, followed by binding of a label comprising a detection binding partner and a detectable label, e.g., fluorescent moiety. Further washes remove unbound label, then the detectable label is released, usually though not necessarily still attached to the detection binding partner. In alternative embodiments, sample and label are added to the capture binding partner without a wash in between, e.g., at the same time. Other variations will be apparent to one of skill in the art.

[00128] In some embodiments, the method for detecting the molecule of interest, e.g., marker of a biological state, uses a sandwich assay with antibodies, e.g., monoclonal antibodies as capture binding partners. The method comprises binding molecules in a sample to a capture antibody that is immobilized on a binding surface, and binding the label comprising a detection antibody to the molecule to form a "sandwich" complex. The label comprises the detection antibody and a fluorescent moiety, as described herein, which is detected, e.g., using the single molecule analyzers of the invention. Both the capture and detection antibodies specifically bind the molecule. Many example of sandwich immunoassays are known, and some are described in U.S. Pat. No. 4,168,146 to Grubb et al. and U.S. Pat. No. 4,366,241 to Tom et al., both of which are incorporated herein by reference. Further examples specific to specific markers are described in the Examples.

[00129] The capture binding partner may be attached to a solid support, e.g., a microtiter plate or paramagnetic beads. In some embodiments, the invention provides a binding partner for a molecule of interest, e.g., marker of a biological state, attached to a paramagnetic bead. Any suitable binding partner that is specific for the molecule that it is wished to capture may be used. The binding partner may be an antibody, e.g., a monoclonal antibody. Production and sources of antibodies are described elsewhere herein. It will be appreciated that antibodies identified herein as useful as a capture antibody may also be useful as detection antibodies, and vice versa.

[00130] The attachment of the binding partner, e.g., antibody, to the solid support may be covalent or noncovalent. In some embodiments, the attachment is noncovalent. An example of a noncovalent attachment well-known in the

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art is biotin-avidin/streptavidin interactions. Thus, in some embodiments, a solid support, e.g., a microtiter plate or a paramagnetic bead, is attached to the capture binding partner, e.g., antibody, through noncovalent attachment, e.g., biotin-avidin/streptavidin interactions. In some embodiments, the attachment is covalent. Thus, in some embodiments, a solid support, e.g., a microtiter plate or a paramagnetic bead, is attached to the capture binding partner, e.g., antibody, through covalent attachment.

[00131] Covalent attachment in which the orientation of the capture antibody is such that capture of the molecule of interest is optimized is especially useful. For example, in some embodiments a solid support, e.g., a microtiter plate or a paramagnetic microparticle, may be used in which the attachment of the binding partner, e.g., antibody, is an oriented attachment, e.g., a covalent oriented attachment.

[00132] An exemplary protocol for oriented attachment of an antibody to a solid support is as follows: IgG is dissolved in 0.1M sodium acetate buffer, pH 5.5 to a final concentration of 1 mg/ml. An equal volume of ice-cold 20 mM sodium periodate in 0.1 M sodium acetate, pH 5.5 is added. The IgG is allowed to oxidize for ½ hour on ice. Excess periodate reagent is quenched by the addition of 0.15 volume of 1 M glycerol. Low molecular weight byproducts of the oxidation reaction are removed by ultrafiltration. The oxidized IgG fraction is diluted to a suitable concentration (typically 0.5 micrograms IgG per ml) and reacted with hydrazide-activated multiwell plates for at least two hours at room temperature. Unbound IgG is removed by washing the multiwell plate with borate buffered saline or another suitable buffer. The plate may be dried for storage, if desired. A similar protocol may be followed for microbeads if the material of the microbead is suitable for such attachment.

[00133] In some embodiments, the solid support is a microtiter plate. In some embodiments, the solid support is a paramagnetic bead. An exemplary paramagnetic bead is Streptavidin C1(Dynal, 650.01-03). Other suitable beads will be apparent to those of skill in the art. Methods for attachment of antibodies to paramagnetic beads are well-known in the art. One example is given in Example 2.

[00134] The molecule of interest is contacted with the capture binding partner, e.g., capture antibody immobilized on a solid support. Some sample preparation may be used; e.g., preparation of serum from blood samples or concentration procedures before the sample is contacted with the capture antibody. Protocols for binding of proteins in immunoassays are well-known in the art and are included in the Examples.

[00135] The time allowed for binding will vary depending on the conditions; it will be apparent that shorter binding times are desirable in some settings, especially in a clinical setting. The use of, e.g., paramagnetic beads can reduce the time required for binding. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less that about 12, 10, 8, 6, 4, 3, 2, or 1 hours, or less than about 60, 50, 40, 30, 25, 20, 15, 10, or 5 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less than about 60 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less that about 40 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less that about 30 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less that about 20 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less that about 15 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less that about 10 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the capture binding partner, e.g., antibody, is less that about 5 minutes.

[00136] In some embodiments, following the binding of the troponin particles to the capture binding partner, e.g., capture antibody, particles that may have bound nonspecifically, as well as other unwanted substances in the sample,

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are washed away leaving substantially only specifically bound troponin particles. In other embodiments, no wash is used between additions of sample and label; it will be appreciated that this reduces sample preparation time even further. Thus, in some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less that about 12, 10, 8, 6, 4, 3, 2, or 1 hours, or less than about 60, 50, 40, 30, 25, 20, 15, 10, or 5 minutes. In some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less that about 60 minutes. In some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less than about 40 minutes. In some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less than about 30 minutes. In some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less than about 20 minutes. In some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less than about 15 minutes. In some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less than about 10 minutes. In some embodiments, the time allowed for both binding of the molecule of interest to the capture binding partner, e.g., antibody, and binding of the label to the molecule of interest, is less than about 5 minutes.

[00137] Some immunoassay diagnostic reagents including the capture and signal antibodies used to measure the molecule of interest may be derived from the sera of animals. Endogenous human heterophilic antibodies, or human anti-animal antibodies, which have the ability to bind to immunoglobulins of other species, are present in the serum or plasma of more than 10% of patients. These circulating heterophile antibodies may interfere with immunoassay measurements. In sandwich immunoassays, these heterophilic antibodies can either bridge the capture and detection (diagnostic) antibodies, thereby producing a false-positive signal, or they may block the binding of the diagnostic antibodies, thereby producing a false-negative signal. In competitive immunoassays, the heterophile antibodies may bind to the analytic antibody and inhibit its binding to the troponin. They also may either block or augment the separation of the antibody-troponin complex from free troponin, especially when antispecies antibodies are used in the separation systems. Therefore, the impact of these heterophile antibody interferences are difficult to predict. Thus, it would be advantageous to block the binding of any heterophilic antibodies. In some embodiments of the invention, the immunoassay includes the step of depleting the sample of heterophile antibodies using one or more heterophile antibody blockers. Methods for removing heterophile antibodies from samples that are to be tested in immunoassays are known and include: heating the specimen in a sodium acetate buffer, pH 5.0, for 15 minutes at 90°C and centrifuging at 1200g for 10 minutes, or the heterophile antibodies can be precipitated using polyethylene glycol (PEG); immunoextracting the interfering heterophile immunoglobulins from the specimen using protein A or protein G; or adding nonimmune mouse IgG. Embodiments of the methods of the invention contemplate preparing the sample prior to analysis with the single molecule detector. The appropriateness of the method of pretreatment may be determined. Biochemicals to minimize immunoassay interference caused by heterophile antibodies are commercially available. For example, a product called MAK33, which is an IgG1 monoclonal antibody to h-CK-MM, may be obtained from Boehringer Mannheim. The MAK33 plus product contains a combination of IgG1 and IgG1-Fab. The polyMAK33 contains IgG1-Fab polymerized with IgG1, and the polyMAC 2b/2a contains IgG2a-Fab polymerized with IgG2b. A second commercial source of biochemicals to neutralize heterophile antibodies is Immunoglobulin Inhibiting Reagent marketed by Bioreclamation Inc, East Meadow, NY. This product is a

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preparation of immunoglobulins (IgG and IgM) from multiple species, mainly murine IgG2a, IgG2b, and IgG3 from Balb/c mice. In some embodiments the heterophile antibody may be immunoextracted from the sample using methods known in the art e.g. depleting the sample of the heterophile antibody by binding the interfering antibody to protein A or G. In some embodiments, the heterophile antibody is neutralized using one or more heterophile antibody blockers. Heterophile blockers may be selected from the group consisting of anti-isotype heterophile antibody blockers, anti-idiotype heterophile antibody blockers, and anti-anti-idiotype heterophile antibody blockers. In some embodiments a combination of heterophile antibody blockers may be used.

[00138] Label is added either with or following the addition of sample and washing. Protocols for binding of antibody and other immunolabels to proteins and other molecules are well-known in the art. If the label binding step is separate from capture binding, the time allowed for label binding can be important, e.g., in the clinical setting. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 12, 10, 8, 6, 4, 3, 2, or 1 hours, or less than about 60, 50, 40, 30, 25, 20, 15, 10, or 5 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 60 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 40 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 30 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 20 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 15 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 10 minutes. In some embodiments, the time allowed for binding of the molecule of interest to the label, e.g., antibody-dye, is less than about 5 minutes. Excess label is removed by washing.

[00139] Label is then eluted from the protein of interest. Preferred elution buffers are effective in releasing the label without generating significant background. It is also useful if the elution buffer is bacteriostatic. Elution buffers of use in the invention include a chaotrope, e.g., urea or a guanidinium compound; a buffer, e.g., borate buffered saline; a protein carrier, e.g., an albumin, such as human, bovine, or fish albumin, or an IgG, to coat the wall of the capillary tube in the detection instrument; and a surfactant, e.g., an ionic or nonionic detergent, selected so as to produce a relatively low background, e.g., Tween 20, Triton X-100, or SDS.

[00140] The elution buffer/label aliquot that is sampled into the single molecule detector is referred to as the "processing sample," to distinguish it from the original sample which was obtained from an individual.

[00141] In another embodiment, the solid phase binding assay may employ a competitive binding assay format. One such method comprises a) competitively binding to a capture antibody immobilized on a binding surface i) a molecule of interest, e.g., marker of a biological state, in a sample and ii) a labeled analog of the molecule comprising a detectable label (the detection reagent) and b) measuring the amount of the label using a single particle analyzer. Another such method comprises a) competitively binding to an antibody having a detectable label (the detection reagent) i) a molecule of interest, e.g., marker of a biological state in a sample and ii) an analog of the molecule that is immobilized on a binding surface (the capture reagent) and b) measuring the amount of the label using a single particle analyzer. An "analog of a molecule" refers, herein, to a species that competes with a molecule for binding to a capture antibody. Examples of competitive immunoassays are disclosed in U.S. Pat. No. 4,235,601 to Deutsch et al., U.S. Pat. No. 4,442,204 to Liotta, and U.S. Pat. No. 5,208,535 to Buechler et al., all of which are incorporated herein by reference.

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C. Detection of molecule of interest and determination of concentration

[00142] Following elution, the label is run through a single molecule detector in e.g., the elution buffer. A processing sample may contain no label, a single label, or a plurality of labels. The number of labels corresponds or is proportional to (if dilutions or fractions of samples are used) the number of molecules of the molecule of interest, e.g., marker of a biological state captured during the capture step.

[00143] Any suitable single molecule detector capable of detecting the label used with the molecule of interest may be used. Suitable single molecule detectors are described herein. Typically the detector will be part of a system that includes an automatic sampler for sampling prepared samples, and, optionally, a recovery system to recover samples.

[00144] In some embodiments, the processing sample is analyzed in a single molecule analyzer that utilizes a capillary flow system, and that includes a capillary flow cell, a laser to illuminate an interrogation space in the capillary through which processing sample is passed, a detector to detect radiation emitted from the interrogation space, and a source of motive force to move a processing sample through the interrogation space. In some embodiments, the single molecule analyzer further comprises a microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space, e.g., a high numerical aperture microscope objective. In some embodiments, the laser and detector are in a confocal arrangement. In some embodiments, the laser is a continuous wave laser. In some embodiments, the detector is a avalanche photodiode detector. In some embodiments, the source of motive force is a pump to provide pressure. In some embodiments, the invention provides an analyzer system that includes a sampling system capable of automatically sampling a plurality of samples providing a fluid communication between a sample container and the interrogation space. In some embodiments, the interrogation space has a volume of between about 0.001 and 500 pL, or between about 0.01 pL and 100 pL, or between about 0.01 pL and 10 pL, or between about 0.01 pL and 5 pL, or between about 0.01 pL and 0.5 pL, or between about 0.02 pL and about 300 pL, or between about 0.02 pL and about 50 pL or between about 0.02 pL and about 5 pL or between about 0.02 pL and about 0.5 pL or between about 0.02 pL and about 2 pL, or between about 0.05 pL and about 50 pL, or between about 0.05 pL and about 5 pL, or between about 0.05 pL and about 0.5 pL, or between about 0.05 pL and about 0.2 pL ,or between about 0.1 pL and about 25 pL. In some embodiments, the interrogation space has a volume between about 0.004 pL and 100 pL. In some embodiments, the interrogation space has a volume between about 0.02 pL and 50 pL. In some embodiments, the interrogation space has a volume between about 0.001 pL and 10 pL. In some embodiments, the interrogation space has a volume between about 0.001 pL and 10 pL. In some embodiments, the interrogation space has a volume between about 0.01 pL and 5 pL. In some embodiments, the interrogation space has a volume between about 0.02 pL and about 5 pL. In some embodiments, the interrogation space has a volume between about 0.05 pL and 5 pL In some embodiments, the interrogation space has a volume between about 0.05 pL and 10 pL. In some embodiments, the interrogation space has a volume between about 0.5 pL and about 5 pL. In some embodiments, the interrogation space has a volume between about 0.02 pL and about 0.5 pL.

[00145] In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.02 pL and about 50 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a

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capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.004 pL and about 100 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.05 pL and about 10 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.05 pL and about 5 pL. In some embodiments, the single molecule detector used in the methods of the invention utilizes a capillary flow system, and includes a capillary flow cell, a continuous wave laser to illuminate an interrogation space in the capillary through which processing sample is passed, a high numerical aperture microscope objective lens that collects light emitted from processing sample as it passes through the interrogation space wherein the lens has a numerical aperture of at least about 0.8, an avalanche photodiode detector to detect radiation emitted from the interrogation space, and a pump to provide pressure to move a processing sample through the interrogation space, where the interrogation space is between about 0.5 pL and about 5 pL. In any of these embodiments the analyzer may contain not more than one interrogation space.

[00146] In some embodiments, the single molecule detector is capable of determining a concentration for a molecule of interest in a sample where sample may range in concentration over a range of at least about 100-fold, or 1000-fold, or 10,000-fold, or 100,000-fold, or 300,00-fold, or 1,000,000-fold, or 10,000,000-fold, or 30,000,000-fold.

[00147] In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 50%, 40%, 30%, 20%, 15%, or 10% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 5, 4, 3, 2, or 1 ul, and wherein the analyte is present at a concentration of less than about 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 5, 4, 3, 2, or 1 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 50% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 100 ul, and wherein the analyte is present at a concentration of less than about 100 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 40% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second

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sample introduced into the analyzer is less than about 50 ul, and wherein the analyte is present at a concentration of less than about 50 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 20% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 20 ul, and wherein the analyte is present at a concentration of less than about 20 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 20% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 10 ul, and wherein the analyte is present at a concentration of less than about 10 femtomolar. In some embodiments, the methods of the invention utilize a single molecule detector capable detecting a difference of less than about 20% in concentration of an analyte between a first sample and a second sample that are introduced into the detector, where the volume of the first sample and said second sample introduced into the analyzer is less than about 5 ul, and wherein the analyte is present at a concentration of less than about 5 femtomolar.

[00148] The single molecule detector and systems are described in more detail below. Further embodiments of single molecule analyzers useful in the methods of the invention, such as detectors with more than one interrogation window, detectors utilize electrokinetic or electrophoretic flow, and the like, may be found in U.S. Patent Application No. 11/048,660, incorporated by reference herein in its entirety.

[00149] Between runs the instrument may be washed. A wash buffer that maintains the salt and surfactant concentrations of the sample may be used in some embodiments to maintain the conditioning of the capillary; i.e., to keep the capillary surface relatively constant between samples to reduce variability.

[00150] A feature that contributes to the extremely high sensitivity of the instruments and methods of the invention is the method of detecting and counting labels, which, in some embodiments, are attached to single molecules to be detected or, more typically, correspond to a single molecule to be detected. Briefly, the processing sample flowing through the capillary is effectively divided into a series of detection events, by subjecting a given interrogation space of the capillary to EM radiation from a laser that emits light at an appropriate excitation wavelength for the fluorescent moiety used in the label for a predetermined period of time, and detecting photons emitted during that time. Each predetermined period of time is a "bin." If the total number of photons detected in a given bin exceeds a predetermined threshold level, a detection event is registered for that bin, i.e., a label has been detected. If the total number of photons is not at the predetermined threshold level, no detection event is registered. In some embodiments, processing sample concentration is dilute enough that, for a large percentage of detection events, the detection event represents only one label passing through the window, which corresponds to a single molecule of interest in the original sample, that is, few detection events represent more than one label in a single bin. In some embodiments, further refinements are applied to allow greater concentrations of label in the processing sample to be detected accurately, i.e., concentrations at which the probability of two or more labels being detected as a single detection event is no longer insignificant.

[00151] Although other bin times may be used without departing from the scope of the present invention, in some embodiments the bin times are selected in the range of about 1 microsecond to about 5 ms. In some embodiments, the bin time is more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 250, 300, 400, 500, 600, 700, 750, 800, 900, 1000, 2000, 3000, 4000, or 5000 microseconds. . In some embodiments, the bin time is less than about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 250, 300, 400, 500, 600, 700, 750, 800, 900, 1000, 2000, 3000, 4000, or 5000 microseconds. In some embodiments, the bin time is about 1 to

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1000 microseconds. In some embodiments, the bin time is about 1 to 750 microseconds. In some embodiments, the bin time is about 1 to 500 microseconds. In some embodiments, the bin time is about 1 to 250 microseconds. In some embodiments, the bin time is about 1 to 100 microseconds. In some embodiments, the bin time is about 1 to 50 microseconds. In some embodiments, the bin time is about 1 to 40 microseconds. In some embodiments, the bin time is about 1 to 30 microseconds. In some embodiments, the bin time is about 1 to 500 microseconds. In some embodiments, the bin time is about 1 to 20 microseconds. In some embodiments, the bin time is about 1 to 10 microseconds. In some embodiments, the bin time is about 1 to 500 microseconds. In some embodiments, the bin time is about 1 to 5 microseconds. In some embodiments, the bin time is about 5 to 500 microseconds. In some embodiments, the bin time is about 5 to 250 microseconds. In some embodiments, the bin time is about 5 to 100 microseconds. In some embodiments, the bin time is about 5 to 50microseconds. In some embodiments, the bin time is about 5 to 20 microseconds. In some embodiments, the bin time is about 5 to 10 microseconds. In some embodiments, the bin time is about 10 to 500 microseconds. In some embodiments, the bin time is about 10 to 250 microseconds. In some embodiments, the bin time is about 10 to 100 microseconds. In some embodiments, the bin time is about 10 to 50 microseconds. In some embodiments, the bin time is about 10 to 30 microseconds. In some embodiments, the bin time is about 10 to 20 microseconds. In some embodiments, the bin time is about 5 microseconds. In some embodiments, the bin time is about 5 microseconds. In some embodiments, the bin time is about 6 microseconds. In some embodiments, the bin time is about 7 microseconds. In some embodiments, the bin time is about 8 microseconds. In some embodiments, the bin time is about 9 microseconds. In some embodiments, the bin time is about 10 microseconds. In some embodiments, the bin time is about 11 microseconds. In some embodiments, the bin time is about 12 microseconds. In some embodiments, the bin time is about 13 microseconds. In some embodiments, the bin time is about 14 microseconds. In some embodiments, the bin time is about 5 microseconds. In some embodiments, the bin time is about 15 microseconds. In some embodiments, the bin time is about 16 microseconds. In some embodiments, the bin time is about 17 microseconds. In some embodiments, the bin time is about 18 microseconds. In some embodiments, the bin time is about 19 microseconds. In some embodiments, the bin time is about 20 microseconds. In some embodiments, the bin time is about 25 microseconds. In some embodiments, the bin time is about 30 microseconds. In some embodiments, the bin time is about 40 microseconds. In some embodiments, the bin time is about 50 microseconds. In some embodiments, the bin time is about 100 microseconds. In some embodiments, the bin time is about 250 microseconds. In some embodiments, the bin time is about 500 microseconds. In some embodiments, the bin time is about 750 microseconds. In some embodiments, the bin time is about 1000 microseconds.

[00152] In some embodiments, the background noise level is determined from the mean noise level, or the root-mean-square noise. In other cases, a typical noise value or a statistical value is chosen. In most cases, the noise is expected to follow a Poisson distribution. Thus, in some embodiments, determining the concentration of a particle-label complex in a sample comprises determining the background noise level.

[00153] Thus, as a label flows through the capillary flow cell, it is irradiated by the laser beam to generate a burst of photons. The photons emitted by the label are discriminated from background light or background noise emission by considering only the bursts of photons that have energy above a predetermined threshold energy level which accounts for the amount of background noise that is present in the sample. Background noise typically comprises low frequency emission produced, for example, by the intrinsic fluorescence of non-labeled particles that are present in the sample, the buffer or diluent used in preparing the sample for analysis, Raman scattering and electronic noise. In some embodiments, the value assigned to the background noise is calculated as the average background signal noise detected in a plurality of bins, which are measurements of photon signals that are detected in an interrogation

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space during a predetermined length of time. Thus in some embodiments, background noise is calculated for each sample as a number specific to that sample.

[00154] Given the value for the background noise, the threshold energy level can be assigned. As discussed above, the threshold value is determined to discriminate true signals (due to fluorescence of a label) from the background noise. Care must be taken in choosing a threshold value such that the number of false positive signals from random noise is minimized while the number of true signals which are rejected is also minimized. Methods for choosing a threshold value include determining a fixed value above the noise level and calculating a threshold value based on the distribution of the noise signal. In one embodiment, the threshold is set at a fixed number of standard deviations above the background level. Assuming a Poisson distribution of the noise, using this method one can estimate the number of false positive signals over the time course of the experiment. In some embodiments, the threshold level is calculated as a value of 4 sigma above the background noise. For example, given an average background noise level of 200 photons, the analyzer system establishes a threshold level of $4\sqrt{200}$ above the average background/noise level of 200 photons to be 256 photons. Thus, in some embodiments, determining the concentration of a label in a sample includes establishing the threshold level above which photon signals represent the presence of a label. Conversely, photon signals that have an energy level that is not greater than that of the threshold level indicate the absence of a label.

[00155] Many bin measurements are taken to determine the concentration of a sample, and the absence or presence of a label is ascertained for each bin measurement. Typically, 60,000 measurements or more can made in one minute (e.g., in embodiments in which the bin size is 1ms—for smaller bin sizes the number of measurements is correspondingly larger, e.g., 6,000,000 measurements per minute for a bin size of 10 microseconds). Thus, no single measurement is crucial and the method provides for a high margin of error. The bins that are determined not to contain a label ("no" bins) are discounted and only the measurements made in the bins that are determined to contain label ("yes" bins) are accounted in determining the concentration of the label in the processing sample. Discounting measurements made in the "no" bins or bins that are devoid of label increases the signal to noise ratio and the accuracy of the measurements. Thus, in some embodiments, determining the concentration of a label in a sample comprises detecting the bin measurements that reflect the presence of a label.

[00156] The signal to noise ratio or the sensitivity of the analyzer system can be increased by minimizing the time that background noise is detected during a bin measurement in which a particle-label complex is detected. For example, in a bin measurement lasting 1 millisecond during which one particle-label complex is detected when passing across an interrogation space within 250 microseconds, 750 microseconds of the 1 millisecond are spent detecting background noise emission. The signal to noise ratio can be improved by decreasing the bin time. In some embodiments, the bin time is 1 millisecond. In other embodiments, the bin time is 750, 500, 250 microseconds, 100 microseconds, 50 microseconds, 25 microseconds or 10 microseconds. Other bin times are as described herein.

[00157] Other factors that affect measurements are the brightness or dimness of the fluorescent moiety, the flow rate, and the power of the laser. Various combinations of the relevant factors that allow for detection of label will be apparent to those of skill in the art. In some embodiments, the bin time is adjusted without changing the flow rate. It will be appreciated by those of skill in the art that as bin time decreases, laser power output directed at the interrogation space must increase to maintain a constant total energy applied to the interrogation space during the bin time. For example, if bin time is decreased from 1000 microseconds to 250 microseconds, as a first approximation, laser power output must be increased approximately four-fold. These settings allow for the detection of the same number of photons in a 250μs as the number of photons counted during the 1000 μs given the

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previous settings, and allow for faster analysis of sample with lower backgrounds and thus greater sensitivity. In addition, flow rates may be adjusted in order to speed processing of sample. These numbers are merely exemplary, and the skilled practitioner can adjust the parameters as necessary to achieve the desired result.

[00158] In some embodiments, the interrogation space encompasses the entire cross-section of the sample stream. When the interrogation space encompasses the entire cross-section of the sample stream, only the number of labels counted and the volume passing through a cross-section of the sample stream in a set length of time are needed to calculate the concentration of the label in the processing sample. In some embodiments, the interrogation space can be defined to be smaller than the cross-sectional area of sample stream by, for example, the interrogation space is defined by the size of the spot illuminated by the laser beam. In some embodiments, the interrogation space can be defined by adjusting the apertures 306 (Figure 1A) or 358 and 359 (Figure 1B) of the analyzer and reducing the illuminated volume that is imaged by the objective lens to the detector. In the embodiments when the interrogation space is defined to be smaller than the cross-sectional area of sample stream, the concentration of the label can be determined by interpolation of the signal emitted by the complex from a standard curve that is generated using one or more samples of known standard concentrations. In yet other embodiments, the concentration of the label can be determined by comparing the measured particles to an internal label standard. In embodiments when a diluted sample is analyzed, the dilution factor is accounted in calculating the concentration of the molecule of interest in the starting sample.

[00159] As discussed above, when the interrogation space encompasses the entire cross-section of the sample stream, only the number of labels counted passing through a cross-section of the sample stream in a set length of time (bin) and the volume of sample that was interrogated in the bin are needed to calculate the concentration the sample. The total number of labels contained in the "yes" bins is determined and related to the sample volume represented by the total number of bins used in the analysis to determine the concentration of labels in the processing sample. Thus, in one embodiment, determining the concentration of a label in a processing sample comprises determining the total number of labels detected "yes" bins and relating the total number of detected labels to the total sample volume that was analyzed. The total sample volume that is analyzed is the sample volume that is passed through the capillary flow cell and across the interrogation space in a specified time interval. Alternatively, the concentration of the label complex in a sample is determined by interpolation of the signal emitted by the label in a number of bins from a standard curve that is generated by determining the signal emitted by labels in the same number of bins by standard samples containing known concentrations of the label.

[00160] In some embodiments, the number of individual labels that are detected in a bin is related to the relative concentration of the particle in the processing sample. At relatively low concentrations, for example at concentrations below about 10^{-16} M the number of labels is proportional to the photon signal that is detected in a bin. Thus, at low concentrations of label the photon signal is provided as a digital signal. At relatively higher concentrations, for example at concentrations greater than about 10^{-16} M, the proportionality of photon signal to a label is lost as the likelihood of two or more labels crossing the interrogation space at about the same time and being counted as one becomes significant. Thus, in some embodiments, individual particles in a sample of a concentration greater than about 10^{-16} M are resolved by decreasing the length of time of the bin measurement.

[00161] Alternatively, in other embodiments, the total the photon signal that is emitted by a plurality of particles that are present in any one bin is detected. These embodiments allow for single molecule detectors of the invention wherein the dynamic range is at least 3, 3.5, 4, 4.5, 5.5, 6, 6.5, 7, 7.5, 8, or more than 8 logs.

[00162] "Dynamic range," as that term is used herein, refers to the range of sample concentrations that may be quantitated by the instrument without need for dilution or other treatment to alter the concentration of successive

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samples of differing concentrations, where concentrations are determined with an accuracy appropriate for the intended use. For example, if a microtiter plate contains a sample of 1 femtomolar concentration for an analyte of interest in one well, a sample of 10,000 femtomolar concentration for an analyte of interest in another well, and a sample of 100 femtomolar concentration for the analyte in a third well, an instrument with a dynamic range of at least 4 logs and a lower limit of quantitation of 1 femtomolar is able to accurately quantitate the concentration of all the samples without the need for further treatment to adjust concentration, e.g., dilution. Accuracy may be determined by standard methods, e.g., using a series of standards of concentrations that span the dynamic range and constructing a standard curve. Standard measures of fit of the resulting standard curve may be used as a measure of accuracy, e.g., an r^2 greater than about 0.7, 0.75, 0.8, 0.85, 0.9, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, or 0.99.

[00163] Increased dynamic range is achieved by altering the manner in which data from the detector is analyzed, and/or by the use of an attenuator between the detector and the interrogation space. At the low end of the range, where processing sample is sufficiently dilute that each detection event, i.e., each burst of photons above a threshold level in a bin (the "event photons"), likely represents only one label, the data is analyzed to count detection events as single molecules. I.e., each bin is analyzed as a simple "yes" or "no" for the presence of label, as described above. For a more concentrated processing sample, where the likelihood of two or more labels occupying a single bin becomes significant, the number of event photons in a significant number of bins is found to be substantially greater than the number expected for a single label, e.g., the number of event photons in a significant number of bins corresponds to two-fold, three-fold, or more, than the number of event photons expected for a single label. For these samples, the instrument changes its method of data analysis to one of integrating the total number of event photons for the bins of the processing sample. This total will be proportional to the total number of labels that were in all the bins. For an even more concentrated processing sample, where many labels are present in most bins, background noise becomes an insignificant portion of the total signal from each bin, and the instrument changes its method of data analysis to one of counting total photons per bin (including background). An even further increase in dynamic range can be achieved by the use of an attenuator between the flow cell and the detector, when concentrations are such that the intensity of light reaching the detector would otherwise exceed the capacity of the detector for accurately counting photons, i.e., saturate the detector.

[00164] The instrument may include a data analysis system that receives input from the detector and determines the appropriate analysis method for the sample being run, and outputs values based on such analysis. The data analysis system may further output instructions to use or not use an attenuator, if an attenuator is included in the instrument.

[00165] By utilizing such methods, the dynamic range of the instrument can be dramatically increased. Thus, in some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than about 1000 (3 log), 10,000 (4 log), 100,000 (5 log), 350,000 (5.5 log), 1,000,000 (6 log), 3,500,000 (6.5 log), 10,000,000 (7 log), 35,000,000 (7.5 log), or 100,000,000 (8 log). In some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than about 100,000 (5 log). In some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than about 1,000,000 (6 log). In some embodiments, the instrument is capable of measuring concentrations of samples over a dynamic range of more than about 10,000,000 (7 log). In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 1000; 10,000; 100,000; 350,000; 1,000,000; 3,500,000; 10,000,000, or 35,000,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 10,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about

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100,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 1,000,000 femtomolar. In some embodiments, the instrument is capable of measuring the concentrations of samples over a dynamic range of from about 1-10 femtomolar to at least about 10,000,000.

[00166] In some embodiments, an analyzer or analyzer system of the invention is capable of detecting an analyte, e.g., a biomarker at a limit of detection of less than 1 nanomolar, or 1 picomolar, or 1 femtomolar, or 1 attomolar, or 1 zeptomolar. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte, or of multiple analytes, e.g., a biomarker or biomarkers, from one sample to another sample of less than about 0.1, 1, 2, 5, 10, 20, 30, 40, 50, 60, or 80% when the biomarker is present at a concentration of less than 1 nanomolar, or 1 picomolar, or 1 femtomolar, or 1 attomolar, or 1 zeptomolar, in the samples, and when the size of each of the sample is less than about 100, 50, 40, 30, 20, 10, 5, 2, 1, 0.1, 0.01, 0.001, or 0.0001 ul. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 1 picomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 100 femtomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 50 femtomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 5 femtomolar, and when the size of each of the samples is less than about 50 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 5 femtomolar, and when the size of each of the samples is less than about 5 µl. In some embodiments, the analyzer or analyzer system is capable of detecting a change in concentration of the analyte from a first sample to a second sample of less than about 20%, when the analyte is present at a concentration of less than about 1 femtomolar, and when the size of each of the samples is less than about 5 µl.

V. Instruments and Systems Suitable for Highly Sensitive Analysis of Molecules

[00167] The methods of the invention utilize analytical instruments of high sensitivity, e.g., single molecule detectors. Such single molecule detectors include embodiments as hereinafter described.

[00168] In some embodiments, the invention provides an analyzer system kit for detecting a single protein molecule in a sample, said system includes an analyzer system for detecting a single protein molecule in a sample and least one label that inlcudes a fluorescent moiety and a binding partner for the protein molecule, where the analyzer includes an electromagnetic radiation source for stimulating the fluorescent moiety; a capillary flow cell for passing the label; a source of motive force for moving the label in the capillary flow cell; an interrogation space defined within the capillary flow cell for receiving electromagnetic radiation emitted from the electromagnetic source; and an electromagnetic radiation detector operably connected to the interrogation space for measuring an electromagnetic characteristic of the stimulated fluorescent moiety, where the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the

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moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules.

[00169] One embodiment of an analyzer kit of the invention is depicted in Fig. 12. The kit includes a label for a protein molecule that includes a binding partner for a protein molecule and a fluorescent moiety. The kit further includes an analyzer system for detecting a single protein molecule (300) that includes an electromagnetic radiation source 301 for stimulating the fluorescent moiety, a capillary flow cell 313 for passing the label; a source of motive force for moving the label in the capillary flow cell (not shown); an interrogation space defined within the capillary flow cell for receiving electromagnetic radiation emitted from the electromagnetic source 314 (Figure 2A) ; and an electromagnetic radiation detector 309 operably connected to the interrogation space for measuring an electromagnetic characteristic of the stimulated fluorescent moiety, where the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and where the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the beam 311 from an electromagnetic radiation source 301 is focused by the microscope objective 315 to form one interrogation space 314 (Figure 2A) within the capillary flow cell 313. the microscope objective may have a numerical aperture of equal to or greater than 0.7, 0.8, 0.9, or 1.0 in some embodiments.

[00170] In some embodiments of the analyzer system kit, the analyzer comprises not more than one interrogation space. In some embodiments, the electromagnetic radiation source is a laser that has a power output of at least about 3, 5, 10, or 20 mW. In some embodiments, the fluorescent moiety comprises a fluorescent molecule. In some embodiments, the fluorescent molecule is a dye molecule, such as a dye molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the fluorescent moiety is a quantum dot. In some embodiments, the electromagnetic radiation source is a continuous wave electromagnetic radiation source, such as a light-emitting diode or a continuous wave laser. In some embodiments, the motive force is pressure. In some embodiments, the detector is an avalanche photodiode detector. In some embodiments, the analyzer utilizes a confocal optical arrangement for deflecting a laser beam onto said interrogation space and for imaging said stimulated dye molecule (shown in Fig. 12), wherein said confocal optical arrangement comprises an objective lens having a numerical aperture of at least about 0.8. In some embodiments, the analyzer further comprises a sampling system capable of automatically sampling a plurality of samples and providing a fluid communication between a sample container and said interrogation space. In some embodiments, the analyzer system further comprises a sample recovery system in fluid communication with said interrogation space, wherein said recovery system is capable of recovering substantially all of said sample. In some embodiments, the kit further includes instructions for use of the system.

A. Apparatus/System

[00171] In one aspect, the methods described herein utilize an analyzer system capable of detecting a single molecule in a sample. In one embodiment, the analyzer system is capable of single molecule detection of a fluorescently labeled particle wherein the analyzer system detects energy emitted by an excited fluorescent label in response to exposure by an electromagnetic radiation source when the single particle is present in an interrogation space defined within a capillary flow cell fluidly connected to the sampling system of the analyzer system. In a further embodiment of the analyzer system, the single particle moves through the interrogation space of the capillary

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flow cell by means of a motive force. In another embodiment of the analyzer system, an automatic sampling system may be included in the analyzer system for introducing the sample into the analyzer system. In another embodiment of the analyzer system, a sample preparation system may be included in the analyzer system for preparing a sample. In a further embodiment, the analyzer system may contain a sample recovery system for recovering at least a portion of the sample after analysis is complete.

[00172] In one aspect, the analyzer system consists of an electromagnetic radiation source for exciting a single particle labeled with a fluorescent label. In one embodiment, the electromagnetic radiation source of the analyzer system is a laser. In a further embodiment, the electromagnetic radiation source is a continuous wave laser.

[00173] In a typical embodiment, the electromagnetic radiation source excites a fluorescent moiety attached to a label as the label passes through the interrogation space of the capillary flow cell. In some embodiments, the fluorescent label moiety includes one or more fluorescent dye molecules. In some embodiments, the fluorescent label moiety is a quantum dot. Any fluorescent moiety as described herein may be used in the label.

[00174] A label is exposed to electromagnetic radiation when the label passes through an interrogation space located within the capillary flow cell. The interrogation space is typically fluidly connected to a sampling system. In some embodiments the label passes through the interrogation space of the capillary flow cell due to a motive force to advance the label through the analyzer system. The interrogation space is positioned such that it receives electromagnetic radiation emitted from the radiation source. In some embodiments, the sampling system is an automated sampling system capable of sampling a plurality of samples without intervention from a human operator.

[00175] The label passes through the interrogation space and emits a detectable amount of energy when excited by the electromagnetic radiation source. In one embodiment, an electromagnetic radiation detector is operably connected to the interrogation space. The electromagnetic radiation detector is capable of detecting the energy emitted by the label, e.g., by the fluorescent moiety of the label.

In a further embodiment of the analyzer system, the system further includes a sample preparation mechanism where a sample may be partially or completely prepared for analysis by the analyzer system. In some embodiments of the analyzer system, the sample is discarded after it is analyzed by the system. In other embodiments, the analyzer system further includes a sample recovery mechanism whereby at least a portion, or alternatively all or substantially all, of the sample may be recovered after analysis. In such an embodiment, the sample can be returned to the origin of the sample. In some embodiments, the sample can be returned to microtiter wells on a sample microtiter plate. The analyzer system typically further consists of a data acquisition system for collecting and reporting the detected signal.

B. Single Particle Analyzer

[00176] As shown in Figure 1A, described herein is one embodiment of an analyzer system 300. The analyzer system 300 includes an electromagnetic radiation source 301, a mirror 302, a lens 303, a capillary flow cell 313, a microscopic objective lens 305, an aperture 306, a detector lens 307, a detector filter 308, a single photon detector 309, and a processor 310 operatively connected to the detector.

[00177] In operation the electromagnetic radiation source 301 is aligned so that its output 311 is reflected off of a front surface 312 of mirror 302. The lens 303 focuses the beam 311 onto a single interrogation space (an illustrative example of an interrogation space 314 is shown in Figure 2A) in the capillary flow cell 313. The microscope objective lens 305 collects light from sample particles and forms images of the beam onto the aperture 306. The aperture 306 affects the fraction of light emitted by the specimen in the interrogation space of the capillary flow cell 313 that can be collected. The detector lens 307 collects the light passing through the aperture 306 and focuses the

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light onto an active area of the detector 309 after it passes through the detector filters 308. The detector filters 308 minimize aberrant noise signals due to light scatter or ambient light while maximizing the signal emitted by the excited fluorescent moiety bound to the particle. The processor 310 processes the light signal from the particle according to the methods described herein.

[00178] In one embodiment, the microscope objective lens 305 is a high numerical aperture microscope objective. As used herein, "high numerical aperture lens" include a lens with a numerical aperture of equal to or greater than 0.6. The numerical aperture is a measure of the number of highly diffracted image-forming light rays captured by the objective. A higher numerical aperture allows increasingly oblique rays to enter the objective lens and thereby produce a more highly resolved image. Additionally, the brightness of an image increases with a higher numerical aperture. High numerical aperture lenses are commercially available from a variety of vendors, and any one lens having a numerical aperture of equal to or greater than approximately 0.6 may be used in the analyzer system. In some embodiments, the lens has a numerical aperture of about 0.6 to about 1.3. In some embodiments, the lens has a numerical aperture of about 0.6 to about 1.0. In some embodiments, the lens has a numerical aperture of about 0.7 to about 1.2. In some embodiments, the lens has a numerical aperture of about 0.7 to about 1.0. In some embodiments, the lens has a numerical aperture of about 0.7 to about 0.9. In some embodiments, the lens has a numerical aperture of about 0.8 to about 1.3. In some embodiments, the lens has a numerical aperture of about 0.8 to about 1.2. In some embodiments, the lens has a numerical aperture of about 0.8 to about 1.0. In some embodiments, the lens has a numerical aperture of at least about 0.6. In some embodiments, the lens has a numerical aperture of at least about 0.7. In some embodiments, the lens has a numerical aperture of at least about 0.8. In some embodiments, the lens has a numerical aperture of at least about 0.9. In some embodiments, the lens has a numerical aperture of at least about 1.0. In some embodiments, the aperture of the microscope objective lens 305 is approximately 1.25. In an embodiment where a microscope objective lens 305 of 0.8 is used, a Nikon 60X/0.8 NA Achromat lens (Nikon, Inc., USA) can be used.

[00179] In some embodiments, the electromagnetic radiation source 301 is a laser that emits light in the visible spectrum. In all embodiments, the electromagnetic radiation source is set such that wavelength of the laser is set such that it is of a sufficient wavelength to excite the fluorescent label attached to the particle. In some embodiments, the laser is a continuous wave laser with a wavelength of 639 nm. In other embodiments, the laser is a continuous wave laser with a wavelength of 532 nm. In other embodiments, the laser is a continuous wave laser with a wavelength of 422 nm. In other embodiments, the laser is a continuous wave laser with a wavelength of 405 nm. Any continuous wave laser with a wavelength suitable for exciting a fluorescent moiety as used in the methods and compositions of the invention may be used without departing from the scope of the invention.

[00180] In a single particle analyzer system 300, as each particle passes through the beam 311 of the electromagnetic radiation source, the particle enters into an excited state. When the particle relaxes from its excited state, a detectable burst of light is emitted. The excitation-emission cycle is repeated many times by each particle in the length of time it takes for it to pass through the beam allowing the analyzer system 300 to detect tens to thousands of photons for each particle as it passes through an interrogation space 314. Photons emitted by fluorescent particles are registered by the detector 309 (Figure 1A) with a time delay indicative of the time for the particle label complex to pass through the interrogation space. The photon intensity is recorded by the detector 309 and sampling time is divided into bins, which are uniform, arbitrary, time segments with freely selectable time channel widths. The number of signals contained in each bin evaluated. One or a combination of several statistical analytical methods are employed in order to determine when a particle is present. Such methods include

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determining the baseline noise of the analyzer system and setting a signal strength for the fluorescent label at a statistical level above baseline noise to eliminate false positive signals from the detector.

[00181] The electromagnetic radiation source 301 is focused onto a capillary flow cell 313 of the analyzer system 300 where the capillary flow cell 313 is fluidly connected to the sample system. An interrogation space 314 is shown in Figure 2A. The beam 311 from the continuous wave electromagnetic radiation source 301 of Figure 1A is optically focused to a specified depth within the capillary flow cell 313. The beam 311 is directed toward the sample-filled capillary flow cell 313 at an angle perpendicular to the capillary flow cell 313. The beam 311 is operated at a predetermined wavelength that is selected to excite a particular fluorescent label used to label the particle of interest. The size or volume of the interrogation space 314 is determined by the diameter of the beam 311 together with the depth at which the beam 311 is focused. Alternatively, the interrogation space can be determined by running a calibration sample of known concentration through the analyzer system.

[00182] When single molecules are detected in the sample concentration, the beam size and the depth of focus required for single molecule detection are set and thereby define the size of the interrogation space 314. The interrogation space 314 is set such that, with an appropriate sample concentration, only one particle is present in the interrogation space 314 during each time interval over which time observations are made.

[00183] It will be appreciated that the detection interrogation volume as defined by the beam is not perfectly spherically shaped, and typically is a "bow-tie" shape. However, for the purposes of definition, "volumes" of interrogation spaces are defined herein as the volume encompassed by a sphere of a diameter equal to the focused spot diameter of the beam. The focused spot of the beam 311 may have various diameters without departing from the scope of the present invention. In some embodiments, the diameter of the focused spot of the beam is about 1 to about 5, 10, 15, or 20 microns, or about 5 to about 10, 15, or 20 microns, or about 10 to about 20 microns, or about 10 to about 15 microns. In some embodiments, the diameter of the focused spot of the beam is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 microns. In some embodiments, the diameter of the focused spot of the beam is about 5 microns. In some embodiments, the diameter of the focused spot of the beam is about 10 microns. In some embodiments, the diameter of the focused spot of the beam is about 12 microns. In some embodiments, the diameter of the focused spot of the beam is about 13 microns. In some embodiments, the diameter of the focused spot of the beam is about 14 microns. In some embodiments, the diameter of the focused spot of the beam is about 15 microns. In some embodiments, the diameter of the focused spot of the beam is about 16 microns. In some embodiments, the diameter of the focused spot of the beam is about 17 microns. In some embodiments, the diameter of the focused spot of the beam is about 18 microns. In some embodiments, the diameter of the focused spot of the beam is about 19 microns. In some embodiments, the diameter of the focused spot of the beam is about 20 microns.

[00184] In an alternate embodiment of the single particle analyzer system, more than one electromagnetic radiation source can be used to excite particles labeled with fluorescent labels of different wavelengths. In another alternate embodiment, more than one interrogation space in the capillary flow cell can be used. In another alternate embodiment, multiple detectors can be employed to detect different emission wavelengths from the fluorescent labels. An illustration incorporating each of these alternative embodiments of an analyzer system is shown in Figure 1B. These embodiments are incorporated by reference from previous U.S. Pat. App. No. 11/048,660.

[00185] In some embodiments of the analyzer system 300, a motive force is required to move a particle through the capillary flow cell 313 of the analyzer system 300. In one embodiment, the motive force can be a form of pressure. The pressure used to move a particle through the capillary flow cell can be generated by a pump. In some embodiments, a Scivex, Inc. HPLC pump can be used. In some embodiments where a pump is used as a motive

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force, the sample can pass through the capillary flow cell at a rate of 1 μL/min to about 20 μL/min, or about 5 μL/min to about 20 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 5 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 10 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 15 μL/min. In some embodiments, the sample can pass through the capillary flow cell at a rate of about 20 μL/min. In some embodiments, an electrokinetic force can be used to move the particle through the analyzer system. Such a method has been previously disclosed and is incorporated by reference from previous U.S. Pat. App. No. 11/048,660.

[00186] In one aspect of the analyzer system 300, the detector 309 of the analyzer system detects the photons emitted by the fluorescent label. In one embodiment, the photon detector is a photodiode. In a further embodiment, the detector is an avalanche photodiode detector. In some embodiments, the photodiodes can be silicon photodiodes with a wavelength detection of 190 nm and 1100 nm. When germanium photodiodes are used, the wavelength of light detected is between 400 nm to 1700 nm. In other embodiments, when an indium gallium arsenide photodiode is used, the wavelength of light detected by the photodiode is between 800 nm and 2600 nm. When lead sulfide photodiodes are used as detectors, the wavelength of light detected is between 1000 nm and 3500 nm.

[00187] In some embodiments, the optics of the electromagnetic radiation source 301 and the optics of the detector 309 are arranged in a conventional optical arrangement. In such an arrangement, the electromagnetic radiation source and the detector are aligned on different focal planes. The arrangement of the laser and the detector optics of the analyzer system as shown in Figures 1A and 1B is that of a conventional optical arrangement.

[00188] In some embodiments, the optics of the electromagnetic radiation source and the optics of the detector are arranged in a confocal optical arrangement. In such an arrangement, the electromagnetic radiation source 301 and the detector 309 are aligned on the same focal plane. The confocal arrangement renders the analyzer more robust because the electromagnetic radiation source 301 and the detector optics 309 do not need to be realigned if the analyzer system is moved. This arrangement also makes the use of the analyzer more simplified because it eliminates the need to realign the components of the analyzer system. The confocal arrangement for the analyzer 300 (Figure 1A) and the analyzer 355 (Figure 1B) are shown in Figures 3A and 3B respectively. Figure 3A shows that the beam 311 from an electromagnetic radiation source 301 is focused by the microscope objective 315 to form one interrogation space 314 (Figure 2A) within the capillary flow cell 313. A dichroic mirror 316, which reflects laser light but passes fluorescent light, is used to separate the fluorescent light from the laser light. Filter 317 that is positioned in front of the detector eliminates any non-fluorescent light at the detector. In some embodiments, an analyzer system configured in a confocal arrangement can comprise two or more interrogations spaces. Such a method has been previously disclosed and is incorporated by reference from previous U.S. Pat. App. No. 11/048,660.

[00189] The laser can be a tunable dye laser, such as a helium-neon laser. The laser can be set to emit a wavelength of 632.8 nm. Alternatively, the wavelength of the laser can be set to emit a wavelength of 543.5 nm or 1523 nm. Alternatively, the electromagnetic laser can be an argon ion laser. In such an embodiment, the argon ion laser can be operated as a continuous gas laser at about 25 different wavelengths in the visible spectrum, the wavelength set between 408.9 and 686.1 nm but at its optimum performance set between 488 and 514.5 nm.

1 Electromagnetic Radiation Source

[00190] In some embodiments of the analyzer system a chemiluminescent label may be used. In such an embodiment, it may not be necessary to utilize an EM source for detection of the particle. In another embodiment, the extrinsic label or intrinsic characteristic of the particle is a light-interacting label or characteristic, such as a

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fluorescent label or a light-scattering label. In such an embodiment, a source of EM radiation is used to illuminate the label and/or the particle. EM radiation sources for excitation of fluorescent labels are preferred.

[00191] In some embodiments, the analyzer system consists of an electromagnetic radiation source 301. Any number of radiation sources may be used in any one analyzer system 300 without departing from the scope of the invention. Multiple sources of electromagnetic radiation have been previously disclosed and are incorporated by reference from previous U.S. Pat. App. No. 11/048,660. In some embodiments, all the continuous wave electromagnetic (EM) radiation sources emit electromagnetic radiation at the same wavelengths. In other embodiments, different sources emit different wavelengths of EM radiation.

[00192] In one embodiment, the EM source(s) 301, 351, 352 are continuous wave lasers producing wavelengths of between 200 nm and 1000 nm. Such EM sources have the advantage of being small, durable and relatively inexpensive. In addition, they generally have the capacity to generate larger fluorescent signals than other light sources. Specific examples of suitable continuous wave EM sources include, but are not limited to: lasers of the argon, krypton, helium-neon, helium-cadmium types, as well as, tunable diode lasers (red to infrared regions), each with the possibility of frequency doubling. The lasers provide continuous illumination with no accessory electronic or mechanical devices, such as shutters, to interrupt their illumination. In an embodiment where a continuous wave laser is used, an electromagnetic radiation source of 3 mW may be of sufficient energy to excite a fluorescent label. A beam from a continuous wave laser of such energy output may be between 2 to 5 μm in diameter. The time of exposure of the particle to laser beam in order to be exposed to 3mW may be a time period of about 1 msec. In alternate embodiments, the time of exposure to the laser beam may be equal to or less than about 500 μsec. In an alternate embodiment, the time of exposure may be equal to or less than about 100 μsec. In an alternate embodiment, the time of exposure may be equal to or less than about 50 μsec. In an alternate embodiment, the time of exposure may be equal to or less than about 10 μsec.

[00193] LEDs are another low-cost, high reliability illumination source. Recent advances in ultra-bright LEDs and dyes with high absorption cross-section and quantum yield support the applicability of LEDs to single particle detection. Such lasers could be used alone or in combination with other light sources such as mercury arc lamps, elemental arc lamps, halogen lamps, arc discharges, plasma discharges, light-emitting diodes, or combination of these.

[00194] In other embodiments, the EM source could be in the form of a pulse wave laser. In such an embodiment, the pulse size of the laser is an important factor. In such an embodiment, the size, focus spot, and the total energy emitted by the laser is important and must be of sufficient energy as to be able to excite the fluorescent label. When a pulse laser is used, a pulse of longer duration may be required. In some embodiments a laser pulse of 2 nanoseconds may be used. In some embodiments a laser pulse of 5 nanoseconds may be used. In some embodiments a pulse of between 2 to 5 nanoseconds may be used.

[00195] The optimal laser intensity depends on the photo bleaching characteristics of the single dyes and the length of time required to traverse the interrogation space (including the speed of the particle, the distance between interrogation spaces if more than one is used and the size of the interrogation space(s)). To obtain a maximal signal, it is desirable to illuminate the sample at the highest intensity which will not result in photo bleaching a high percentage of the dyes. The preferred intensity is one such that no more that 5% of the dyes are bleached by the time the particle has traversed the interrogation space.

[00196] The power of the laser is set depending on the type of dye molecules that need to be stimulated and the length of time the dye molecules are stimulated, and/or the speed with which the dye molecules pass through the capillary flow cell. Laser power is defined as the rate at which energy is delivered by the beam and is measured in

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units of Joules/second, or Watts. It will be appreciated that the greater the power output of the laser, the shorter the time that the laser illuminates the particle may be, while providing a constant amount of energy to the interrogation space while the particle is passing through the space. Thus, in some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is more than about 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is less than about 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, or 110 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 0.1 and 100 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 1 and 100 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 1 and 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 2 and 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 60 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 40 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is between about 3 and 30 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 1 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 3 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 5 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 10 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 15 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 20 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 30 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 40 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 50 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 60 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about

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70 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 80 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 90 microJoule. In some embodiments, the combination of laser power and time of illumination is such that the total energy received by the interrogation space during the time of illumination is about 100 microJoule.

[00197] In some embodiments, the laser power output is set to at least about 1 mW, 2 mW, 3mW, 4mW, 5 mW, 6, mw, 7 mW, 8 mW, 9 mW, 10 mW, 13 mW, 15 mW, 20 mW, 25 mW, 30 mW, 40 mW, 50 mW, 60 mW, 70 mW, 80 mW, 90 mW, 100 mW, or more than 100 mW. In some embodiments, the laser power output is set to at least about 1 mW. In some embodiments, the laser power output is set to at least about 3 mW. In some embodiments, the laser power output is set to at least about 5 mW. In some embodiments, the laser power output is set to at least about 10 mW. In some embodiments, the laser power output is set to at least about 15 mW. In some embodiments, the laser power output is set to at least about 20 mW. In some embodiments, the laser power output is set to at least about 30 mW. In some embodiments, the laser power output is set to at least about 40 mW. In some embodiments, the laser power output is set to at least about 50 mW. In some embodiments, the laser power output is set to at least about 60 mW. In some embodiments, the laser power output is set to at least about 90 mW.

[00198] The time that the laser illuminates the interrogation space can be set to no less than about 1, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 150, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 microseconds. The time that the laser illuminates the interrogation space can be set to no more than about 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 150, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 1500, or 2000 microseconds. The time that the laser illuminates the interrogation space can be set between about 1 and 1000 microseconds. The time that the laser illuminates the interrogation space can be set between about 5 and 500 microseconds. The time that the laser illuminates the interrogation space can be set between about 5 and 100 microseconds. The time that the laser illuminates the interrogation space can be set between about 10 and 100 microseconds. The time that the laser illuminates the interrogation space can be set between about 10 and 50 microseconds. The time that the laser illuminates the interrogation space can be set between about 10 and 20 microseconds. The time that the laser illuminates the interrogation space can be set between about 5 and 50 microseconds. The time that the laser illuminates the interrogation space can be set between about 1 and 100 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 1 microsecond. In some embodiments, the time that the laser illuminates the interrogation space is about 5 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 10 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 25 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 50 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 100 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 250 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 500 microseconds. In some embodiments, the time that the laser illuminates the interrogation space is about 1000 microseconds.

[00199] For example, the time that the laser illuminates the interrogation space can be set to 1 millisecond, 250 microseconds, 100 microseconds, 50 microseconds, 25 microseconds or 10 microseconds with a laser that provides a power output of 3 mW, 4 mw, 5 mW, or more than 5 mW. In some embodiments, a label is illuminated with a laser that provides a power output of 3mW and illuminates the label for about 1000 microseconds. In other

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embodiments, a label is illuminated for less than 1000 milliseconds with a laser providing a power output of not more than about 20 mW. In other embodiments, the label is illuminated with a laser power output of 20 mW for less than or equal to about 250 microseconds. In some embodiments, the label is illuminated with a laser power output of about 5 mW for less than or equal to about 1000 microseconds.

2. Capillary Flow Cell

[00200] The capillary flow cell is fluidly connected to the sample system. In one embodiment, the interrogation space 314 of an analyzer system, is determined by the cross sectional area of the corresponding beam 311 and by a segment of the beam within the field of view of the detector 309. In one embodiment of the analyzer system, the interrogation space 314 has a volume, as defined herein, of between about between about 0.01 and 500 pL, or between about 0.01 pL and 100 pL, or between about 0.01 pL and 10 pL, or between about 0.01 pL and 1 pL, or between about 0.01 pL and 0.5 pL, or between about 0.02 pL and about 300 pL, or between about 0.02 pL and about 50 pL or between about 0.02 pL and about 5 pL or between about 0.02 pL and about 0.5 pL or between about 0.02 pL and about 2 pL, or between about 0.05 pL and about 50 pL, or between about 0.05 pL and about 5 pL, or between about 0.05 pL and about 0.5 pL, or between about 0.05 pL and about 0.2 pL ,or between about 0.1 pL and about 25 pL. In some embodiments, the interrogation space has a volume between about 0.01 pL and 10 pL. In some embodiments, the interrogation space 314 has a volume between about 0.01 pL and 1 pL. In some embodiments, the interrogation space 314 has a volume between about 0.02 pL and about 5 pL. In some embodiments, the interrogation space 314 has a volume between about 0.02 pL and about 0.5 pL. In some embodiments, the interrogation space 314 has a volume between about 0.05 pL and about 0.2 pL. In some embodiments, the interrogation space 314 has a volume of about 0.1 pL. Other useful interrogation space volumes are as described herein. It should be understood by one skilled in the art that the interrogation space 314 can be selected for maximum performance of the analyzer. Although very small interrogation spaces have been shown to minimize the background noise, large interrogation spaces have the advantage that low concentration samples can be analyzed in a reasonable amount of time. In embodiments in which two interrogation spaces 370 and 371 are used, volumes such as those described herein for a single interrogation space 314 may be used.

[00201] In one embodiment of the present invention, the interrogation spaces are large enough to allow for detection of particles at concentrations ranging from about 1000 femtomolar (fM) to about 1 zeptomolar (zM). In one embodiment of the present invention, the interrogation spaces are large enough to allow for detection of particles at concentrations ranging from about 1000 fM to about 1 attomolar (aM). In one embodiment of the present invention, the interrogation spaces are large enough to allow for detection of particles at concentrations ranging from about 10 fM to about 1 attomolar (aM). In many cases, the large interrogation spaces allow for the detection of particles at concentrations of less than about 1 fM without additional pre-concentration devices or techniques. One skilled in the art will recognize that the most appropriate interrogation space size depends on the brightness of the particles to be detected, the level of background signal, and the concentration of the sample to be analyzed.

[00202] The size of the interrogation space 314 can be limited by adjusting the optics of the analyzer. In one embodiment, the diameter of the beam 311 can be adjusted to vary the volume of the interrogation space 314. In another embodiment, the field of view of the detector 309 can be varied. Thus, the source 301 and the detector 309 can be adjusted so that single particles will be illuminated and detected within the interrogation space 314. In another embodiment, the width of aperture 306 (Figure 1A) that determine the field of view of the detector 309 is variable. This configuration allows for altering the interrogation space, in near real time, to compensate for more or

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less concentrated samples, ensuring a low probability of two or more particles simultaneously being within an interrogation space. Similar alterations for two or more interrogation spaces, 370 and 371, may performed.

[00203] In another embodiment, the interrogation space can be defined through the use of a calibration sample of known concentration that is passed through the capillary flow cell prior to the actual sample being tested. When only one single particle is detected at a time in the calibration sample as the sample is passing through the capillary flow cell, the depth of focus together with the diameter of the beam of the electromagnetic radiation source determines the size of the interrogation space in the capillary flow cell.

[00204] Physical constraints to the interrogation spaces can also be provided by a solid wall. In one embodiment, the wall is one or more of the walls of a flow cell 313 (Figure 2A), when the sample fluid is contained within a capillary. In one embodiment, the cell is made of glass, but other substances transparent to light in the range of about 200 to about 1,000 nm or higher, such as quartz, fused silica, and organic materials such as Teflon, nylon, plastics, such as polyvinylchloride, polystyrene, and polyethylene, or any combination thereof, may be used without departing from the scope of the present invention. Although other cross-sectional shapes (e.g., rectangular, cylindrical) may be used without departing from the scope of the present invention, in one embodiment the capillary flow cell 313 has a square cross section. In another embodiment, the interrogation space may be defined at least in part by a channel (not shown) etched into a chip (not shown). Similar considerations apply to embodiments in which two interrogation spaces are used (370 and 371 in Fig. 2B).

[00205] The interrogation space is bathed in a fluid. In one embodiment, the fluid is aqueous. In other embodiments, the fluid is non-aqueous or a combination of aqueous and non-aqueous fluids. In addition the fluid may contain agents to adjust pH, ionic composition, or sieving agents, such as soluble macroparticles or polymers or gels. It is contemplated that valves or other devices may be present between the interrogation spaces to temporarily disrupt the fluid connection. Interrogation spaces temporarily disrupted are considered to be connected by fluid.

[00206] In another embodiment of the invention, an interrogation space is the single interrogation space present within the flow cell 313 which is constrained by the size of a laminar flow of the sample material within a diluent volume, also called sheath flow. In these and other embodiments, the interrogation space can be defined by sheath flow alone or in combination with the dimensions of the illumination source or the field of view of the detector. Sheath flow can be configured in numerous ways, including: The sample material is the interior material in a concentric laminar flow, with the diluent volume in the exterior; the diluent volume is on one side of the sample volume; the diluent volume is on two sides of the sample material; the diluent volume is on multiple sides of the sample material, but not enclosing the sample material completely; the diluent volume completely surrounds the sample material; the diluent volume completely surrounds the sample material concentrically; the sample material is the interior material in a discontinuous series of drops and the diluent volume completely surrounds each drop of sample material.

[00207] In some embodiments, single molecule detectors of the invention comprise no more than one interrogation space. In some embodiments, multiple interrogation spaces are used. Multiple interrogation spaces have been previously disclosed and are incorporated by reference from U.S. Pat. App. No. 11/048,660. One skilled in the art will recognize that in some cases the analyzer will contain 2, 3, 4, 5, 6 or more distinct interrogation spaces.

3. Motive Force

[00208] In one embodiment of the analyzer system, the particles are moved through the interrogation space by a motive force. In some embodiments, the motive force for moving particles is pressure. In some embodiments, the pressure is supplied by a pump, and air pressure source, a vacuum source, a centrifuge, or a combination thereof. In

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some embodiments, the motive force for moving particles is an electrokinetic force. The use of an electrokinetic force as a motive force has been previously disclosed in a prior application and is incorporated by reference from U.S. Pat. App. No. 11/048,660.

[00209] In one embodiment, pressure can be used as a motive force to move particles through the interrogation space of the capillary flow cell. In a further embodiment, pressure is supplied to move the sample by means of a pump. Suitable pumps are known in the art. In one embodiment, pumps manufactured for HPLC applications, such as those made by Scivax, Inc. can be used as a motive force. In other embodiments, pumps manufactured for microfluidics applications can be used when smaller volumes of sample are being pumped. Such pumps are described in U.S. Pat. Nos. 5,094,594, 5,730,187, 6,033,628, and 6,533,553, which discloses devices which can pump fluid volumes in the nanoliter or picoliter range. Preferably all materials within the pump that come into contact with sample are made of highly inert materials, e.g., polyetheretherketone (PEEK), fused silica, or sapphire.

[00210] A motive force is necessary to move the sample through the capillary flow cell to push the sample through the interrogation space for analysis. A motive force is also required to push a flushing sample through the capillary flow cell after the sample has been passed through. A motive force is also required to push the sample back out into a sample recovery vessel, when sample recovery is employed. Standard pumps come in a variety of sizes, and the proper size may be chosen to suit the anticipated sample size and flow requirements. In some embodiments, separate pumps are used for sample analysis and for flushing of the system. The analysis pump may have a capacity of approximately 0.000001 mL to approximately 10 mL, or approximately 0.001 mL to approximately 1 mL, or approximately 0.01 mL to approximately 0.2 mL, or approximately 0.005, 0.01, 0.05, 0.1, or 0.5 mL. Flush pumps may be of larger capacity than analysis pumps. Flush pumps may have a volume of about 0.01 mL to about 20 mL, or about 0.1 mL to about 10 mL, or about 0.1 mL to about 2 mL, or about or about 0.05, 0.1, 0.5, 1, 5, or 10 mL. These pump sizes are illustrative only, and those of skill in the art will appreciate that the pump size may be chosen according to the application, sample size, viscosity of fluid to be pumped, tubing dimensions, rate of flow, temperature, and other factors well known in the art. In some embodiments, pumps of the system are driven by stepper motors, which are easy to control very accurately with a microprocessor.

[00211] In preferred embodiments, the flush and analysis pumps are used in series, with special check valves to control the direction of flow. The plumbing is designed so that when the analysis pump draws up the maximum sample, the sample does not reach the pump itself. This is accomplished by choosing the ID and length of the tubing between the analysis pump and the analysis capillary such that the tubing volume is greater than the stroke volume of the analysis pump.

4. Detectors

[00212] In one embodiment, light (e.g., light in the ultra-violet, visible or infrared range) emitted by a fluorescent label after exposure to electromagnetic radiation is detected. The detector 309 (Figure 1A), or detectors (364, 365, Figure 1B), is capable of capturing the amplitude and duration of photon bursts from a fluorescent moiety, and further converting the amplitude and duration of the photon burst to electrical signals. Detection devices such as CCD cameras, video input module cameras, and Streak cameras can be used to produce images with contiguous signals. In another embodiment, devices such as a bolometer, a photodiode, a photodiode array, avalanche photodiodes, and photomultipliers which produce sequential signals may be used. Any combination of the aforementioned detectors may also be used. In one embodiment, avalanche photodiodes are used for detecting photons.

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[00213] Using specific optics between an interrogation space 314 (Figure 2A) and its corresponding detector 309 (Figure 1A), several distinct characteristics of the emitted electromagnetic radiation can be detected including: emission wavelength, emission intensity, burst size, burst duration, and fluorescence polarization. In some embodiments, the detector 309 is a photodiode that is used in reverse bias. A photodiode set in reverse bias usually has an extremely high resistance. This resistance is reduced when light of an appropriate frequency shines on the P/N junction. Hence, a reverse biased diode can be used as a detector by monitoring the current running through it. Circuits based on this effect are more sensitive to light than ones based on zero bias.

[00214] In one embodiment of the analyzer system, the photodiode can be an avalanche photodiode, which can be operated with much higher reverse bias than conventional photodiodes, thus allowing each photo-generated carrier to be multiplied by avalanche breakdown, resulting in internal gain within the photodiode, which increases the effective responsiveness (sensitivity) of the device. The choice of photodiode is determined by the energy or emission wavelength emitted by the fluorescently labeled particle. In some embodiments, the photodiode is a silicon photodiode that detects energy in the range of 190-1100 nm; in another embodiment the photodiode is a germanium photodiode that detects energy in the range of 800-1700 nm; in another embodiment the photodiode is an indium gallium arsenide photodiode that detects energy in the range of 800-2600 nm; and in yet other embodiments, the photodiode is a lead sulfide photodiode that detects energy in the range of between less than 1000 nm to 3500 nm. In some embodiments, the avalanche photodiode is a single-photon detector designed to detect energy in the 400 nm to 1100 nm wavelength range. Single photon detectors are commercially available (for example Perkin Elmer, Wellesley, MA).

[00215] In some embodiments the detector is a avalanche photodiode detector that detects energy between 300 nm and 1700 nm. In one embodiment, silicon avalanche photodiodes can be used to detect wavelengths between 300 nm and 1100 nm. Indium gallium arsenic photodiodes can be used to detect wavelengths between 900nm and 1700 nm. In some embodiments, an analyzer system can comprise at least one detector; in other embodiments, the analyzer system can comprise at least two detectors, and each detector can be chosen and configured to detect light energy at a specific wavelength range. For example, two separate detectors can be used to detect particles that have been tagged with different labels, which upon excitation with an EM source, will emit photons with energy in different spectra. In one embodiment, an analyzer system can comprise a first detector that can detect fluorescent energy in the range of 450-700 nm such as that emitted by a green dye (e.g. Alexa 546); and a second detector that can detect fluorescent energy in the range of 620-780 nm such as that emitted by a far-red dye (e.g. Alexa 647). Detectors for detecting fluorescent energy in the range of 400-600 nm such as that emitted by blue dyes (e.g. Hoechst 33342), and for detecting energy in the range of 560-700 nm such as that emitted by red dyes (Alexa 546 and Cy3) can also be used.

[00216] A system comprising two or more detectors can be used to detect individual particles that are each tagged with two or more labels that emit light in different spectra. For example, two different detectors can detect an antibody that has been tagged with two different dye labels. Alternatively, an analyzer system comprising two detectors can be used to detect particles of different types, each type being tagged with a different dye molecules, or with a mixture of two or more dye molecules. For example, two different detectors can be used to detect two different types of antibodies that recognize two different proteins, each type being tagged with a different dye label or with a mixture of two or more dye label molecules. By varying the proportion of the two or more dye label molecules, two or more different particle types can be individually detected using two detectors. It is understood that three or more detectors can be used without departing from the scope of the invention.

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[00217] It should be understood by one skilled in the art that one or more detectors can be configured at each interrogation space, whether one or more interrogation spaces are defined within a flow cell, and that each detector may be configured to detect any of the characteristics of the emitted electromagnetic radiation listed above. The use of multiple detectors, e.g., for multiple interrogation spaces, has been previously disclosed in a prior application and is incorporated by reference here from U.S. Pat. App. No. 11/048,660. Once a particle is labeled to render it detectable (or if the particle possesses an intrinsic characteristic rendering it detectable), any suitable detection mechanism known in the art may be used without departing from the scope of the present invention, for example a CCD camera, a video input module camera, a Streak camera, a bolometer, a photodiode, a photodiode array, avalanche photodiodes, and photomultipliers producing sequential signals, and combinations thereof. Different characteristics of the electromagnetic radiation may be detected including: emission wavelength, emission intensity, burst size, burst duration, fluorescence polarization, and any combination thereof.

C. Sampling System

[00218] In a further embodiment, the analyzer system may include a sampling system to prepare the sample for introduction into the analyzer system. The sampling system included is capable of automatically sampling a plurality of samples and providing a fluid communication between a sample container and a first interrogation space.

[00219] In some embodiments, the analyzer system of the invention includes a sampling system for introducing an aliquot of a sample into the single particle analyzer for analysis. Any mechanism that can introduce a sample may be used. Samples can be drawn up using either a vacuum suction created by a pump or by pressure applied to the sample that would push liquid into the tube, or by any other mechanism that serves to introduce the sample into the sampling tube. Generally, but not necessarily, the sampling system introduces a sample of known sample volume into the single particle analyzer; in some embodiments where the presence or absence of a particle or particles is detected, precise knowledge of the sample size is not critical. In preferred embodiments the sampling system provides automated sampling for a single sample or a plurality of samples. In embodiments where a sample of known volume is introduced into the system, the sampling system provides a sample for analysis of more than about 0.0001, 0.001, 0.01, 0.1, 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, 1000, 1500, or 2000 μl. In some embodiments the sampling system provides a sample for analysis of less than about 2000, 1000, 500, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2, 1, 0.1, 0.01, or 0.001 μl. In some embodiments the sampling system provides a sample for analysis of between about 0.01 and 1500 μl, or about 0.1 and 1000 μl, or about 1 and 500 μl, or about 1 and 100 μl, or about 1 and 50 μl, or about 1 and 20 μl. In some embodiments, the sampling system provides a sample for analysis between about 5 μl and 200 μl, or about 5 μl and about 100 μl, or about 5 μl and 50 μl. In some embodiments, the sampling system provides a sample for analysis between about 10 μl and 200 μl, or between about 10 μl and 100 ul, or between about 10 μl and 50 μl. In some embodiments, the sampling system provides a sample for analysis between about 0.5 μl and about 50 μl.

[00220] In some embodiments, the sampling system provides a sample size that can be varied from sample to sample. In these embodiments, the sample size may be any one of the sample sizes described herein, and may be changed with every sample, or with sets of samples, as desired.

[00221] Sample volume accuracy, and sample to sample volume precision of the sampling system, is required for the analysis at hand. In some embodiments, the precision of the sampling volume is determined by the pumps used, typically represented by a CV of less than about 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.1, 0.05, or 0.01% of sample volume. In some embodiments, the sample to sample precision of the sampling system is represented by a CV of

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less than about 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.1, 0.05, or 0.01%. In some embodiments, the intra-assay precision of the sampling system is represented by a CV of less than about 10, 5, 1, 0.5, or 0.1%. In some embodiments, the intra-assay precision of the sampling system shows a CV of less than about 5%. In some embodiments, the interassay precision of the sampling system is represented by a CV of less than about 10, 5, or 1%. In some embodiments, the interassay precision of the sampling system shows a CV of less than about 5%.

[00222] In some embodiments, the sampling system provides low sample carryover, advantageous in that an additional wash step is not required between samples. Thus, in some embodiments, sample carryover is less than about 1, 0.5, 0.1, 0.05, 0.04, 0.03, 0.02, 0.01, 0.005, or 0.001%. In some embodiments, sample carryover is less than about 0.02%. In some embodiments, sample carryover is less than about 0.01%.

[00223] In some embodiments the sampler provides a sample loop. In these embodiments, multiple samples are drawn into tubing sequentially and each is separated from the others by a "plug" of buffer. The samples typically are read one after the other with no flushing in between. Flushing is done once at the end of the loop. In embodiments where a buffer "plug" is used, the plug may be recovered ejecting the buffer plug into a separate well of a microtiter plate.

[00224] The sampling system may be adapted for use with standard assay equipment, for example, a 96-well microtiter plate, or, preferably, a 384-well plate. In some embodiments the system includes a 96 well plate positioner and a mechanism to dip the sample tube into and out of the wells, e.g., a mechanism providing movement along the X, Y, and Z axes. In some embodiments, the sampling system provides multiple sampling tubes from which samples may be stored and extracted from, when testing is commenced. In some embodiments, all samples from the multiple tubes are analyzed on one detector. In other embodiments, multiple single molecule detectors may be connected to the sample tubes. Samples may be prepared by steps that include operations performed on sample in the wells of the plate prior to sampling by the sampling system, or sample may be prepared within the analyzer system, or some combination of both.

D. **Sample preparation system**

[00225] Sample preparation includes the steps necessary to prepare a raw sample for analysis. These steps can involve, by way of example, one or more steps of: separation steps such as centrifugation, filtration, distillation, chromatography; concentration, cell lysis, alteration of pH, addition of buffer, addition of diluents, addition of reagents, heating or cooling, addition of label, binding of label, cross-linking with illumination, separation of unbound label, inactivation and/or removal of interfering compounds and any other steps necessary for the sample to be prepared for analysis by the single particle analyzer. In some embodiments, blood is treated to separate out plasma or serum. Additional labeling, removal of unbound label, and/or dilution steps may also be performed on the serum or plasma sample.

[00226] In some embodiments, the analyzer system includes a sample preparation system that performs some or all of the processes needed to provide a sample ready for analysis by the single particle analyzer. This system may perform any or all of the steps listed above for sample preparation. In some embodiments samples are partially processed by the sample preparation system of the analyzer system. Thus, in some embodiments, a sample may be partially processed outside the analyzer system first. For example, the sample may be centrifuged first. The sample may then be partially processed inside the analyzer by a sample preparation system. Processing inside the analyzer includes labeling the sample, mixing the sample with a buffer and other processing steps that will be known to one in the art. In some embodiments, a blood sample is processed outside the analyzer system to provide a serum or plasma sample, which is introduced into the analyzer system and further processed by a sample preparation system

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to label the particle or particles of interest and, optionally, to remove unbound label. In other embodiments preparation of the sample can include immunodepletion of the sample to remove particles that are not of interest or to remove particles that can interfere with sample analysis. In yet other embodiments, the sample can be depleted of particles that can interfere with the analysis of the sample. For example, sample preparation can include the depletion of heterophilic antibodies, which are known to interfere with immunoassays that use non-human antibodies to directly or indirectly detect a particle of interest. Similarly, other proteins that interfere with measurements of the particles of interest can be removed from the sample using antibodies that recognize the interfering proteins.

[00227] In some embodiments, the sample can be subjected to solid phase extraction prior to being assayed and analyzed. For example, a serum sample that is assayed for cAMP can first be subjected to solid phase extraction using a c18 column to which it binds. Other proteins such as proteases, lipases and phosphatases are washed from the column, and the cAMP is eluted essentially free of proteins that can degrade or interfere with measurements of cAMP. Solid phase extraction can be used to remove the basic matrix of a sample, which can diminish the sensitivity of the assay. In yet other embodiments, the particles of interest present in a sample may be concentrated by drying or lyophilizing a sample and solubilizing the particles in a smaller volume than that of the original sample.

[00228] In some embodiments the analyzer system provides a sample preparation system that provides complete preparation of the sample to be analyzed on the system, such as complete preparation of a blood sample, a saliva sample, a urine sample, a cerebrospinal fluid sample, a lymph sample, a BAL sample, a biopsy sample, a forensic sample, a bioterrorism sample, and the like. In some embodiments the analyzer system provides a sample preparation system that provides some or all of the sample preparation. In some embodiments, the initial sample is a blood sample that is further processed by the analyzer system. In some embodiments, the sample is a serum or plasma sample that is further processed by the analyzer system. The serum or plasma sample may be further processed by, e.g., contacting with a label that binds to a particle or particles of interest; the sample may then be used with or without removal of unbound label.

[00229] In some embodiments, sample preparation is performed, either outside the analysis system or in the sample preparation component of the analysis system, on one or more microtiter plates, such as a 96-well plate. Reservoirs of reagents, buffers, and the like can be in intermittent fluid communication with the wells of the plate by means of tubing or other appropriate structures, as are well-known in the art. Samples may be prepared separately in 96 well plates or tubes. Sample isolation, label binding and, if necessary, label separation steps may be done on one plate. In some embodiments, prepared particles are then released from the plate and samples are moved into tubes for sampling into the sample analysis system. In some embodiments, all steps of the preparation of the sample are done on one plate and the analysis system acquires sample directly from the plate. Although this embodiment is described in terms of a 96-well plate, it will be appreciated that any vessel for containing one or more samples and suitable for preparation of sample may be used. For example, standard microtiter plates of 384 or 1536 wells may be used. More generally, in some embodiments, the sample preparation system is capable of holding and preparing more than about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 500, 1000, 5000, or 10,000 samples. In some embodiments, multiple samples may be sampled for analysis in multiple analyzer systems. Thus, in some embodiments, 2 samples, or more than about 2, 3, 4, 5, 7, 10, 15 20, 50, or 100 samples are sampled from the sample preparation system and run in parallel on multiple sample analyzer systems.

[00230] Microfluidics systems may also be used for sample preparation and as sample preparation systems that are part of analyzer systems, especially for samples suspected of containing concentrations of particles high enough that detection requires smaller samples. Principles and techniques of microfluidic manipulation are known in the art.

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See, e.g., U.S. Patent Nos. 4,979,824; 5,770,029; 5,755,942; 5,746,901; 5,681,751; 5,658,413; 5,653,939; 5,653,859; 5,645,702; 5,605,662; 5,571,410; 5,543,838; 5,480,614, 5,716,825; 5,603,351; 5,858,195; 5,863,801; 5,955,028; 5,989,402; 6,041,515; 6,071,478; 6355,420; 6,495,104; 6,386,219; 6,606,609; 6,802,342;6, 749,734; 6,623,613; 6,554,744; 6,361,671; 6,143,152; 6,132,580; 5,274,240; 6,689,323; 6,783,992; 6,537,437; 6,599,436; 6,811,668 and published PCT patent application no. WO9955461(A1). Samples may be prepared in series or in parallel, for use in a single or multiple analyzer systems.

[00231] Preferably, the sample comprises a buffer. The buffer may be mixed with the sample outside the analyzer system, or it may be provided by the sample preparation mechanism. While any suitable buffer can be used, the preferable buffer has low fluorescence background, is inert to the detectably labeled particle, can maintain the working pH and, in embodiments wherein the motive force is electrokinetic, has suitable ionic strength for electrophoresis. The buffer concentration can be any suitable concentration, such as in the range from about 1 to about 200 mM. Any buffer system may be used as long as it provides for solubility, function, and delectability of the molecules of interest. Preferably, for application using pumping, the buffer is selected from the group consisting of phosphate, glycine, acetate, citrate, acidulate, carbonate/bicarbonate, imidazole, triethanolamine, glycine amide, borate, MES, Bis-Tris, ADA, aces, PIPES, MOPSO, Bis-Tris Propane, BES, MOPS, TES, HEPES, DIPSO, MOBS, TAPSO, Trizma, HEPPSO, POPSO, TEA, EPPS, Tricine, Gly-Gly, Bicine, HEPBS, TAPS, AMPD, TABS, AMPSO, CHES, CAPSO, AMP, CAPS, and CABS. The buffer can also be selected from the group consisting of Gly-Gly, bicine, tricine, 2-morpholine ethanesulfonic acid (MES), 4-morpholine propanesulfonic acid (MOPS) and 2-amino-2-methyl-1-propanol hydrochloride (AMP). A useful buffer is 2 mM Tris/borate at pH 8.1, but Tris/glycine and Tris/HCl are also acceptable. Other buffers are as described herein.

[00232] Buffers useful for electrophoresis are disclosed in a prior application and are incorporated by reference herein from U.S. Pat. App. No. 11/048,660.

E. Sample recovery

[00233] One highly useful feature of embodiments of the analyzers and analysis systems of the invention is that the sample can be analyzed without consuming it. This can be especially important when sample materials are limited. Recovering the sample also allows one to do other analyses or reanalyze it. The advantages of this feature for applications where sample size is limited and/or where the ability to reanalyze the sample is desirable, e.g., forensic, drug screening, and clinical diagnostic applications, will be apparent to those of skill in the art.

[00234] Thus, in some embodiments, the analyzer system of the invention further provides a sample recovery system for sample recovery after analysis. In these embodiments, the system includes mechanisms and methods by which the sample is drawn into the analyzer, analyzed and then returned, e.g., by the same path, to the sample holder, e.g., the sample tube. Because no sample is destroyed and because it does not enter any of the valves or other tubing, it remains uncontaminated. In addition, because all the materials in the sample path are highly inert, e.g., PEEK, fused silica, or sapphire, there is little contamination from the sample path. The use of the stepper motor controlled pumps (particularly the analysis pump) allows precise control of the volumes drawn up and pushed back out. This allows complete or nearly complete recovery of the sample with little if any dilution by the flush buffer. Thus, in some embodiments, more than about 50%, 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 99.9% of the sample is recovered after analysis. In some embodiments, the recovered sample is undiluted. In some embodiments, the recovered sample is diluted less than about 1.5-fold, 1.4-fold, 1.3-fold, 1.2-fold, 1.1-fold, 1.05-fold, 1.01-fold, 1.005-fold, or 1.001-fold.

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[00235] For sampling and/or sample recovery, any mechanism for transporting a liquid sample from a sample vessel to the analyzer may be used. In some embodiments the inlet end of the analysis capillary has attached a short length of tubing, e.g., PEEK tubing that can be dipped into a sample container, e.g. a test tube or sample well, or can be held above a waste container. When flushing, to clean the previous sample from the apparatus, this tube is positioned above the waste container to catch the flush waste. When drawing a sample in, the tube is put into the sample well or test tube. Typically the sample is drawn in quickly, and then pushed out slowly while observing particles within the sample. Alternatively, in some embodiments, the sample is drawn in slowly during at least part of the draw-in cycle; the sample may be analyzed while being slowly drawn in. This can be followed by a quick return of the sample and a quick flush. In some embodiments, the sample may be analyzed both on the inward (draw-in) and outward (pull out) cycle, which improves counting statistics, e.g., of small and dilute samples, as well as confirming results, and the like. If it is desired to save the sample, it can be pushed back out into the same sample well it came from, or to another. If saving the sample is not desired, the tubing is positioned over the waste container.

VI. Methods Using Highly Sensitive Analysis of Molecules

[00236] The systems, system kits, and methods of the present invention make possible measurement of molecules in samples at concentrations far lower than previously measured. The high sensitivity of the instruments, kits, and methods of the invention allows the establishment of markers, e.g., biological markers, that have not previously been possible because of a lack of sensitivity of detection. The invention also includes the use of the compositions and methods described herein for the discovery of new markers.

[00237] There are numerous markers currently available which, while potentially of use in determining a biological state, are not currently of practical use because their lower ranges are unknown. In some cases, abnormally high levels of the marker are detectable by current methodologies, but normal ranges have not been established. In some cases, upper normal ranges of the marker are detectable, but not lower normal ranges, or levels below normal. In some cases, for example, markers specific to tumors, or markers of infection, any level of the marker indicates the potential presence of the biological state, and enhancing sensitivity of detection is an advantage for early diagnosis. In some cases, the rate of change, or lack of change, in the concentration of the marker over multiple timepoints provides the most useful information, but present methods of analysis do not permit determination of levels of the marker at timepoint sampling in the early stages of a condition, when it is typically at its most treatable. In many cases, the marker may be detected at clinically useful levels only through the use of cumbersome methods that are not practical or useful in a clinical setting, such as methods that require complex sample treatment and time-consuming analysis.

[00238] In addition, there are potential markers of biological states that exist in sufficiently low concentrations that their presence remains extremely difficult or impossible to detect by current methods.

[00239] The analytical methods and compositions of the present invention provide levels of sensitivity and precision that allow the detection of markers for biological states at concentrations at which the markers have been previously undetectable, thus allowing the "repurposing" of such markers from confirmatory markers, or markers useful only in limited research settings, to diagnostic, prognostic, treatment-directing, or other types of markers useful in clinical settings and/or in large-scale clinical settings such as clinical trials. Such methods allow, e.g., the determination of normal and abnormal ranges for such markers.

[00240] The markers thus repurposed can be used for, e.g., detection of normal state (normal ranges), detection of responder/non-responder (e.g., to a treatment, such as administration of a drug); early disease or pathological

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occurrence detection (e.g., detection of cancer in its earliest stages, early detection of cardiac ischemia); disease staging (e.g., cancer); disease monitoring (e.g., diabetes monitoring, monitoring for recurrence of cancer after treatment); study of disease mechanism; and study of treatment toxicity, such as toxicity of drug treatments (e.g., cardiotoxicity).

A. Methods

[00241] The invention thus provides methods and compositions for the sensitive detection of markers, and further methods of establishing values for normal and abnormal levels of the markers. In further embodiments, the invention provides methods of diagnosis, prognosis, and/or treatment selection based on values established for the markers. The invention also provides compositions for use in such methods, e.g., detection reagents for the ultrasensitive detection of markers.

[00242] In some embodiments, the invention provides a method of establishing a marker for a biological state, by establishing a range of concentrations for the marker in biological samples obtained from a first population by measuring the concentrations of the marker the biological samples by detecting single molecules of the marker (e.g., by detecting a label that has been attached to a single molecule of the marker). In some embodiments, the marker is a polypeptide or small molecule. The samples may be any sample type described herein, e.g., blood, plasma, or serum; or urine.

[00243] The method may utilize samples from a first population where the population is a population that does not exhibit the biological state. In the case where the biological state is a disease state, the first population may be a population that does not exhibit the disease, e.g., a "normal" population. In some embodiments the method may further comprise establishing a range of range of levels for the marker in biological samples obtained from a second population, where the members of the second population exhibit the biological state, by measuring the concentrations of the marker the biological samples by detecting single molecules of the marker. In some embodiments, e.g., cross-sectional studies, the first and second populations are different. In some embodiments, at least one member of the second population is a member of the first population, or at least one member of said the population is a member of the second population. In some embodiments, e.g., longitudinal studies, substantially all the members of the second population are members of the first population who have developed the biological state (e.g., a disease or pathological state).

[00244] The detecting of single molecules of the marker is performed using a method as described herein, e.g., a method with a limit of detection for said marker of less than about 1000, 100, 50, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, or 0.001 femtomolar of the marker in the samples, by detecting single molecules of the marker.

[00245] The biological state may be a phenotypic state; a condition affecting the organism; a state of development; age; health; pathology; disease; disease process; disease staging; infection; toxicity; or response to chemical, environmental, or drug factors (such as drug response phenotyping, drug toxicity phenotyping, or drug effectiveness phenotyping).

[00246] In some embodiments, the biological state is a pathological state, including but not limited to inflammation, abnormal cell growth, and abnormal metabolic state. In some embodiments, the state is a disease state. Disease states include, but are not limited to, cancer, cardiovascular disease, inflammatory disease, autoimmune disease, neurological disease, infectious disease and pregnancy related disorders. In some embodiments the state is a disease stage state, e.g., a cancer disease stage state.

[00247] The methods may also be used for determination of a treatment response state. In some embodiments, the treatment is a drug treatment. The response may be a therapeutic effect or a side effect, e.g., an

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adverse effect. Markers for therapeutic effects will be based on the disease or condition treated by the drug. Markers for adverse effects typically will be based on the drug class and specific structure and mechanism of action and metabolism. A common adverse effect is drug toxicity. An example is cardiotoxicity, which can be monitored by the marker cardiac troponin. In some embodiments one or more markers for the disease state and one or more markers for one or more adverse effects of a drug are monitored, typically in a population that is receiving the drug. Samples may be taken at intervals and the respective values of the markers in the samples may be evaluated over time.

[00248] The detecting of single molecules of the marker may comprise labeling the marker with a label comprising a fluorescent moiety capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, where the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules. In some embodiments, the fluorescent moiety comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the fluorescent moiety may comprise a dye selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700. In some embodiments, the moiety comprises AlexaFluor 647. In some embodiments, the label further comprises a binding partner for the marker, e.g., an antibody specific for said marker, such as a polyclonal antibody or a monoclonal antibody. Binding partners for a variety of markers are described herein.

[00249] The method may further include establishing a threshold level for the marker based on the first range, or the first and second ranges, where the presence of marker in a biological sample from an individual at a level above or below the threshold level indicates an increased probability of the presence of the biological state in said individual. An example of a threshold determined for a normal population is the suggested threshold for cardiac troponin of greater than the 99th percentile value in a normal population. See Example 3. Other threshold levels may be determined empirically, i.e., based on data from the first and second populations regarding marker levels and the presence, absence, severity, rate of progression, rate of regression, and the like, of the biological state being monitored. It will be appreciated that threshold levels may be established at either end of a range, e.g., a minimum below which the concentration of the marker in a sample indicates an increased probablity of a biological state, and/or a maximum above which the concentration of the marker in a sample indicates an increased probablity of a biological state. In some embodiments, a risk stratification may be produced in which two or more ranges of marker concentrations correspond to two or more levels of risk. Other methods of analyzing data from two populations and for markers and producing clinically-relevant values for use by, e.g., physicians and other health care professionals, are well-known in the art.

[00250] For some biological markers, the presence of any marker at all is an indication of a disease or pathological state, and the threshold is essentially zero. An example is the use of prostate specific antigen (PSA) to monitor cancer recurrence after removal of the prostate gland. As PSA is produced only by the prostate gland, and as the prostate gland and all tumor are presumed to be removed, PSA after removal is zero. Appearance of PSA at any level signals a possible recurrence of the cancer, e.g., at a metastatic site. Thus, the more sensitive the method of detection, the earlier an intervention may be made should such recurrence occur.

[00251] Other evaluations of marker concentration may also be made, such as in a series of samples, where change in value, rate of change, spikes, decrease, and the like may all provide useful information for determination of a biological state. In addition, panels of markers may be used if it is found that more than one marker provides information regarding a biological state. If panels of markers are used, the markers may be measured separately in

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separate samples (e.g., aliquots of a common sample) or simultaneously by multiplexing. Examples of panels of markers and multiplexing are given in, e.g., U.S. Patent Application No. 11/048,660.

[00252] The establishment of such markers and, e.g., reference ranges for normal and/or abnormal states, allow for sensitive and precise determination of the biological state of an organism. Thus, in some embodiments, the invention provides a method for detecting the presence or absence of a biological state of an organism, comprising i) measuring the concentration of a marker in a biological sample from the organism, wherein said marker is a marker established through establishing a range of concentrations for said marker in biological samples obtained from a first population by measuring the concentrations of the marker the biological samples by detecting single molecules of the marker; and ii) determining the presence of absence of said biological state based on said concentration of said marker in said organism.

[00253] In some embodiments, the invention provides a method for detecting the presence or absence of a biological state in an organism, comprising i) measuring the concentrations of a marker in a plurality of biological samples from said organism, wherein said marker is a marker established through establishing a range of concentrations for said marker in biological samples obtained from a first population by measuring the concentrations of the marker the biological samples by detecting single molecules of the marker; and ii) determining the presence of absence of said biological state based on said concentrations of said marker in said plurality of samples. In some embodiments, the samples are of different types, e.g., are samples from different tissue types. In this case, the determining is based on a comparison of the concentrations of said marker in said different types of samples. More commonly, the samples are of the same type, and the samples are taken at intervals. The samples may be any sample type described herein, e.g., blood, plasma, or serum; or urine. Intervals between samples may be minutes, hours, days, weeks, months, or years. In an acute clinical setting, the intervals may be minutes or hours. In settings involving the monitoring of an individual, the intervals may be days, weeks, months, or years.

[00254] In many cases, the biological state whose presence or absence is to be detected is a disease phenotype. Thus, in one embodiment, a phenotypic state of interest is a clinically diagnosed disease state. Such disease states include, for example, cancer, cardiovascular disease, inflammatory disease, autoimmune disease, neurological disease, respiratory disease, infectious disease and pregnancy related disorders.

[00255] Cancer phenotypes are included in some aspects of the invention. Examples of cancer herein include, but are not limited to: breast cancer, skin cancer, bone cancer, prostate cancer, liver cancer, lung cancer, brain cancer, cancer of the larynx, gallbladder, pancreas, rectum, parathyroid, thyroid, adrenal, neural tissue, head and neck, colon, stomach, bronchi, kidneys, basal cell carcinoma, squamous cell carcinoma of both ulcerating and papillary type, metastatic skin carcinoma, osteo sarcoma, Ewing's sarcoma, veticulum cell sarcoma, myeloma, giant cell tumor, small-cell lung tumor, non-small cell lung carcinoma gallstones, islet cell tumor, primary brain tumor, acute and chronic lymphocytic and granulocytic tumors, hairy-cell tumor, adenoma, hyperplasia, medullary carcinoma, pheochromocytoma, mucosal neuronms, intestinal ganglloneuromas, hyperplastic corneal nerve tumor, marfanoid habitus tumor, Wilm's tumor, seminoma, ovarian tumor, leiomyomater tumor, cervical dysplasia and in situ carcinoma, neuroblastoma, retinoblastoma, soft tissue sarcoma, malignant carcinoid, topical skin lesion, mycosis fungoide, rhabdomyosarcoma, Kaposi's sarcoma, osteogenic and other sarcoma, malignant hypercalcemia, renal cell tumor, polycythermia vera, adenocarcinoma, glioblastoma multiforma, leukemias, lymphomas, malignant melanomas, epidermoid carcinomas, and other carcinomas and sarcomas.

[00256] Cardiovascular disease may be included in other applications of the invention. Examples of cardiovascular disease include, but are not limited to, congestive heart failure, high blood pressure, arrhythmias, atherosclerosis, cholesterol, Wolff-Parkinson-White Syndrome, long QT syndrome, angina pectoris, tachycardia, bradycardia, atrial

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fibrillation, ventricular fibrillation, congestive heart failure, myocardial ischemia, myocardial infarction, cardiac tamponade, myocarditis, pericarditis, arrhythmogenic right ventricular dysplasia, hypertrophic cardiomyopathy, Williams syndrome, heart valve diseases, endocarditis, bacterial, pulmonary atresia, aortic valve stenosis, Raynaud's disease, Raynaud's disease, cholesterol embolism, Wallenberg syndrome, Hippel-Lindau disease, and telangiectasis.

[00257] Inflammatory disease and autoimmune disease may be included in other embodiments of the invention. Examples of inflammatory disease and autoimmune disease include, but are not limited to, rheumatoid arthritis, non-specific arthritis, inflammatory disease of the larynx, inflammatory bowel disorder, psoriasis, hypothyroidism (e.g., Hashimoto thyroidism), colitis, Type 1 diabetes, pelvic inflammatory disease, inflammatory disease of the central nervous system, temporal arteritis, polymyalgia rheumatica, ankylosing spondylitis, polyarteritis nodosa, Reiter's syndrome, scleroderma, systemic lupus and erythematosus.

[00258] The methods and compositions of the invention can also provide laboratory information about markers of infectious disease including markers of Adenovirus, Bordella pertussis, Chlamydia pneumoiea, Chlamydia trachomais, Cholera Toxin, Cholera Toxin β, Campylobacter jejuni, Cytomegalovirus, Diptheria Toxin, Epstein-Barr NA, Epstein-Barr EA, Epstein-Barr VCA, Helicobacter Pylori, Hepatitis B virus (HBV) Core, Hepatitis B virus (HBV) Envelope, Hepatitis B virus (HBV) Survace (Ay), Hepatitis C virus (HCV) Core, Hepatitis C virus (HCV) NS3, Hepatitis C virus (HCV) NS4, Hepatitis C virus (HCV) NS5, Hepititis A, Hepititis D, Hepatitis E virus (HEV) orf2 3KD, Hepatitis E virus (HEV) orf2 6KD, Hepatitis E virus (HEV) orf3 3KD, Human immunodeficiency virus (HIV)-1 p24, Human immunodeficiency virus (HIV)-1 gp41, Human immunodeficiency virus (HIV)-1 gp120, Human papilloma virus (HPV), Herpes simplex virus HSV-1/2, Herpes simplex virus HSV-1 gD, Herpes simplex virus HSV-2 gG, Human T-cell leukemia virus (HTLV)-1/2, Influenza A, Influenza A H3N2, Influenza B, Leishmanina donovani, Lyme disease, Mumps, M. pneumoniae, M. teberculosis, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Polio Virus, Respiratory syncytial virus (RSV), Rubella, Rubeola, Streptolysin O, Tetanus Toxin, T. pallidum 15kd, T. pallidum p47, T. cruzi, Toxoplasma, and Varicella Zoater.

B. Exemplary markers

[00259] The instruments, labels, and methods of the invention have been used to establish ranges for markers in, e.g., serum and urine, at levels 10- to 100-fold lower than previous levels, or lower. The markers are indicative of a wide variety of biological states, e.g., cardiac disease and cardiotoxicity (troponin), infection (TREM-1), inflammation and other conditions (LTE4, IL-6 and IL-8), asthma (LTE4), cancer (Akt1, TGF-beta, Fas ligand), and allograft rejection and degenerative disease (Fas ligand).

[00260] Markers include protein and non-protein markers. The markers are described briefly here and procedures and results given in the Examples.

1. Cardiac damage

[00261] Cardiac troponin is an example of a marker that is currently detectable only in abnormally high amounts. Cardiac troponin is a marker of cardiac damage, useful in diagnosis, prognosis, and determination of method of treatment in a number of diseases and conditions, e.g., acute myocardial infarct. In addition, cardiac troponin is a useful marker of cardiotoxicity due to treatment, e.g., drug treatment.

[00262] The troponin complex in muscle consists of troponin I, C and T. Troponin C exists as two isoforms, one from cardiac and slow-twitch muscle and one from fast-twitch muscle; because it is found in virtually all striated muscle, its use as a specific marker is limited. In contrast, troponin I and T are expressed as different isoforms in slow-twitch, fast-twitch and cardiac muscle The unique cardiac isoforms of troponin I and T allow them to be

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distinguished immunologically from the other troponins of skeletal muscle. Therefore, the release into the blood of cardiac troponin I and T is indicative of damage to cardiac muscle, and provides the basis for their use as diagnostic or prognostic markers, or to aid in determination of treatment.

[00263] Currently used markers for cardiac damage suffer disadvantages that limit their clinical usefulness. Cardiac enzyme assays have formed the basis for determining whether or not there is damage to the cardiac muscle. Unfortunately, the standard creatine kinase-MB (CK-MB) assay is not reliable in excluding infarction until 10 to 12 hours after the onset of chest pain. Earlier diagnosis would have very specific advantages with regard to fibrinolytic therapy and triage.

[00264] Because the level of troponin found in the circulation of healthy individuals is very low, and cardiac specific troponins do not arise from extra-cardiac sources, the troponins are very sensitive and specific markers of cardiac injury. In addition to cardiac infarct, a number of other conditions can cause damage to the heart muscle, and early detection of such damage would prove useful to clinicians. However, present methods of detection and quantitation of cardiac troponin do not possess sufficient sensitivity to detect the release of cardiac troponin into the blood until levels have reached abnormally high concentrations, e.g., 0.1 ng/ml or greater.

[00265] The methods and compositions of the invention thus include methods and compositions for the highly sensitive detection and quantitation of cardiac troponin, and compositions and methods for diagnosis, prognosis, and/or determination of treatment based on such highly sensitive detection and quantitation. A standard curve for cardiac troponin I was established with a limit of detection less than about 1 pg/l (Example 1). Levels of cardiac troponin I were established in normal individuals, and a threshold value at the 99th percentile of normal established (Example 3). Serial samples from individuals who suffered acute myocardial infarct were analyzed, and time courses for cardiac troponin I concentrations, including deviations from baseline, were determined (Example 4). Thus, cardiac troponin I serves as an example of a marker that can be detected by the systems and methods of the invention at levels to provide diagnostic and prognostic information of use in clinical and research settings. See also U.S. Patent Application No. _____, entitled "Highly Sensitive System and Methods for Analysis of Troponin," filed on even date herewith, which is incorporated by reference herein in its entirety.

2. Infection

[00266] TREM-1 is a marker of bacterial or fungal infections. Assays of the invention suggest that TREM-1 may routinely be measured at a concentration of 100 fM

3. Cytokines

[00267] The normal level of many cytokines, chemokines and growth factors is not known primarily because of the inability of existing technology to detect levels that are below those found in samples from diseased patients. For example, the basal level of other cytokines such as IL-10, TNF-alpha, IL-4, IL-1beta, IL-2, IL-12 and IFN-gamma cannot be detected by routine assays that are performed in a clinical setting, whereas the analyzer systems of the invention can readily determine the level of these and other cytokines. Knowing the level of cytokines and growth factors aids clinicians with the diagnosis, prognosis and treatment of a variety of diseases including cancer, and respiratory, infectious, and cardiovascular diseases. Early cytokine detection to monitor normal and disease states in clinical specimens can be achieved using the analyzer systems of the invention to analyze samples such as plasma, serum, and urine as well as other fluid samples to provide for better translational medicine . For example, determining levels of cytokines for which a normal range of concentration is not known, would aid clinicians with diagnosis and treatment of the following conditions and diseases. Bone Morphogenetic Proteins would be useful to

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monitor the treatment for fractures, spinal fusions, orthopedic surgery, and oral surgery; Interleukin-10 (IL-10) would be useful for detecting and monitoring for the presence of cancers including non-Hodgkin's lymphoma, multiple myeloma, melanoma, and ovarian cancer, as well as for detecting and monitoring the effect of anti-inflammatory therapy, organ transplantation, immunodeficiencies, and parasitic infections; Interleukin-11 (IL-11) is useful for the detection and monitoring for the presence of cancers such as breast cancer; Interleukin-12 (IL-12) for cancer and HIV infections; TNFα, an inflammatory cytokine, alone or in combination with IL-6, can be used as a good predictor of sepsis, acute pancreatitis, tuberculosis, and autoimmune disease such as rheumatoid arthritis and lupus.

[00268] Alternatively, databases may already exist for normal and abnormal values but present methods may not be practical for screening individuals on a routine basis to determine with sufficient sensitivity whether the value of the individual for the marker is within the normal range. For example, most present methods for the determination of IL-6 concentration in a sample are capable of detecting IL-6 only down to a concentration of about 5 pg/ml; the normal range of IL-6 values is about 1 to about 10 pg/ml; hence, present methods are able to detect IL-6 only in the upper part of normal ranges. In contrast, the analyzers and analyzer systems of the invention allow the detection of IL-6 down to a concentration below about 0.1 pg/ml, or less than one-tenth of normal range values. The lower limit of quantitation (LLOQ) is about 0.01pg/ml. Thus, the analyzers and analyzer systems of the invention allow a far broader and more nuanced database to be produced for a biomarker, e.g., for IL-6, and also allow screening for that biomarker both within and outside of the normal range, allowing earlier detection.

[00269] Thus, the analyzers and analyzer systems of the invention allow a far broader and more nuanced database to be produced for a biomarker, e.g., for IL-6, and also allow screening for that biomarker both within and outside of the normal range, allowing earlier detection of conditions in which IL-6 is implicated. IL-6-related disorders include but are not limited to sepsis, peripheral arterial disease, and chronic obstructive pulmonary disease. Interleukin-6 mediated inflammation is also the common causative factor and therapeutic target for atherosclerotic vascular disease and age-related disorders including osteoporosis and type 2 diabetes. In addition, IL-6 can be measured in combination with other cytokines, for example TNFα to diagnose additional diseases such as septic shock.

4. Inflammatory markers

[00270] Other cytokines that can be useful in detecting early onset of inflammatory disease include markers and panels of markers of inflammation as described herein. Examples of cytokines that can be used to detect an inflammatory disorders are Leukotriene 4 (LTE4), which can be an early marker of asthma and TGFβ, which can be used to detect and monitor the status of inflammatory disorders including fibrosis, sclerosis. Some markers can be used to detect more than one disorder, for example TGFβ can also be used to detect the presence of cancer.

[00271] Leukotriene E4 Cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) play an important role in the pathogenesis of asthma. Leukotrienes are produced by mast cells, eosinophils, and other airway inflammatory cells and increase vascular permeability, constrict bronchial smooth muscle, and mediate bronchial hyperresponsiveness. Levels of urinary LTE_4, the stable metabolite of LTC_4 and LTD_4, are increased in children and adults with asthma compared with healthy controls and in asthmatics after bronchial challenge with antigen, after oral challenge with aspirin in aspirin sensitive asthmatic subjects, and during exercise induced bronchospasm. The importance of leukotrienes in the pathology of asthma has been further demonstrated in large clinical trials with agents that block the actions of leukotrienes. For example, montelukast, a potent leukotriene receptor antagonist taken orally once daily,

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significantly improves asthma control in both children (aged 2–14 years) and adults and attenuates exercise induced bronchoconstriction.

[00272] Activation of the leukotriene pathways is accompanied by rises in urinary levels of LTE4, and acute exacerbations of asthma are accompanied by increased levels of LTE4 in urine followed by a significant decrease during resolution. The degree of airflow limitation correlates with levels of urinary LTE4 during the exacerbation and follow up periods, thus indicating that the leukotriene pathway is activated during acute asthma. In addition, inhalation of bronchoconstricting doses of LTC4 or LTE4 alter urinary LTE4 excretion in a dose-dependent manner thus indicating that urinary LTE4 can be used as a marker of sulphidopeptide leukotriene synthesis in the lungs of patients with asthma.

[00273] The methods of the invention can be used to detect changes in LTE4 in biological samples such as urinary samples (Examples). Measurements of subnanogram levels of LTE4 can be useful as a references for detecting and monitoring sulphidopeptide leukotriene synthesis in the lungs of patients with chronic or acute asthma.

6. **TGFβ**

[00274] The methods of the invention can also be performed to detect the early onset of diseases for which TGFβ is a marker. Examples of TGFβ-related diseases include fibrotic diseases. Fibrosis refers to the excessive and persistent formation of scar tissue, which is responsible for morbidity and mortality associated with organ failure in a variety of chronic diseases affecting the lungs, kidneys, eyes, heart, liver, and skin. TGFβ is well known for its role as a mediator of chronic fibrotic effects. For example, TGFβ is implicated in promoting fibroblastic proliferation and matrix accumulation in fibrotic lung disease. Inhibition of TGFβ has been proposed as a potential therapeutic avenue for the management of lung fibrosis. TGFβ not only stimulates the synthesis of many extracellular matrix molecules, including fibronectin and type I collagen and their receptors, but also decreases matrix degradation via differential effects on the expression of proteases and their inhibitors, strongly promoting generation of extracellular matrix. Thus the analyzer systems of the invention can detect abnormal levels of TGFβ, e.g., associated with fibrotic diseases, including but not limited to idiopathic pulmonary fibrosis, diabetic nephropathy, progressive nephropathies including glomerulosclerosis and IgA nephropathy (causes of kidney failure and the need for dialysis and retransplant); diabetic retinopathy and advanced macular degeneration (fibrotic diseases of the eye and leading causes of blindness); cirrhosis and biliary atresia (leading causes of liver fibrosis and failure); and congestive heart failure, myocardiopathy associated with progressive fibrosis in Chagas disease; lung fibrosis; and scleroderma.

[00275] TGFβ is also a marker for cancers including prostate cancer, cervical cancer, lung carcinoma, and Hodgkin's disease. Plasma levels of TGFβ in patients with lung cancer are often elevated. It has been shown that in patients with an elevated plasma TGF beta 1 level at diagnosis, monitoring this level may be useful in detecting both disease persistence and recurrence after radiotherapy.

[00276] Transforming growth factor-beta (TGF-beta) is a multipotent growth factor affecting development, homeostasis, and tissue repair. In addition, increased expression of TGF-beta has been reported in different malignancies, suggesting a role for this growth factor in tumorigenesis. In particular, it has been demonstrated that the presence of TGF-beta in the endothelial and perivascular layers of small vessels in the tumor stroma of osteosarcomas suggests an angiogenic activity of this growth factor, and that increased expression of TGF-beta isoforms have been suggested to play a role in the progression of osteosarcoma (Kloen et al., Cancer, 80:2230-9 (1997)). TGFβ is one of the few known proteins that can inhibit cell growth, however, although the notion is controversial, some researchers believe that some human malignancies such as breast cancer subvert TGFβ for their

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own purposes. In a paradox that is not understood, these cancers make TGFβ and steadily increase its expression until it becomes a marker of advancing metastasis and decreased survival. For example, levels of plasma TGFß are markedly elevated in men with prostate cancer metastatic to regional lymph nodes and bone. In men without clinical or pathologic evidence of metastases, the preoperative plasma TGF-ß level is a strong predictor of biochemical progression after surgery, presumably because of an association with occult metastatic disease present at the time of radical prostatectomy.

[00277] Other markers of abnormal cell growth that are detected by the methods of the invention include Akt1, Fas ligand and IL-6.

[00278] Diagnosis of cancers often depends on the use of crude measurements of tumor growth, such as visualization of the tumor itself, that are either inaccurate or that must reach high levels before they become detectable, e.g., in a practical clinical setting by present methods. At the point of detection, the tumor has often grown to sufficient size that intervention is unlikely to occur before metastasis. For example, detection of lung cancer by X-ray requires a tumor of > 1cm in diameter, and by CT scan of > 2-3 mm. Alternatively, a biomarker of tumor growth may be used, but, again, often the tumor is well-advanced by the time the biomarker is detectable at levels accessible to current clinical technology. Furthermore, after intervention (e.g., surgery, chemotherapy, or radiation to shrink or remove the tumor or tumors), it is often not possible to measure the tumor marker with sufficient sensitivity to determine if there has been a recurrence of the cancer until residual disease has progressed to the point where further intervention is unlikely to be successful. Using the analyzers, systems, and methods of the present invention, it is possible to both detect onset of tumor growth and return of tumor growth at a point where intervention is more likely to be successful, e.g., due to lower probability of metastasis. Markers for cancer that can be detected at levels not previously shown include markers disclosed above. Examples of assays for the detection of markers that can be repurposed to diagnostic markers include TGFβ discussed above, Akt1, Fas ligand and IL-6, which are given in Examples.

7. **Akt1**

[00279] Akt1 is v-akt murine thymoma viral oncogene homolog 1 and is a serine-threonine protein kinase encoded by the AKT1gene. Akt kinases have been implicated in disparate cell responses, including inhibition of apoptosis and promotion of cell proliferation, angiogenesis, and tumor cell invasiveness.

[00280] Best known for its ability to inhibit apoptotic and non-apoptotic cell death, Akt can be monitored to predict tumor response to anticancer treatment. Predicting tumor response by assessing the influence of apoptosis and nonapoptotic cell death, would allow for developing a more efficient strategy for enhancing the therapeutic effect of anticancer treatment. Anticancer treatment-induced apoptosis is regulated by the balance of proapoptotic and antiapoptotic proteins through mitochondria, and resistance to apoptosis is mediated by Akt-dependent and Bcl-2-dependent pathways. Bcl-2 partially inhibits nonapoptotic cell death as well as apoptosis, whereas Akt inhibits both apoptotic and nonapoptotic cell death through several target proteins. Since drug sensitivity is likely correlated with the accumulation of apoptotic and nonapoptotic cell deaths, which may influence overall tumor response in anticancer treatment. the ability to predict overall tumor response from the modulation of several important cell death-related proteins may result in a more efficient strategy for improving the therapeutic effect.

[00281] Akt1 is also involved in Epithelial-mesenchymal transition (EMT), which is an important process during development and oncogenesis by which epithelial cells acquire fibroblast-like properties and show reduced intercellular adhesion and increased motility. AKT is activated in many human carcinomas, and the AKT-driven

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EMT may confer the motility required for tissue invasion and metastasis. Thus future therapies based on AKT inhibition may complement conventional treatments by controlling tumor cell invasion and metastasis. Akt is constitutively activated in most melanoma cell lines and tumor samples of different progression stages, and activation of AKT has been linked to the expression of invasion/metastasis-related melanoma cell adhesion molecule (MelCAM), which in turn is strongly associated with the acquisition of malignancy by human melanoma. Akt1 is also activated in pancreatic cancer, and AKT activation has been shown to correlate with higher histologic tumor grade. Thus, AKT activation is associated with tumor grade, an important prognostic factor. Akt1 is also upregulated in prostate cancer and that expression is correlated with tumor progression. Thus, Akt1 could be targeted for therapeutic intervention of cancer while at its earliest stages. In some embodiments, the analyzer systems of the invention provide a method for providing an early diagnosis of a cancer by determining the presence or concentration of Akt1 in a sample from a patient when the level of Akt 1is less than about 100, 50, or 25 pg/ml. See Example 6.

[00282] 8. Fas ligand

[00283] Fas Ligand (FasL), also known as CD95L, is a member of the TNF family and induces apoptosis via binding to Fas (CD95). The protein exists in two forms; either membrane FasL or soluble FasL, which migrate at molecular weight of 45 kDa and 26 kDa, respectively. FasL is expressed on a variety of cells including activated lymphocytes, natural killer cells and monocytes. Interaction of FasL and Fas plays an important role in physiological apoptotic processes. Malfunction of the Fas-FasL system causes hyperplasia in peripheral lymphoid organs and accelerates autoimmune disease progression and tumorigenesis. There are limited data about the levels of soluble apoptotic factors in general, and more specifically about their modulation with therapeutic regimens.

[00284] The systems and methods of the invention can detect concentrations of Fas ligand that are as low as 2.4 pg/ml. Thus, in some embodiments, the analyzer systems and methods of the invention provide for the detection of Fas ligand to identify pathological conditions such as abnormal levels of apoptosis. Measurements of Fas in patient samples can be used to diagnose conditions such as polycystic ovarian syndrome, tumors such as testicular germ cell tumors, bladder cancer, lung cancer, and rare tumors such as follicular dendritic cell tumors. In addition, Fas measurements of Fas ligand can be used t diagnose allograft rejection.; and degenerative disease such as osteoarthritis. Thus, in some embodiments, the analyzer systems and methods of the invention can be used to determine the concentration of Fas ligand in a sample from a patient suspected of suffering from Fas ligand related disorder to diagnose the disorder, or the concentration of Fas ligand can be used to monitor the progress or status of a Fas ligand related disorder in a patient undergoing therapy for the disorder. In some embodiments, the assay is capable of determining the level of Fas ligand in the sample at a concentration less than about 100, 50, 25, 10, or 5 pg/ml. See Example 8.

C. Business Methods

[00285] The present invention relates to systems and methods (including business methods) for establishing markers that can be used for diagnosing a biological state or a condition in an organism, preparing diagnostics based on such markers, and commercializing/marketing diagnostics and services utilizing such diagnostics

[00286] In one embodiment, the business methods herein comprise: establishing one or more markers using a method comprising: establishing a range of concentrations for said marker or markers in biological samples obtained from a first population by measuring the concentrations of the marker or markers in the biological samples by detecting single molecules of the marker or markers; and commercializing the one or more markers identified in the

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above step, e.g., in a diagnostic product. The biomarkers identified are preferably polypeptides or small molecules. Such polypeptides can be previously known or unknown. The diagnostic product herein can include one or more antibodies that specifically binds to the marker (e.g., polypeptide).

[00287] In one embodiment, the business methods herein comprise: establishing one or more markers using a system comprising: establishing a range of concentrations for said marker in biological samples obtained from a first population by measuring the concentrations of the marker the biological samples by detecting single molecules of the marker; and providing a diagnostic service to determine if an organism has or does not have a biological state or condition of interest. A diagnostic service herein may be provided by a CLIA approved laboratory that is licensed under the business or the business itself. The diagnostic services herein can be provided directly to a health care provider, a health care insurer, or a patient. Thus the business methods herein can make revenue from selling e.g., diagnostic services or diagnostic products.

[00288] The business methods herein also contemplate providing diagnostic services to, for example, health care providers, insurers, patients, etc. The business herein can provide diagnostic services by either contracting out with a service lab or setting up a service lab (under Clinical Laboratory Improvement Amendment (CLIA) or other regulatory approval). Such service lab can then carry out the methods disclosed herein to identify if a particular marker or pattern of markers is within a sample.

[00289] The one or more markers are polypeptides or small molecules, or new chemical entities.

Kits

[00290] The invention further provides kits. In some embodiments, kits include an analyzer system and a label, as previously described. Kits of the invention include one or more compositions useful for the sensitive detection of a molecule, such as a marker, as described herein, in suitable packaging. In some embodiments, kits of the invention provide a label, as described herein, together with other components such as instructions, reagents, or other components. In some embodiments, the kit provides the label as separate components, in separate containers, such as an antibody and a fluorescent moiety, for attachment before use by the consumer. In some embodiments kits of the invention provide binding partner pairs, e.g., antibody pairs, that are specific for a molecule, e.g., a marker, where at least one of the binding partners is a label for the marker, as described herein. In some embodiments, the binding partners, e.g., antibodies, are provided in separate containers. In some embodiments, the binding partners, e.g., antibodies, are provided in the same container. In some embodiments, one of the binding partners, e.g., antibody, is immobilized on a solid support, e.g., a microtiter plate or a paramagnetic bead. In some of these embodiments, the other binding partner, e.g., antibody, is labeled with a fluorescent moiety as described herein.

[00291] Binding partners, e.g., antibodies, solid supports, and fluorescent labels for components of the kits may be any suitable such components as described herein.

[00292] The kits may additionally include reagents useful in the methods of the invention, e.g., buffers and other reagents used in binding reactions, washes, buffers or other reagents for preconditioning the instrument on which assays will be run, filters for filtering reagents, and elution buffers or other reagents for running samples through the instrument.

[00293] Kits may include one or more standards, e.g., standards for use in the assays of the invention, such as standards of highly purified, e.g., recombinant, protein markers, or various fragments, complexes, and the like, thereof. Kits may further include instructions.

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EXAMPLES

[00294] The following examples are offered by way of illustration and not by way of limiting the remaining disclosure.

[00295] Unless otherwise specified, processing samples in the Examples were analyzed in a single molecule detector (SMD) as described herein, with the following parameters: Laser: continuous wave gallium arsenite diode laser of wavelength 639 nm (Blue Sky Research, Milpitas, CA), focused to a spot size of approximately 2 microns (interrogation space of .004 pL as defined herein); flow rate = 5 microliter/min through a fused silica capillary of 100 micron square ID and 300 micron square OD; non-confocal arrangement of lenses (see, e.g., Fig. 1A); focusing lens of 0.8 numerical aperture (Olympus); silicon avalanche photodiode detector (Perkin Elmer, Waltham, MA).

Example 1. Sandwich assays for biomarkers: cardiac Troponin I (cTnI)

[00296] The assay: The purpose of this assay was to detect the presence of cardiac Troponin I (cTNI) in human serum. The assay format was a two-step sandwich immunoassay based on a mouse monoclonal capture antibody and a goat polyconal detection antibody. Ten microliters of sample were required. The working range of the assay is 0 – 900 pg/ml with a typical analytical limit of detection of 1 – 3 pg/ml. The assay required about four hours of bench time to complete.

[00297] Materials: the following materials were used in the procedure described below: Assay plate: Nunc Maxisorp, product 464718, 384 well, clear, passively coated with monoclonal antibody, BiosPacific A34440228P Lot # A0316 (5μg/ml in 0.05 M sodium carbonate pH 9.6, overnight at room temperature); blocked with 5% sucrose, 1% BSA in PBS, and stored at 4oC. For the standard curve, Human cardiac Troponin I (BiosPacific Cat # J34000352) was used. The diluent for the standard concentrations was human serum that was immonodepleted of endogenous cTNI, aliquoted and stored at - 20oC. Dilution of the standards was done in a 96 well, conical, polypropylene, (Nunc product # 249944). The following buffers and solutions were used: (a) assay buffer: BBS with 1% BSA and 0.1% TritonX-100; (b) passive blocking solution in assay buffer containing 2mg/ml mouse IgG , (Equitech Bio); 2mg/ml goat IgG, (Equitech Bio); and 2mg/ml MAK33 poly, Roche# 11939661; (c) detection Antibody (Ab): Goat Polyclonal antibody affinity purified to Peptide 3, (BiosPacific G129C), which was label with a fluorescent dye AlexaFluor 647, and stored at 4oC; detection antibody diluent: 50% assay buffer, 50% passive blocking solution; wash buffer: borate buffer saline Triton Buffer (BBST) (1.0 M borate, 15.0 M sodium chloride, 10% Triton X-100, pH 8.3); elution buffer: BBS with 4M urea, 0.02% Triton X-100 and 0.001% BSA.

[00298] Preparation of AlexaFluor 647 labeled antibodies: the detection antibody G-129-C was conjugated to AlexaFluor 647 by first dissolving 100ug of G-129-C in 400uL of the coupling buffer (0.1M NaHCO3). The antibody solution was then concentrated to 50ul by transferring the solution into YM-30 filter and subjecting the solution and filter to centrifugation. The YM-30 filter and antibody was then washed three times by adding 400ul of the coupling buffer. The antibody was recovered by adding 50□l to the filter, inverting the filter, and centrifuging for 1 minute at 5,000 x g. The resulting antibody solution was 1-2ug/ul. AlexaFluor 647 NHS ester was reconstituted by adding 20ul DMSO to one vial of AlexaFluor 647, this solution was stored at -20oC for up to one month. 3ul of AlexaFluor 647 stock solution was added to the antibody solution, which was then mixed and incubated in the dark for one hour. After the one hour, 7.5ul 1M tris was added to the antibody AlexaFluor 647 solution and mixed. The solution was ultrafiltered with YM-30 to remove low molecular weight components. The volume of the retentate, which contained the antibody conjugated to AlexaFluor 647, was adjusted to 200-400□l by

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adding PBS. 3ul 10% NaN3 was added to the solution, the resulting solution was transferred to an Ultrafree 0.22 centrifugal unit and spun for 2 minutes at 12,000 x g. The filtrate containing the conjugated antibody was collected and used in the assays.

[00299] Procedure: cTnI standard and sample preparation and analysis:

[00300] The standard curve was prepared as follows: working standards were prepared (0 – 900 pg/ml) by serial dilutions of the stock of cTnI into standard diluent or to achieve a range of cTnI concentrations of between 1.2 pg/ml – 4.3 μg/ml.

[00301] 10 μl passive blocking solution and 10 μl of standard or of sample were added to each well. Standards were run in quadruplicate. The plate was sealed with Axyseal sealing film, centrifuged for 1min at 3000 RPM, and incubated for 2 hours at 25oC with shaking. The plate was washed five times, and centrifuged until rotor reached 3000 RPM in an inverted position over a paper towel. A 1nM working dilution of detection antibody was prepared, and 20 μl detection antibody were added to each well. The plate was sealed and centrifuged, and the assay incubated for 1hour at 25oC with shaking. 30 μl elution buffer were added per well, the plate was sealed and the assay incubated for ½ hour at 25oC. The plate was either stored for up to 48 hours at 4oC prior to analysis, or the sample was analyzed immediately.

[00302] For analysis, 20 μl per well were acquired at 40 μl/minute, and 5 μl were analyzed at 5 μl/minute. The data were analyzed based on a threshold of 4 sigma. Raw signal versus concentration of the standards was plotted. A linear fit was performed for the low concentration range, and a non- linear fit was performed for the full standard curve. The limit of detection (LoD) was calculated as LOD = (3 x standard deviation of zeros) / slope of linear fit. The concentrations of the samples were determined from the equation (linear or non-linear) appropriate for the sample signal.

[00303] An aliquot was pumped into the analyzer. Individually-labeled antibodies were measured during capillary flow by setting the interrogation volume such that the emission of only 1 fluorescent label was detected in a defined space following laser excitation. With each signal representing a digital event, this configuration enables extremely high analytical sensitivities. Total fluorescent signal is determined as a sum of the individual digital events. Each molecule counted is a positive data point with hundreds to thousands of DMC events/sample. The limit of detection the cTnI assay of the invention was determined by the mean +3 SD method.

[00304] Results: Data for a typical cTnI standard curve measured in quadruplicate using the assay protocol is shown in Table 3.

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Table 3

Standard Curve for cTnI

cTnI (pg/ml)	Signal	Standard Deviation	% CV
0	233	25	10.8
1.5625	346	31	8.9
3.125	463	35	7.5
6.25	695	39	5.6
12.5	1137	61	5.3
25	1988	139	7.0
50	3654	174	4.8
100	5493	350	6.4
200	8264	267	3.2
400	9702	149	1.5
800	9976	50	0.5

[00305] The sensitivity of the analyzer system was tested in 15 runs and was found routinely to detect sub femtomol/l (fM) levels of calibrator, as shown by the data in Table 4. The precision was 10% at 4 and 12 pg/ml cTnI.

Table 4

Instrument Sensitivity

Calibrator (fM)	Signal counts	CV
0	11	
12	302	9
60	1341	8
300	4784	7

[00306] Linearized standard curve for the range concentrations of cTnI are shown in Figure 5.

[00307] The analytical limit of detection (LoD) was determined across 15 sequential assays. The LoD was the mean of the 0 std + 3 SD (n=4) intra-assay determinations. The average LoD was 1.7 pg/ml (range 0.4-2.8 pg/ml).

[00308] The recovery of the sample was determined by analyzing samples of serum that had been immunodepleted of cTnI and spiked with known amounts of cTnI. Table 5 shows the data for sample recovery by the system analyzed over 3 days.

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Table 5

Sample Recovery

Spike (pg/ml)	Recovery (mean)	Standard Deviation	% CV
5	5.7	0.9	16
15	13.7	0.2	2
45	43	0.6	2
135	151	6.2	4

[00309] The linearity of the assay was determined in pooled human serum that was spiked with cTnI and diluted with standard diluent. The results in Table 6 show the dilutions and % of the signal expected for the corresponding dilution.

Table 6

Assay Linearity

Serum Dilution	% of expected
1:2	79
1:4	87
1:8	96

[00310] These data show that the analyzer system of the invention allows for performing highly sensitive laser-induced immunoassay for sub-femtomolar concentrations of cTnI.

Example 2: Sandwich Bead-based Assays for TnI

[00311] The assays described above use the same microtiter plate format where the plastic surface is used to immobilize target molecules. The single particle analyzer system also is compatible with assays done in solution using microparticles or beads to achieve separation of bound from unbound entities.

[00312] Materials: MyOne Streptavidin C1 microparticles (MPs) are obtained from Dynal (650.01-03, 10 mg/ml stock). Buffers use in the assay include: 10X borate buffer saline Triton Buffer (BBST) (1.0 M borate, 15.0 M sodium chloride, 10% Triton X-100, pH 8.3); assay buffer (2 mg/ml normal goat IgG, 2 mg/ml normal mouse IgG, and 0.2 mg/ml MAB-33-IgG-Polymer in 0.1 M Tris (pH 8.1), 0.025 M EDTA, 0.15 M NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.1% NaN3, stored at 4C); .and elution buffer (BBS with 4 M urea, 0.02% Triton X-100, and 0.001% BSA, stored at 2-8C). Antibodies used in the sandwich bead-based assay include: Bio-Ab (A34650228P (BiosPacific) with 1-2 biotins per IgG) and Det-Ab (G-129-C (BiosPacific) conjugated to A647, 2-4 fluors per IgG). The standard is recombinant human cardiac troponin I (BiosPacific, cat # J34120352). The calibrator diluent is 30 mg/ml BSA in TBS wEDTA.

[00313] Microparticles Coating: 100 ul of the MPs stock is placed in an eppendorf tube. The MPs are washed three times with 100 ul of BBST wash buffer by applying a magnet, removing the supernatant, removing the magnet, and

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resuspending in wash buffer. After the washes the MPs are resuspended in 100 ul of assay buffer and 15 ug of Bio-Ab are added. The mixture is then incubated for an hour at room temperature with constant mixing. The MPs are washed five times with 1 ml wash buffer as described above. After the washes the MPs are resuspended in 15 ml of assay buffer (or 100 ul to store at 4 0C).

[00314] Preparation of Standard and Samples: The standard is diluted with calibrator diluent to prepare proper standard curve (usually 200 pg/ml down to 0.1 pg/ml). Frozen serum and plasma samples need to be centrifuged 10 minutes at room temperature at 13K rpm. Clarified serum/plasma is removed carefully to avoid taking any possible pellets or floaters and put into fresh tubes. 50 ul of each standard or sample is pippetted into appropriate wells.

[00315] Capture Target: 150 ul of MPs (after resuspension to 15 ml in assay buffer + 400 mM NaCl) are added to each well. The mixture is incubated on JitterBug, 5 at room temperature for 1 hr.

[00316] Washes and Detection: The plate is placed on a magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 250 ul of wash buffer are added after removing the plate from the magnet. The plate is then placed on the magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 20 ul Det-Ab are added per well (Det-Ab to 500 ng/ml is diluted in assay buffer + 400 mM NaCl)). The mixture is incubated on JitterBug, 5 at room temperature for 30 min.

[00317] Washes and Elution: The plate is placed on a magnet and washed three times with wash buffer. The supernatant is removed after ensuring that all MPs are captured by the magnet and 250 ul of wash buffer are added. After the washes the samples are transferred into a new 96-well plate. The new plate is then placed on the magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 250 ul of wash buffer are then added after removing the plate from the magnet. The plate is then placed on the magnet and the supernatant is removed after ensuring that all MPs are captured by the magnet. 20 ul of elution buffer are then added and the mixture is incubated on JitterBug, 5 at room temperature for 30 min.

[00318] Filter out MPs and transfer to 384-well plate: The standard and samples are transferred into a 384-well filter plate placed on top of a 384-well assay plate. The plate is then centrifuged at room temperature at 3000 rpm with a plate rotor. The filter plate is removed and the appropriate calibrators are added. The plate is covered and is ready to be run on SMD.

[00319] SMD: An aliquot is pumped into the analyzer. Individually-labeled antibodies are measured during capillary flow by setting the interrogation volume such that the emission of only 1 fluorescent molecule is detected in a defined space following laser excitation. With each signal representing a digital event, this configuration enables extremely high analytical sensitivities. Total fluorescent signal is determined as a sum of the individual digital events. Each molecule counted is a positive data point with hundreds to thousands of DMC events/sample. The limit of detection the cTnI assay of the invention is determined by the mean +3 SD method.

Example 3. Concentration range for cTnI in a population of normal non-diseased subjects.

[00320] A reference range or normal range for cTnI concentrations in human serum was established using serum samples from 88 apparently healthy subjects (non-diseased). A sandwich immunoassay as described in Example 1 was performed and the number of signals or events as described above were counted using the single particle analyzer system of the invention. The concentration of serum troponin I was determined by correlating the signals detected by the analyzer with the standard curve as described above. All assays were perfumed in quadruplicate.

[00321] In accordance with recommendations by the current European and American Cardiology Societies (ESC/ACC) troponin assays should quantify accurately the 99th percentile of the normal range with an assay

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imprecision (CV) of less than 10% in order to distinguish reliably between patients with ACS and patients without ischemic heart disease, and risk stratification for adverse cardiac events. The assay showed that the biological threshold (cutoff concentration) for TnI is at a TnI concentration of 7pg/ml, which is established at the 99th percentile with a corresponding CV of 10% (Figure 5). At the 10% CV level the precision profile points at a TnI concentration of 4 and 12 pg/ml.

[00322] In addition, the assay correlates well with the Troponin-I standard measurements provided by the National Institute of Standards and Technology (Figure 6).

[00323] The assay of the invention is sufficiently sensitive and precise to fulfill the requirements of the ESC/ACC, and it is the most sensitive assay for cardiac troponin I when compared to assays such as those described by Koerbin et al.(Ann Clin Biochem, 42:19-23 (2005). The assay of the invention has a 10-20 fold greater sensitivity than that currently available assays, which has determined the biological threshold range to be 111-333 pg/ml cTnI.

Example 4. Detection of early release of TnI into the circulation of patients with acute myocardial infarction (AMI)

[00324] Study 1: 47 samples were obtained serially from 18 patients that presented with chest pain in the emergency department (ED). These patients all had non-ST elevated ECG were, and were diagnosed with AMI. The concentration of cTnI in the initial samples from all 18 patients was determined according to a commercial assay at the time of admission to the emergency room to be <350 pg/ml (10% cutpoint), and 12 were <100 pg/ml (99th%) percentile. These samples were tested at later times using the same commercial assay, and were determined to test positive for cTnI. The same serum samples were also assayed for TnI according to the assay of the invention as described in Examples 1 and 3, and the results compared to the results obtained using the commercial assay.

[00325] Blood was drawn for the first time at the time the patient presented with chest pain (sample 1), and subsequently at intervals between 4-8 hours (samples 2 at 12 hours; sample 3 at 16 hours; sample 4 at 24 hours; sample 5 at 30 hours; sample 6 at 36 hours; sample 7 at 42 hours; and sample 8 at 48 hours). The serum was analyzed by the methods of the invention and by a current commercial method, and the results obtained are shown in Figure 7. The analyzer of the invention detected TnI at the time the patient presented with chest pain (sample 1), while the commercial assay first detected cTnI at a much later time (sample 6 at 36 hours). The concentration of TnI in sample 3 exceeded the biological threshold level that was established using the analyzer of the invention (7 pg/ml, see Figure 5), and indicated that sample 3 is positive for TnI to suggest the incidence of a cardiac event. The biological threshold for the commercial assay lies between 111 and 333 pg/ml of TnI. Accordingly, sample 3 would not have been considered to indicate a possible cardiac event.

[00326] In addition, the methods and compositions of the present invention allow for much earlier diagnosis and possible intervention based on cardiac troponin levels, as evidenced by results for the first sample taken from the patients. In the 3 cases that had initial commercial assay cTnI values of between 100 and 350 ng/ml, all were positive for cTnI by the analytical methods of the invention (i.e., cTnI over 7 pg/ml). In the 12 cases that had initial commercial cTnI values of less than 100 pg/ml, 5 were determined to be positive for a cardiovascular event according to the assay of the invention (i.e., cTnI over 7 pg/ml). The prospective use of the assay of the invention would have detected 53% more AMI cases than the current commercial assay when the admission sample was tested.

[00327] Study 2: 50 additional serum samples, which tested negative according to the commercial assay, were tested using the analyzer and assay of the invention. The results are shown in Figure 8. Of the 50 samples, 36 were

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within the 99th% and determined to be within the normal range established by the assay of the invention. However, the remaining 14 samples that were determined to be within the commercial "normal" or non-diseased range, tested above the biological threshold established by the invention.

[00328] Therefore, the high sensitivity cTnI assay of the invention allows for the detection of myocardial damage in patients when cTnI serum levels are below threshold values by commercially available technology. The use of the highly sensitive and precise cTnI assay of the invention enables detection of AMI earlier than with existing cTnI assays, and thereby provides the opportunity for appropriate diagnosis and early medical intervention to improve the outcome.

[00329] Example 5. Detection of Leukotriene T4 (LTE4)

[00330] The assay was developed to quantify Leukotriene E_4 (LTE_4) in buffer as a preliminary assay for assays using, e.g., urine specimens. The assay format was a one-step single antibody competitive immunoassay. Fifty microliters of sample were required. The typical working range of this assay was 0 – 300 pg/ml with a typical limit of detection of 2-3 pg/ml (0.1-0.15 pg/sample). The assay required about four hours of bench time to complete.

[00331] The following materials were prepared and used in the procedure described below: Mouse anti-rabbit IgG coated plate provided in Cayman Chemical Leukotriene E_4 (EIA Kit, Catalog # 520411); stock LTE_4 Standard (purified LTE4 at 100 ng/ml in ethanol (Cayman Chemical Leukotriene E_4 EIA Kit, Catalog # 520411)); assay buffer (10X EIA buffer concentrate (Cayman Chemical Leukotriene E_4 EIA Kit, Catalog # 520411)) diluted 1:10 with 90 ml Nanopure water; buffer for dilution of standards (3% ethanol); anti-LTE_4 antibody (Leukotriene E_4 EIA antiserum (Cayman Chemical Leukotriene E_4 EIA Kit, Catalog # 520411) diluted with 30 ml EIA buffer; streptavidin-Alexa detection reagent stock solution of 31 μM (streptavidin labeled with AlexaFluor™ 647); tracer (LTE_4-biotin conjugate) was made compatible for detection by the analyzer; wash buffer (400X concentrate (Cayman Chemical Leukotriene E_4 EIA Kit, Catalog # 520411)) diluted 1:40; elution buffer (borate buffered saline, pH 8.3 with 4M urea, 0.02% Triton X-100 and 0.001% BSA). The matrix of the tracer and the antiserum concentrations were tested to identify the most sensitive assay conditions.

[00332] A standard curve was prepared as follows: working standards were prepared by making serial dilutions of the 100 ng/ml stock into assay buffer to achieve a range of concentrations between 0.005 pg/ml and 3000 pg/ml. 50 μl standard (or sample) were added per well of the assay plate. All standards were run in duplicate. Working tracer was prepared by diluting the tracer stock to 1 pg/ml with assay buffer. 50 μl tracer (or buffer) were added per well of the assay plate. A 10% working antiserum solution was prepared by diluting 100% stock (made according to the kit instructions) into assay buffer. 50 μl antiserum (or buffer) were added per well of the assay plate; the plate was sealed and incubated overnight at 25 °C with shaking. A working streptavidin-Alexa detection reagent was prepared by diluting stock to 140 pM with assay buffer. 15 ul of detection reagent were added to each well, and the plate was incubated for 30 min at 25 °C with shaking. The plate was washed 5 times. 50 μl of elution buffer were added to each well, and the plate was incubated for ½ hour at 25°C with shaking. The plate was use immediately or stored for up to 48 hours at 4°C prior to analysis.

[00333] 20 μl were pumped into the analyzer at a rate of 40 μl/minute, and 5 μl of sample were analyzed at 5 μl/minute. The data files were analyzed using a threshold = 4 sigma, and a cross correlation of between 0 – 8 msec. Raw signal versus concentration was plotted for the standards, and a linear fit was used for low range standards, while a non-linear fit was used for full standard curve. The limit of detection was calculated as LOD = 80% of the maximum signal (no target control) (the concentration at which $B/B_0 = 80\%$). The concentrations of samples were calculated from the equation (linear or non-linear) appropriate for the sample signal.

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[00334] The competition curve of LTE4 is shown in Figure 9. The LOD was calculated to be 80% B/Bo = 1.5 pg/ml (approximately 5 pM). The LTE4 assay performed using a commercially available kit can detect LTE4 only if present at a concentration of at least 30 pg/ml.

[00335] Therefore, the analyzer system can be used to detect levels of LTE4 to indicate the presence of an LTE4-related disorder e.g. asthma at the onset of disease, and alert clinicians to the need for therapeutic intervention at an early stage of the disease to improve the clinical outcome.

Example 6. Detection of human Akt1

[00336] A sandwich immunoassay was developed for the quantification of low levels of Akt1 in serum samples. A standard curve was generated by dilution of a concentrated standard into a buffered protein solution. Ten microliters (µl) of assay buffer and 10 µl of sample or standard were added to each well of a 384-well plate that had been coated with an antibody specific for Akt1 and incubated for two hours. More specifically antibody 841660 (R&D Systems) was coated onto Nunc Maxisorp plates @ 2.5 micrograms/ml. The plate was washed, and 20 µl of labeled detection antibody specific for Akt1, AF1775 (R&D Systems), labeled with AlexaFluor 647, 2 – 4 fluors/IgG, was added to each well. After one hour of incubation the plate was washed to remove unbound detection antibody. Bound detection antibody was eluted and measured in the analyzer instrument.

[00337] The following materials were used in the assay procedure described below. Coated 384 well plate; assay buffer; resuspension buffer; dilution buffer; standard diluent; Akt1 standard; detection antibody reagent for Akt1; wash buffer (10mM Borate, 150mM NaCl, 0.1% TritonX-100, pH 8.3); elution buffer (4 M urea with 0.02% Triton X-100 and 0.001% BSA), .Microplate shaker, set at "7", Microplate washer, Plate centrifuge, Axyseal sealing film, Axygen product 321-31-051, Nunc pierceable sealing tape, Nunc product 235306.

Materials

Provided Reagents
Capture antibody: 841660 (R&D Systems), coated onto Nunc Maxisorp plates @ 2.5 micrograms/ml (384 well plate)
Assay buffer
Resuspension Buffer
Dilution Buffer
Standard diluent
Akt 1 standard
Detection antibody reagent for Akt1, AF1775 (R&D Systems), labeled with AlexaFluor 647, 2 – 4 fluors/IgG
Other Required Reagents
TritonX-100 Wash buffer (10mM Borate, 150mM NaCl, 0.1% TritonX-100, pH 8.3)
Elution buffer (4 M urea with 0.02% Triton X-100 and 0.001% BSA)
Microplate shaker, set at "7"
Microplate washer
Plate centrifuge
Axyseal sealing film, Axygen product 321-31-051
Nunc pierceable sealing tape, Nunc product 235306

Procedure

Akt1 standard preparation
Resuspend standard in 0.5ml Resuspension Buffer, final concentration = 170ng/ml
Dilute standard 1:3 in Dilution Buffer = 57ng/ml
Dilute standard 1: 19 in Standard Diluent = 3ng/ml
Do serial 3 fold dilutions down to 4.1pg/ml in Standard Diluent
Add 10 µl Assay Buffer per well
Add 10 µl standard or sample per well
Seal plate with Axyseal sealing film
Spin 1 min at 3000 RPM

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Incubate 2 hours at 25oC with shaking
Wash plate five times
Spin plate inverted on a paper towel 1 min at 3000 RPM
Add 20 µl detection antibody reagent per well
Seal plate with Axyseal sealing film
Spin plate inverted on a paper towel 1min at 3000 RPM
Incubate 1 hour at 25oC with shaking
Wash plate five times
Spin plate inverted on a paper towel 1min at 3000 RPM
Add 30 µl elution buffer per well
Spin 1min at 3000 RPM
Seal with Nunc pierceable sealing tape, secure tight seal with roller
Incubate ½ hour at 25oC with shaking
The plate may be stored for up to 48 hours at 4oC prior to analysis
Analyze on Zeptx instrument

[00338] The Akt1 standard curve was generated as follows. Akt1 standards were prepared to achieve a range of between 4.1 pg/ml to 170 ng/ml Akt1. 10 µl of each standard dilution (or sample) were added to the assay plate wells. The plate was sealed and incubated for 2 hours at 25°C with shaking. The plate was washed and centrifuged dry. 20 µl detection antibody reagent was added per well and incubated for1 hour at 25°C with shaking. The antibody-Akt1 complex was disrupted by adding 30 µl elution buffer per well and incubating for ½ hour at 25°C with shaking. The plate was either used immediately or stored for up to 48 hours at 4°C prior to analysis. Eluate was pumped into the analyzer.

[00339] Data for a typical Akt1 standard curve measured in quadruplicate using the assay protocol is given in Table 14, and the graphed data is shown in Figure 10.

Table 14

Standard curve for Akt1

Concentration Akt1 standard (pg/ml)	Average Signal	Standard deviation	%CV
0	113	16	14
4.1	126	10	8
12.4	133	1	0
37	151	34	22
111	173	15	8
333	350	74	21
1000	733	136	19
3000	1822	243	13

[00340] Intra-Assay Precision was tested using 36 replicate samples of the 1000 pg/ml standard by assaying the samples on a single plate. The average signal was 1822 ± 243 with a % CV = 13. The limit of detection of the assay (LoD) was determined by adding two standard deviations to the mean signal of thirty six zero standard replicates and calculating the corresponding Akt1 concentration from the standard curve. The LoD was calculated to be 25 pg/ml.

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[00341] Therefore, the analyzer system can be used to detect levels of Akt1 to determine the presence or absence of an Akt1-related disorder, e.g. cancer.

Example 7. Detection of TGF-β

[00342] A sandwich immunoassay was developed for the quantification of low levels of TGFβ in serum. A standard curve was generated by dilution of a concentrated standard into a buffered protein solution. Ten microliters (µl) of assay buffer and 10 µl of sample or standard were added to each well of a 384-well plate coated with an antibody specific for TGFβ and incubated for two hours. The plate was washed and 20 µl of labeled detection antibody specific for TGFβ was added to each well. After one hour of incubation the plate was washed to remove unbound detection antibody. Bound detection antibody was eluted and measured in the analyzer instrument.

[00343] The following materials were used in the assay procedure described below. Coated 384 well plate; assay buffer; standard diluent; 10ug/ml stock solution of TGFβ standard; detection antibody reagent for TGFβ; TritonX-100 Wash buffer (10mM Borate, 150mM NaCl, 0.1% TritonX-100, pH 8.3); elution buffer (4 M urea with 0.02% Triton X-100 and 0.001% BSA).

[00344] The TGF-β standard curve was generated as follows. TGF-β standards were prepared to achieve a range of between 100 ng/ml to 4.1 pg/ml TGFβ. 10 µl assay buffer and 10 µl standard or sample were added to each well. The plate was sealed and incubated for 2 hours at 25°C with shaking. The plate was sealed and incubated for 2 hours at 25°C with shaking. The plate was washed and centrifuged dry. 20 µl detection antibody reagent was added per well and incubated for1 hour at 25°C with shaking. The antibody- TGF-β complex was disrupted by adding 30 µl elution buffer per well and incubating for ½ hour at 25°C with shaking. The plate was either used immediately or stored for up to 48 hours at 4°C prior to analysis. Eluate was pumped into the analyzer.

[00345] Data for a typical TGF-β standard curve measured in quadruplicate using the assay protocol is given in Table 15, and the graphed data is shown in Figure 11.

Table 15

Standard curve for TGF-β

Concentration (pg/ml)	Average Signal	Standard deviation	%CV
0	1230	114	9
4	1190	68	6
12	1261	132	10
37	1170	158	14
111	1242	103	8
333	1364	135	10
1000	1939	100	5
3000	3604	497	14

[00346] The limit of detection of the assay (LoD) was determined by adding two standard deviations to the mean signal of twenty zero standard replicates and calculating the corresponding TGFβ concentration from the standard curve. The LoD = 350 pg/ml.

[00347] Therefore, the analyzer system can be used to detect levels of TGFβ to determine the presence or absence of an TGFβ -related disorder, e.g. cancer.

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[00348] Example 8. Detection of Fas ligand

[00349] A sandwich immunoassay for the quantification of low levels of Fas ligand in serum. A standard curve was generated by dilution of a concentrated standard into a buffered protein solution. Ten microliters (µl) of assay buffer and 10 µl of sample or standard were added to each well of a 384-well plate coated with an antibody specific for Fas ligand and incubated for 2 hours. The plate was washed and 20 µl of labeled detection antibody specific for Fas ligand was added to each well. After a 1 hour incubation the plate was washed to remove unbound detection antibody. Bound detection antibody was eluted and measured in the $Zept_X$ instrument.

[00350] The Fas ligand standard curve was generated as follows. Fas ligand standards were prepared to achieve a range of between 100 ng/ml to 4.1 pg/ml Fas ligand. 10 µl assay buffer and 10 µl standard or sample were added to each well. The plate was sealed and incubated for 2 hours at 25°C with shaking. The plate was sealed and incubated for 2 hours at 25°C with shaking. The plate was washed and centrifuged dry. 20 µl detection antibody reagent was added per well and incubated forl hour at 25°C with shaking. The antibody-Fas ligand complex was disrupted by adding 30 µl elution buffer per well and incubating for ½ hour at 25°C with shaking. The plate was either used immediately or stored for up to 48 hours at 4°C prior to analysis.

[00351] Data for a typical Fas ligand standard curve measured in quadruplicate using the assay protocol is given in Table 16.

Table 16

Standard curve for Fas ligand

Concentration Fas ligand standard (pg/ml)	Average Signal	Standard deviation	%CV
0	935	82	9
1.2	1007	44	4
3.4	1222	56	5
11	1587	70	4
33	2869	52	2
100	5939	141	2
300	9276	165	2
900	11086	75	1

Intra-Assay Precision was tested using 12 replicate samples of 3 standard concentrations by assaying the samples on a single plate. The mean, standard deviation and CV for the 12 values for each of the three points are shown in Table 17.

Table 17

Intra-assay precision for Fas ligand

Concentration (pg/ml)	Average Signal	Standard deviation	*%CV*
11	**1717**	**128**	**7**
33	**3031**	**262**	**9**
100	**6025**	**257**	**4**

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[00352] The limit of detection of the assay (LoD) was determined by adding two standard deviations to the mean signal of twenty zero standard replicates and calculating the corresponding Fas ligand concentration from the standard curve. The LoD was calculated to be 2.4 pg/ml

[00353] Therefore, the analyzer system of the invention can detect levels of Fas ligand to indicate the presence of a Fas ligand-related disorder e.g. cancer, allograft rejection and degenerative diseases such as osteoarthritis.

[00354] Example 9. Sandwich assays for biomarker TREM-1.

[00355] Recent reports have established TREM-1 as a biomarker of bacterial or fungal infections (see, e.g., Bouchon et al. (2000) J. Immunol. 164:4991-5; Colonna (2003) Nat. Rev. Immunol. 3:445-53; Gibot et al. (2004) N. Engl. J. Med. 350:451-8; Gibot et al. (2004) Ann. Intern. Med. 141:9-15. Assays for TREM-1 have been developed using a sandwich assay format (Sandwich Assay for Detection of Individual Molecules, US Provisional Patent Application No. 60/624,785). Assay reagents for TREM-1 detection are available commercially (R&D Systems, Minneapolis, MN). The assay was done in a 96 well plate. A monoclonal antibody was used as the capture reagent, and either another monoclonal or a polyclonal antibody was used for detection. The detection antibody was labeled with AlexaFluorA647®.

[00356] The assay protocol was as follows:

[00357] 1. Coat plates with the capture antibody, washed 5X,

[00358] 2. Block in 1% BSA, 5% sucrose in PBS,

[00359] 3. Add the target diluted in serum, incubate, wash 5X,

[00360] 4. Add the detection antibody, incubate, wash 5X

[00361] 5. Add 0.1 M glycine pH 2.8 to release the bound assay components from the plate.

[00362] 6. Transfer samples from the processing plate to the detection plate, bring the pH of the sample to neutral and run on the single particle analyzer system.

[00363] Figure 13 shows a standard curve of TREM-1 generated using the assay. The assay was linear in the measured range of 100-1500 femtomolar. An ELISA assay from R&D Systems has recently been introduced. The standard curve reported for their ELISA assay is between 60-4000 pg/ml. This Example suggests we can routinely measure 100 fM (4.7 pg/ml) in a standard curve, allowing for about 10X more sensitive measurements. In addition, standard curves for chemokines, T cell activation molecules, cell adhesion molecules and signal transduction molecules have been generated **(Figure 15)**. The results show that the detection by the detection of analyte using the single particle analyzer is consistently between 10 and 100 fold more sensitive than detection using ELISA assays.

[00364] Example 10. Sandwich assays for biomarkers: IL-6 and IL-8 levels in serum

[00365] The assay: This protocol describes a sandwich immunoassay for the quantification of low levels of IL-6 in serum using the single particle analyzer system of the invention. A standard curve was generated by dilution of a concentrated standard into a buffered protein solution. Ten microliters (µl) of assay buffer and 10 µl of sample or standard were added to each well of a 384-well plate coated with an antibody specific for IL-6 and incubated for two hours. The plate was washed, and 20 µl of labeled detection antibody specific for IL-6 was added to each well. After one hour of incubation the plate was washed to remove unbound detection antibody. Bound detection antibody was eluted and measured in the single particle analyzer instrument.

[00366] Materials: the following materials were used in the procedure described below: coated 384 well plate; assay buffer; standard diluent; 100 ng/ml stock solution of IL-6 standard; detection antibody for IL-6 (R&D

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Systems) labeled with AlexaFluor 647 dye; TritonX-100 Wash buffer (10mM Borate, 150mM NaCl, 0.1% TritonX-100, pH 8.3); Elution buffer (4 M urea with 0.02% Triton X-100 and 0.001% BSA); Microplate shaker, set at "7"; Microplate washer; Plate centrifuge; Axyseal sealing film, Axygen product 321-31-051; and Nunc pierceable sealing tape, Nunc product 235306.

[00367] Procedure: IL-6 standard and sample preparation and analysis

A standard curve for IL-6 was prepared as follows: 100 ng/ml stock solution were thawed, and the stock solution was diluted 1:1000 to 100 pg/ml in standard diluent by doing six serial, 3 fold dilutions to obtain a range of concentration having the lowest standard concentration of 0.14 pg/ml. 10 µl assay buffer and 10 µl standard or sample were added to each well per well of the coated 384 well plate. The plate was sealed with Axyseal sealing film, and centrifuged for one minute at 3000 RPM. The assay plate was incubated for 2 hours at 25°C with shaking; washed five times; and centrifuged while inverted on a paper towel for one minute at 3000 RPM. 20 µl detection antibody reagent was added to each well; the plate was sealed with Axyseal sealing film, and centrifuged for one minute at 3000 RPM. The assay plate was incubated for one hour at 25°C with shaking, washed five times, and centrifuged while inverted on a paper towel for one minute at 3000 RPM. 30 µl elution buffer was added to each well; the plate was sealed with Nunc pierceable sealing tape, and a tight seal was secured using with roller. The assay plate was centrifuged for one minute at 3000 RPM, and incubated for ½ hour at 25°C with shaking. Analysis of the assay was performed immediately. Alternatively, the plate was stored for up to 48 hours at 4°C prior to analysis.

[00368] Samples of serum from EDTA treated whole blood of 32 blood bank donors were analyzed for IL-6.

[00369] Results: Data for a typical IL-6 standard curve measured in quadruplicate using the assay protocol is shown in Table 18.

Table 18

Standard Curve for IL-6

Concentration (pg/ml)	Average Signal	Standard deviation	CV
370	11035	206	2%
125	9983	207	2%
41	8522	95	1%
14	5023	108	2%
4.5	2577	124	5%
1.7	1178	114	10%
0.5	577	36	6%
0	106	15	14%

[00370] Linearized standard curves for higher and low range concentrations of IL-6 are shown in Figures 15 A-B, respectively. The assay allowed for detection of IL-6 at less than 0.5 pg/ml **(Figures 14 A-B)**. The limit of detection (LoD) was calculated to be 0.06 pg/ml. The limit of detection of the assay (LoD) was determined by adding two standard deviations to the mean signal of the zero standard replicates and calculating the corresponding IL-6 concentration from the standard curve. This level of sensitivity is excellent for detection of even normal levels of IL-6 which range between 0.5 and 10 pg/ml.

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[00371] Assays to detect IL-6 and IL-8 in serum of blood samples from blood bank donors were performed, and the results of the analysis are shown in **Figures 14 C and D**. IL-6 was quantified in 100% of the samples (32/32). The average concentration of IL-6 was 2.3 pg/ml, and the range of concentration was 0.2 to > 26 pg/ml **(Figure14 C)**. The same samples were also assayed for IL-8 essentially using the procedure described for IL-6. IL-8 standards and IL-8 specific antibodies were used. A standard curve for IL-8 was established (not shown) and used to determine the concentration of IL-8 in the samples **(Figure 14D)**. IL-8 was quantified in 100% (32/32) samples. The average concentration for IL-8 was 7.3 pg/ml, and the range of concentration was 1.2 to > 26 pg/ml.

[00372] Measurements of IL-6 or any particle of interest can be measured at low and higher concentrations **(Figures 14 A and B)** by switching the detection of the analyzer from counting molecules (digital signal) to detecting the sum of photons (analog signal) that are generated at the higher concentrations of analyte. This is shown in a general way in **Figure 14E**. The single particle analyzer has an expanded linear dynamic range of 6 logs. The ability to increase the dynamic range for detecting the concentration of a particle in a sample allows for the determination of the concentration of a particle for normal (lower concentration range) and disease levels (higher concentration range). The range of detection for normal and disease levels of IL-6 is shown in Figure **15F**.

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WHAT IS CLAIMED IS:

1. An analyzer system kit for detecting a single protein molecule in a sample, said kit comprising an analyzer and at least one label comprising a fluorescent moiety and a binding partner for said protein molecule, and wherein said analyzer comprises:

a) an electromagnetic radiation source for stimulating said fluorescent moiety;

b) a capillary flow cell for passing said label;

c) a source of motive force for moving said label in said capillary flow cell;

d) an interrogation space defined within said capillary flow cell for receiving electromagnetic radiation emitted from said electromagnetic source; and

e) an electromagnetic radiation detector operably connected to said interrogation space for measuring an electromagnetic characteristic of said stimulated fluorescent moiety;

wherein said fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

2. The analyzer system of claim 1, wherein said electromagnetic radiation source is a laser, and wherein said laser has a power output of at least about 3, 5, 10, or 20 mW.

3. The analyzer system of claim 1, wherein said fluorescent moiety comprises a fluorescent molecule.

4. The analyzer system of claim 3, wherein said fluorescent molecule is a dye molecule.

5. The analyzer system of claim 4, wherein said dye molecule comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance.

6. The analyzer system kit of claim 1, wherein the analyzer comprises not more than one interrogation space.

7. The analyzer system of claim 1 wherein said electromagnetic radiation source is a continuous wave electromagnetic radiation source.

8. The analyzer system of claim 7, wherein said continuous wave electromagnetic radiation source is a light-emitting diode or a continuous wave laser.

9. The analyzer system of claim 1, wherein said motive force is pressure.

10. The analyzer system of claim 1, wherein said detector is an avalanche photodiode detector.

11. The system of claim 1, further comprising a confocal optical arrangement for deflecting a laser beam onto said interrogation space and for imaging said stimulated dye molecule, wherein said confocal optical arrangement comprises an objective lens having a numerical aperture of at least about 0.8.

12. The analyzer system of claim 1 further comprising a sampling system capable of automatically sampling a plurality of samples and providing a fluid communication between a sample container and said interrogation space.

13. The analyzer system of claim 1 further comprising a sample recovery system in fluid communication with said interrogation space, wherein said recovery system is capable of recovering substantially all of said sample.

14. A method for determining the presence or absence of a single molecule of a protein in a biological sample, comprising labeling said molecule with a label and detecting the presence or absence of said label in a single molecule detector, wherein said label comprises a fluorescent moiety that is capable of emitting at least about 200

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photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

15. The method of claim 14 wherein the limit of detection of said single molecule in said sample is less than about 10, 1, 0.1, 0.01, or 0.001 femtomolar.

16. The method of claim 14 wherein the limit of detection is less than about 1 femtomolar.

17. The method of claim 14 wherein said detecting comprises detecting electromagnetic radiation emitted by said fluorescent moiety.

18. The method of claim 14 wherein said single molecule detector comprises not more than one interrogation space.

19. The method of claim 18 further comprising exposing said fluorescent moiety to electromagnetic radiation, wherein said electromagnetic radiation is provided by a laser.

20. The method of claim 19, wherein said laser stimulates said moiety at a power output of less than about 20 mW.

21. The method of claim 20, wherein said laser stimulates said moiety for a duration of less than about 1000, 250, 100, 50, 25 or 10 microseconds.

22. The method of claim 14 wherein said label further comprises a binding partner specific for binding said molecule.

23. The method of claim 22 wherein said binding partner is an antibody.

24. The method of claim 14, wherein said fluorescent moiety comprises a fluorescent dye molecule.

25. The method of claim 24, wherein said dye molecule comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance.

26. The method of claim 26 wherein said dye molecule is an AlexFluor molecule selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700.

27. The method of claim 24, wherein said dye molecule is an AlexaFluor647 dye molecule.

28. The method of claim 24, wherein said fluorescent moiety comprises a plurality of AlexaFluor 647 molecules.

29. The method of claim 28, wherein said plurality of AlexaFluor 647 molecules comprises about 2-4 AlexaFluor 647 molecules.

30. The method of claim 14, wherein said fluorescent moiety is a quantum dot.

31. The method of claim 14 further comprising measuring the concentration of said protein in the sample.

32. The method of claim 14 wherein said detecting the presence or absence of said label comprises:

(i) passing a portion of said sample through an interrogation space; and

(ii) subjecting said interrogation space to exposure to electromagnetic radiation, said electromagnetic radiation being sufficient to stimulate said fluorescent moiety to emit photons, if said label is present; and

(iii) detecting photons emitted during said exposure of step (ii).

33. The method of claim 32, further comprising determining a background photon level in said interrogation space, wherein said background level represents the average photon emission of the interrogation space when it is subjected to electromagnetic radiation in the same manner as in step (ii), but without label in the interrogation space.

34. The method of claim 33 further comprising comparing the amount of photons detected in step (iii) to a threshold photon level, wherein said threshold photon level is a function of said background photon level, wherein an amount of photons detected in step (iii) greater that the threshold level indicates the presence of said label, and an amount of photons detected in step (iii) equal to or less than the threshold level indicates the absence of said label.

35. A method for determining the presence or absence of a biological state in a subject, comprising:

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a) performing an immunoassay on a sample from said subject, wherein said immunoassay comprises binding a plurality of labels for a marker to a plurality of molecules of said marker in said sample, wherein said label is specific to said marker and wherein one label binds to one molecule of marker, and wherein said label comprises an antibody attached to a fluorescent moiety that is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules;

b) detecting said labels wherein said detecting comprises detecting single labels with a single molecule detector; and

c) determining a concentration for said marker in said sample based on the number of labels detected in step b).

36. The method of claim 35 further comprising comparing said concentration obtained in step b) to a concentration or range of concentrations for said marker that are known to be indicative of the presence or absence of said biological state.

37. A label for detecting a biological molecule comprising a binding partner for said biological molecule that is attached to a fluorescent moiety, wherein said fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules, wherein said moiety comprises about 2 to 4 fluorescent entities.

38. The label of claim 37 wherein the biological molecule is a protein or a small molecule.

39. The label of claim 38 wherein the biological molecule is a protein.

40. The label of claim 37 wherein the fluorescent entities comprise fluorescent dye molecules.

41. The label of claim 37 wherein the fluorescent dye molecules comprise at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance.

42. The label of claim 40 wherein said dye molecules are AlexFluor molecules selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700.

43. The label of claim 42 wherein said dye molecules are AlexaFluor647 dye molecules.

44. The label of claim 37 wherein said binding partner is an antibody.

45. The labeled protein of claim 37, wherein said dye molecules comprise a first type and a second type of dye molecules.

46. The labeled protein of claim 45, wherein said first type and second type of dye molecules have different emission spectra.

47. The labeled protein of claim 46, wherein the ratio of the number of first type to second type of dye molecule is about 3:1, 2: 1, 1:1, 1:2, or 1:3.

48. An analyzer for determining the concentration of species in a sample, wherein the analyzer is capable of determining said concentration over a dynamic range of concentrations of at least about 10^5.

49. The analyzer of claim 48 wherein said dynamic range is from about 1 femtomolar to about 100 picomolar.

50. A method of establishing a marker for a biological state, comprising

i) establishing a range of concentrations for said marker in biological samples obtained from a first population, wherein said establishing comprises measuring the concentrations of said marker in said biological samples by detecting single molecules of said marker.

51. The method of claim 50 wherein said first population is a population that does not exhibit said biological state.

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52. The method of claim 50 further comprising establishing a range of range of levels for said marker in biological samples obtained from a second population, wherein the members of said second population exhibit the biological state, and wherein said establishing comprises measuring the levels of said marker in said biological samples by detecting single molecules of said marker.

53. The method of claim 52 wherein said first and second populations are different.

54. The method of claim 52 wherein at least one member of said second population is a member of said first population, or at least one member of said first population is a member of said second population.

55. The method of claim 54 wherein substantially all the members of the second population are members of the first population who have developed the biological state.

56. The method of claim 50 wherein said detecting of single molecules of said marker is performed using a method with a limit of detection for said marker of less than about 1000, 100, 50, 20, 10, 5, 1, or 0.5 femtomolar of said marker in said samples.

57. The method of claim 50 wherein said biological state is a phenotypic states; a condition affecting the organism; a state of development; age; health; pathology; disease; disease process; disease staging; infection; toxicity; or response to chemical, environmental, or drug factors (such as drug response phenotyping, drug toxicity phenotyping, or drug effectiveness phenotyping).

58. The method of claim 57 wherein said biological state is a pathology.

59. The method of claim 50 wherein the marker is a polypeptide or small molecule.

60. The method of claim 57 wherein said state is a disease state.

61. The method of claim 60 wherein said disease state is selected from the group consisting of cancer, cardiovascular disease, inflammatory disease, autoimmune disease, neurological disease, infectious disease and pregnancy related disorders

62. The method of claim 61 wherein said state is a disease stage state.

63. The method of claim 62 wherein said disease stage state is a cancer disease stage state.

64. The method of claim 57 wherein the state is a treatment response state.

65. The method of claim 64 wherein the treatment is a drug treatment.

66. The method of claim 65 wherein said response is selected from the group consisting of a therapeutic effect and a side effect.

67. The method of claim 66 wherein the response is a therapeutic effect.

68. The method of claim 66 wherein the response is a side effect.

69. The method of claim 68 wherein said side effect is an adverse effect.

70. The method of claim 50 wherein the detecting comprises labeling said marker with a label comprising a fluorescent moiety capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

71. The method of claim 70 wherein said fluorescent moiety comprises a molecule that comprises at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. group.

72. The method of claim 70 wherein said fluorescent moiety comprises a dye selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700.

73.. The method of claim 70wherein said fluorescent moiety comprises AlexaFluor 647.

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74. The method of claim 70 wherein said label further comprises a binding partner for said marker.

75. The method of claim 74 wherein said binding partner comprises an antibody specific for said marker.

76. The method of claim 75 wherein said antibody comprises a polyclonal antibody.

77. The method of claim 75 wherein said antibody is a monoclonal antibody.

78 The method of claim 50 further comprising establishing a threshold level for said marker based on said first and second ranges, wherein the presence of said marker in a biological sample from an individual at a level above said threshold level indicates a substantially increased probability of the presence of the biological state in said individual.

79. A method for detecting the presence or absence of a biological state of an organism, comprising

i) measuring the concentration of a marker in a biological sample from said organism, wherein said marker is a marker established by the method of claim 50; and

ii) determining the presence of absence of said biological state based on said concentration of said marker in said organism.

80. A method for detecting the presence or absence of a biological state in an organism, comprising

i) measuring the concentrations of a marker in a plurality of biological samples from said organism, wherein said marker is a marker established by the method of claim 50; and

ii) determining the presence of absence of said biological state based on said concentrations of said marker in said plurality of samples.

81. The method of claim 80 wherein said samples are of different types.

82. The method of claim 81 wherein said determining is based on a comparison of the concentrations of said marker in said different types of samples.

83. The method of claim 80 wherein the samples are of the same type, and wherein the samples are taken at intervals.

84. The method of claim 50 wherein the biological sample is blood, plasma, or serum.

85. A business method comprising: identifying one or more markers using the system of claim 1 that is associated with a biological state of an organism; and commercializing said one or more markers in a diagnostic to detect the presence or absence of said biological state in said organism.

86. The business method of claim 85 wherein said one or more markers are polypeptides or small molecules.

87. The business method of claim 85 wherein said one or more markers are new chemical entities.

88. The business method of claim 85 wherein said business method further comprises performing a diagnostic service for fee.

89. The business method of claim 85 wherein said business method further comprises selling a diagnostic product based on said one or more biomarkers.

90. A business method comprising: identifying one or more markers using a system of claim 50 1 that is associated with a biological state of an organism; and providing a diagnostic service of determining if said organism has or does not have said biological state.

91. The business method of claim 90 wherein said diagnostic service is provided by a CLIA approved laboratory.

92. The business method of claim 91 wherein said diagnostic service is provided to a health care provider, insurer or patient.

93. A label for the detection of a marker, wherein the label comprises a binding partner for the marker and a fluorescent moiety, wherein the fluorescent moiety is capable of emitting at least about 200 photons when simulated by a laser emitting light at the excitation wavelength of the moiety, wherein the laser is focused on a spot not less

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than about 5 microns in diameter that contains the moiety, and wherein the total energy directed at the spot by the laser is no more than about 3 microJoules.

94. The label of claim 93 wherein the fluorescent moiety comprises a fluorescent molecule.

95. The label of claim 94 wherein the fluorescent moiety comprises a plurality of fluorescent molecules.

96. The label of claim 95 wherein the label comprises about 2-10, 2-8, 2-6, 2-4, 3-10, 3-8, 3-6 fluorescent molecules.

97. The label of claim 96 wherein the label comprises about 2-4 fluorescent molecules.

98. The label of claim 97wherein the fluorescent dye molecules comprise at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance.

99. The label of claim 97 wherein the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700.

1001. The label of claim 9 wherein the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 680 or AlexaFluor 700.

101.. The label of claim 93 wherein the binding partner comprises an antibody.

102. The label of claim 101 wherein said antibody is a monoclonal antibody.

103. The label of claim 102wherein said antibody is a polyclonal antibody.

104. The label of claim 101 wherein said antibody is specific for a marker of inflammation.

105. The label of claim 104 wherein said antibody is specific for a marker selected from the group consisting of chemokines, growth factors, proteases and inhibitors, cell adhesion molecules, stem cell factors, signal transduction and apoptosis molecules, neurotrophic factors, and developmental proteins.

106. The label of claim 105 wherein the antibody is specific for a growth factor.

107. The label of claim 101 wherein the antibody is specific for a cytokine.

108. The label of claim 108 wherein the cytokine

109. The label of claim 101 wherein the fluorescent moiety comprises a fluorescent molecule.

110. The label of claim 110 wherein the fluorescent moiety comprises a plurality of fluorescent molecules.

111. The label of claim 111wherein the label comprises about 2-10, 2-8, 2-6, 2-4, 3-10, 3-8, or 3-6 fluorescent molecules.

112. The label of claim 112wherein the label comprises about 2-4 fluorescent molecules.

113. The label of claim 112 wherein the fluorescent dye molecules comprise at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance.

114. The label of claim 112 wherein the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680 or AlexaFluor 700

115. The label of claim 101 wherein the fluorescent molecules are selected from the group consisting of AlexaFluor 488, AlexaFluor 532, AlexaFluor 680 or AlexaFluor 700.

116. The label of claim 101 wherein the fluorescent molecules are AlexaFluor 647 molecules.

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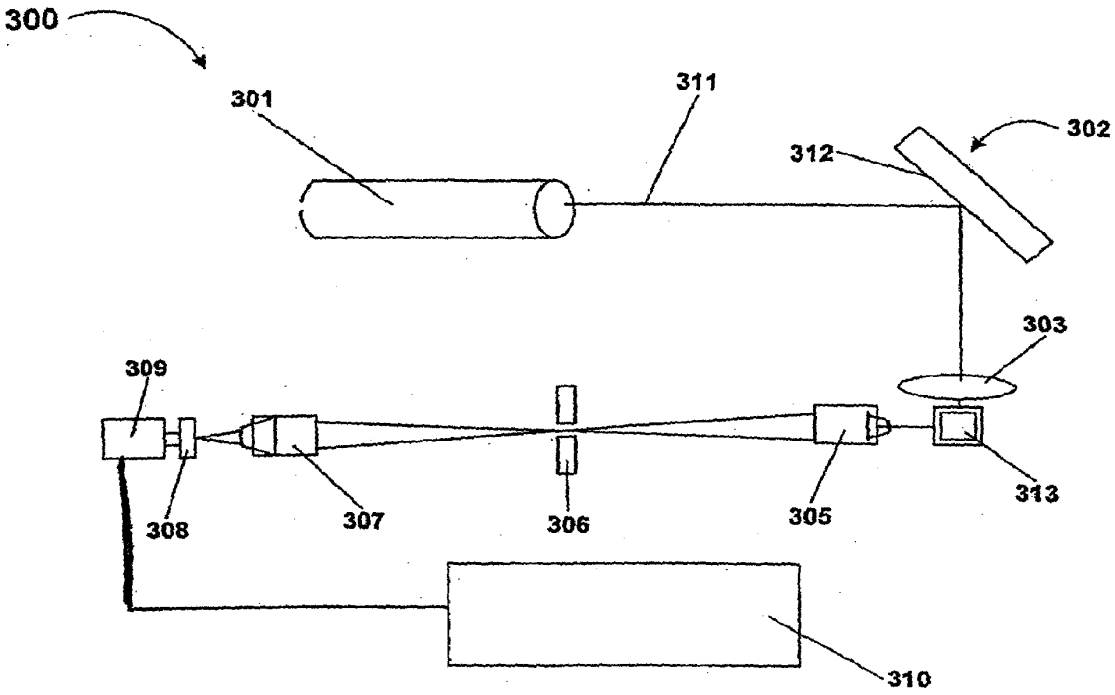


Figure 1A

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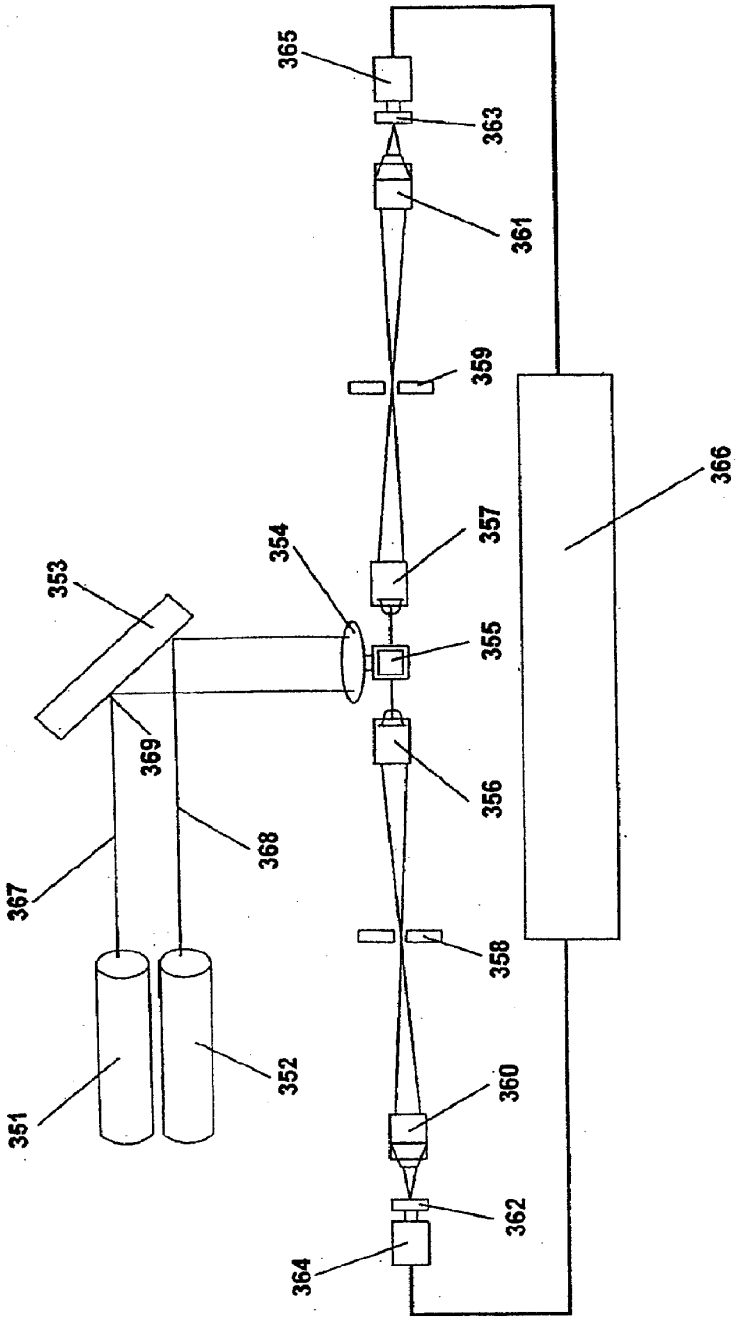


Figure 1B

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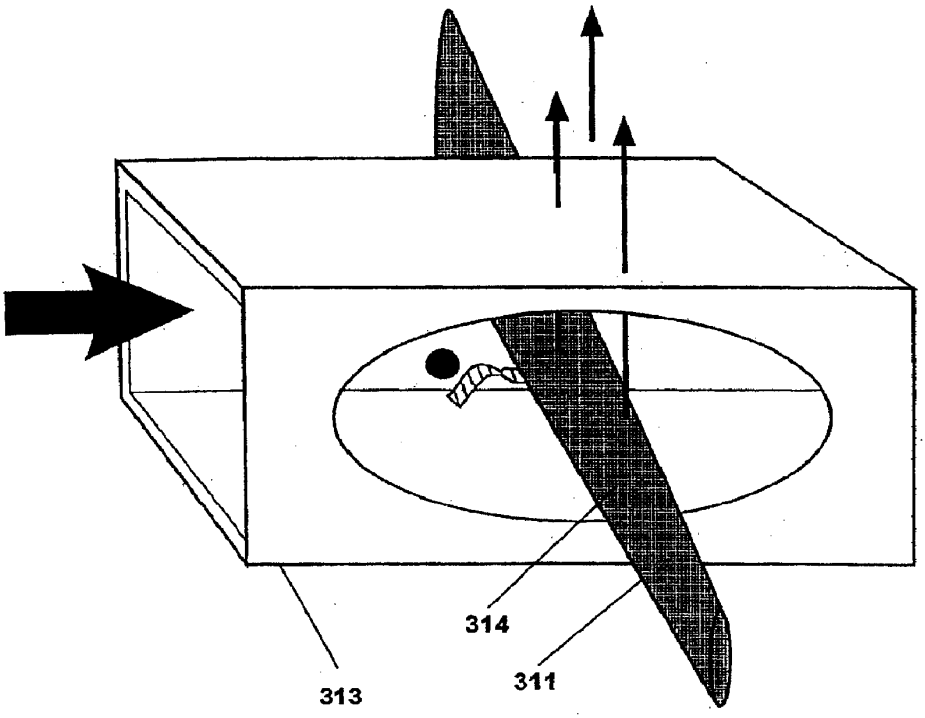


Figure 2A

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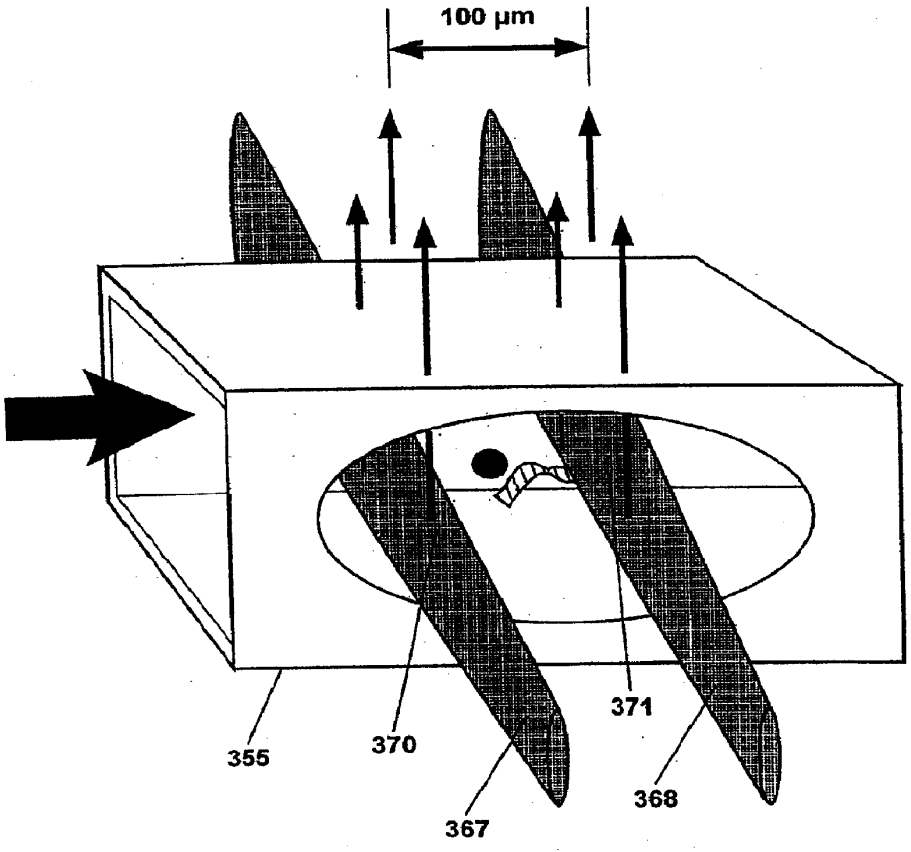


Figure 2B

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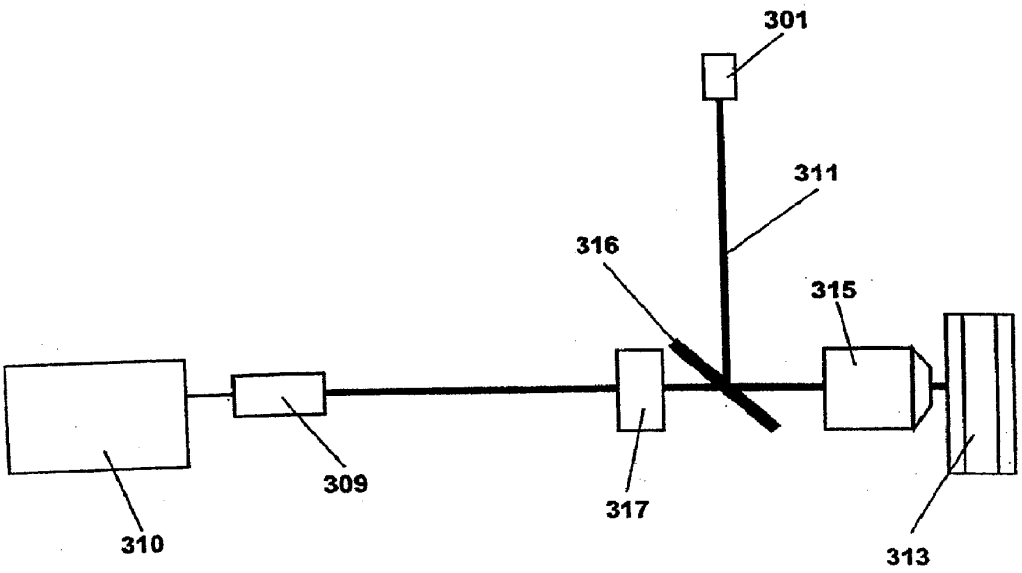


Figure 3A

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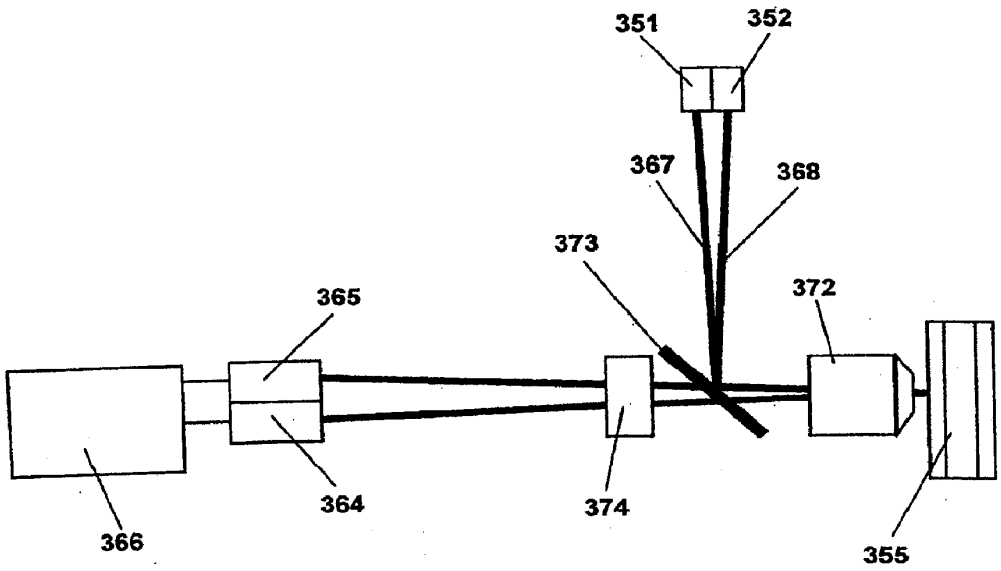


Figure 3B

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2158

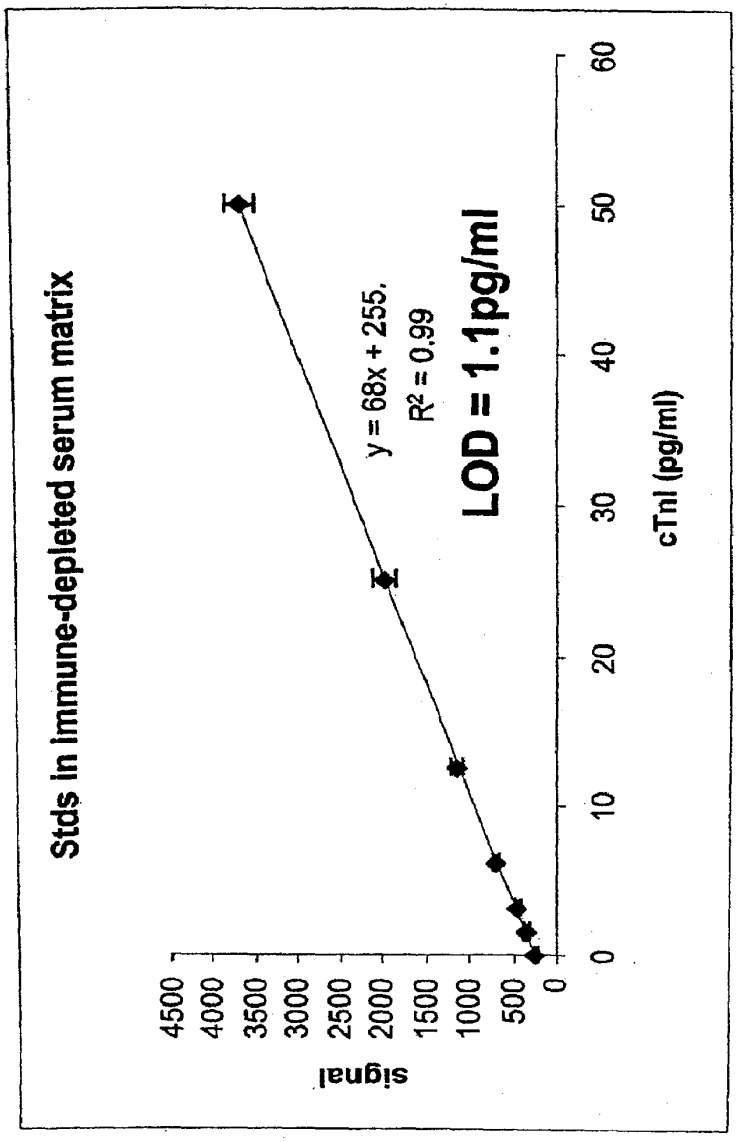


Figure 4

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2159

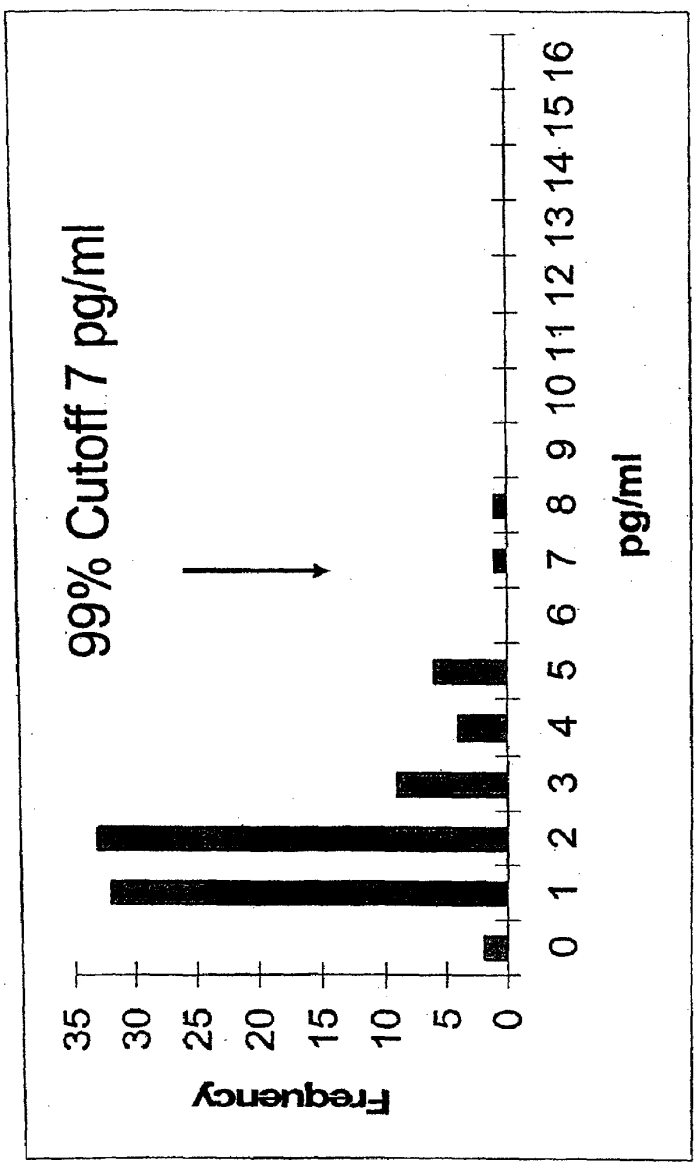


Figure 5

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2160

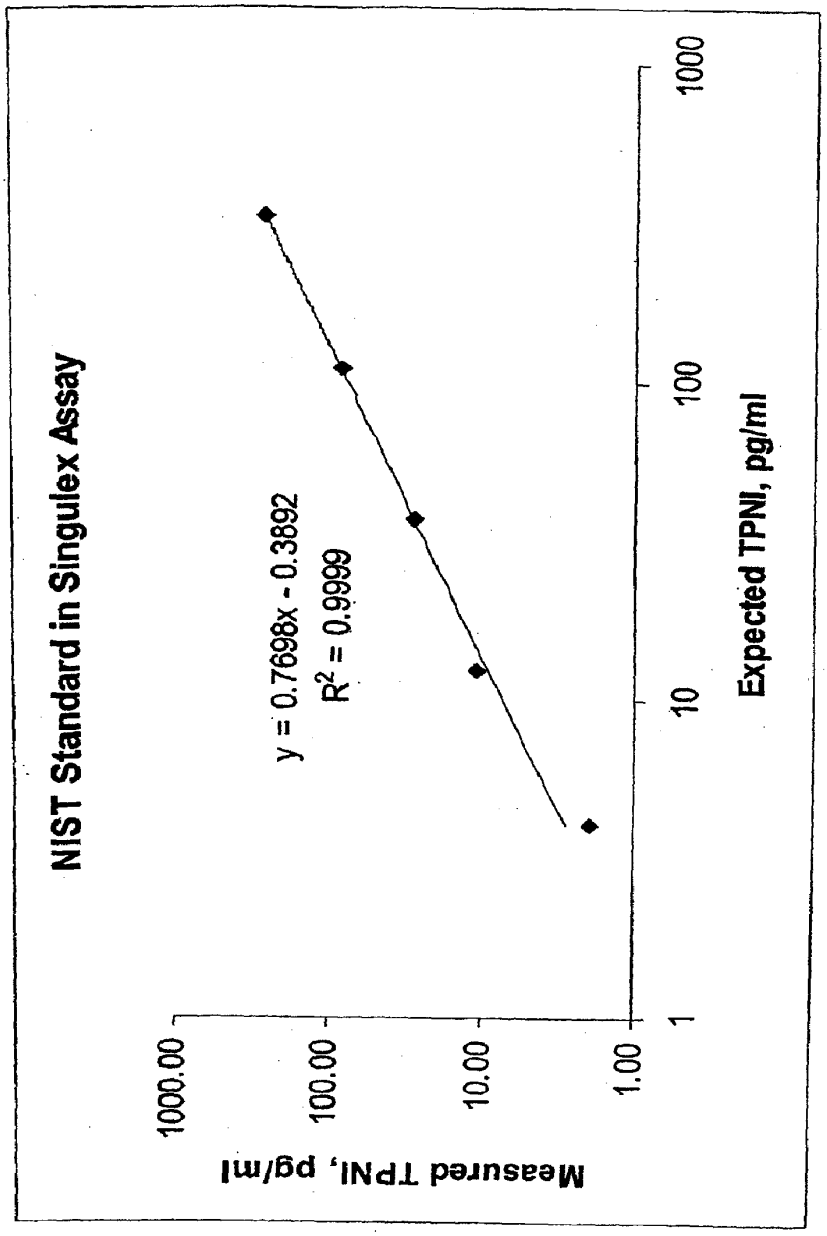


Figure 6

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2161

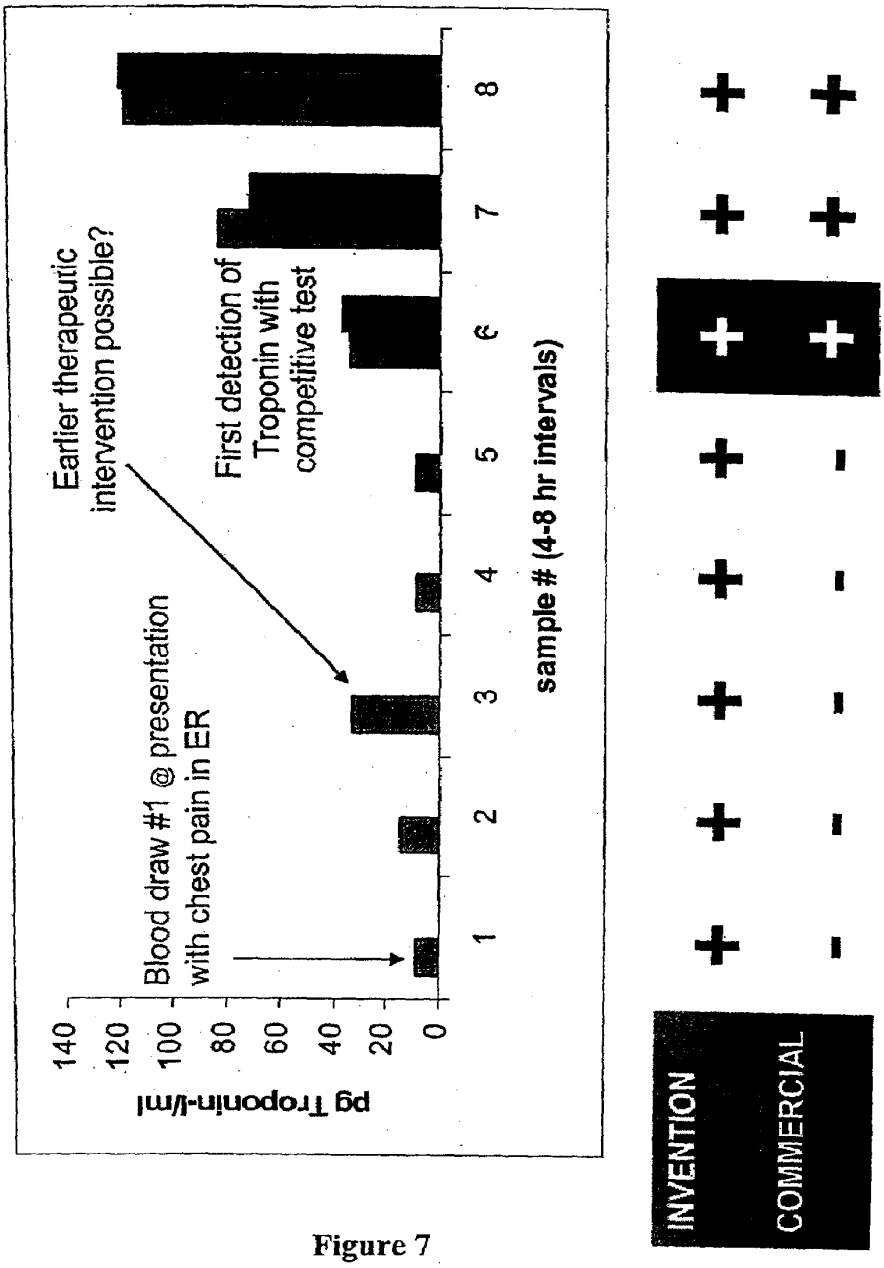


Figure 7

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2162

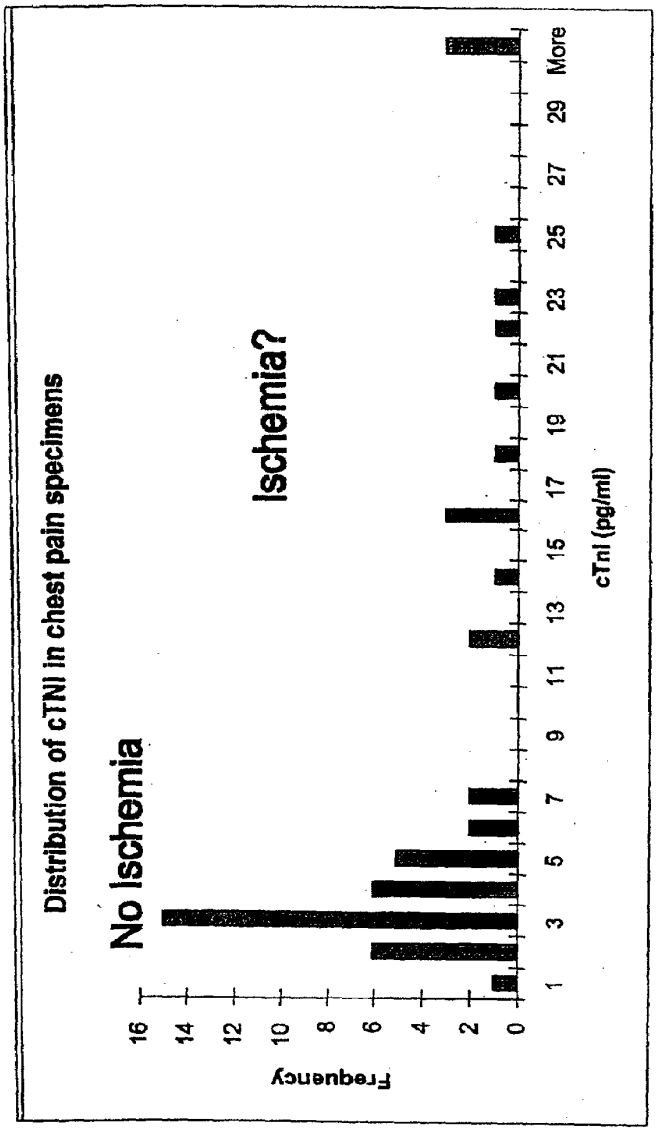


Figure 8

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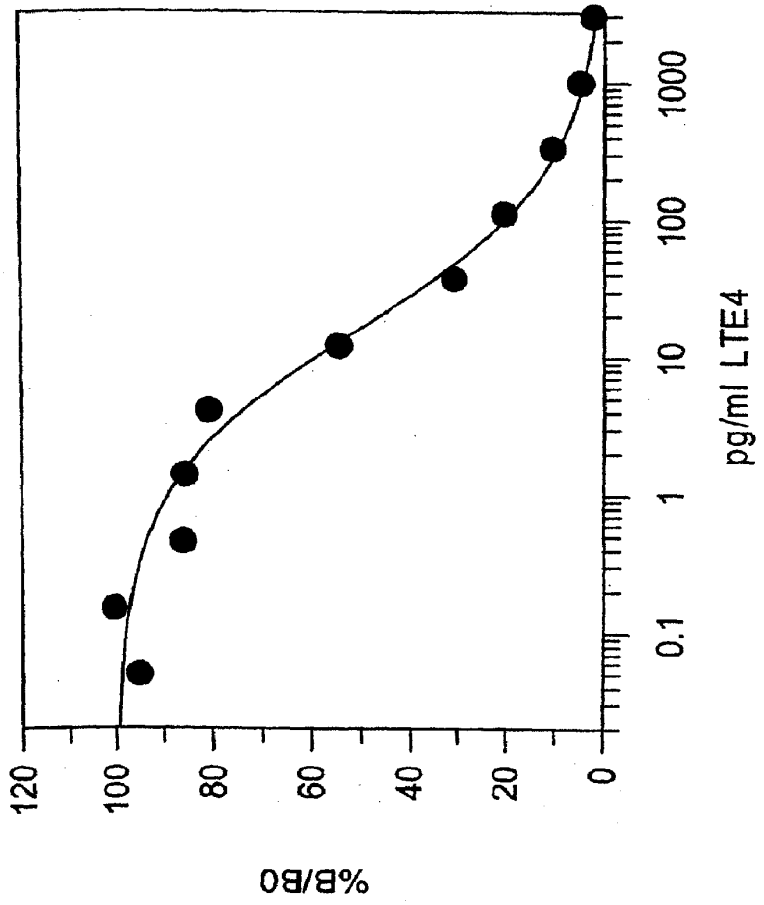


Figure 9

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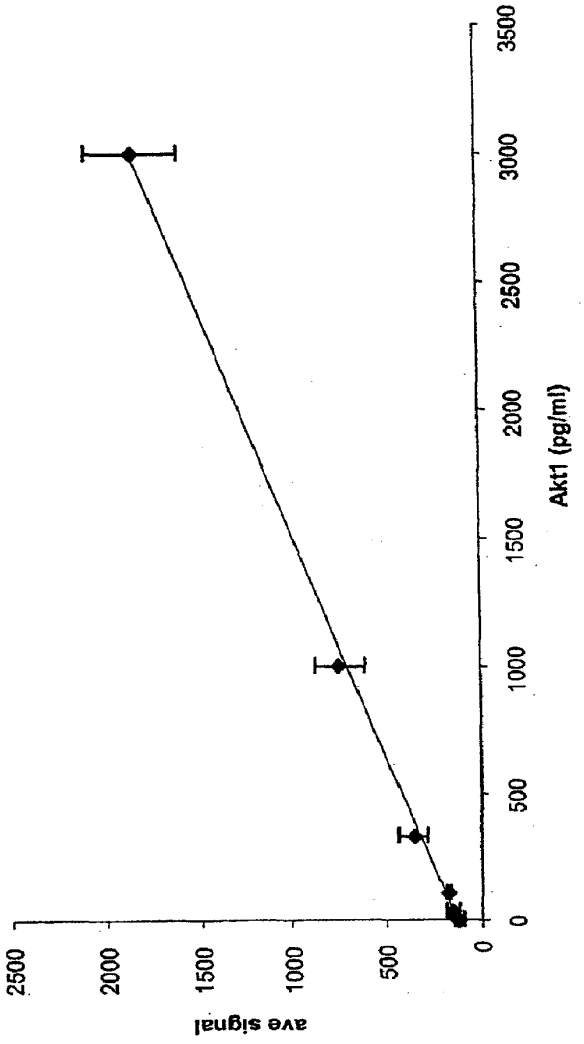


Figure 10

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2165

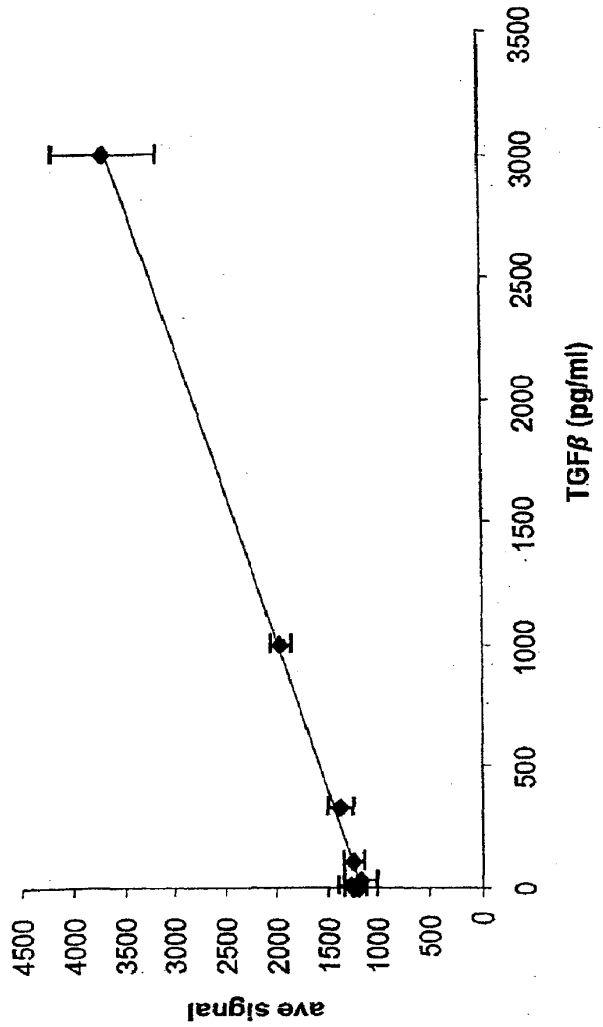


Figure 11

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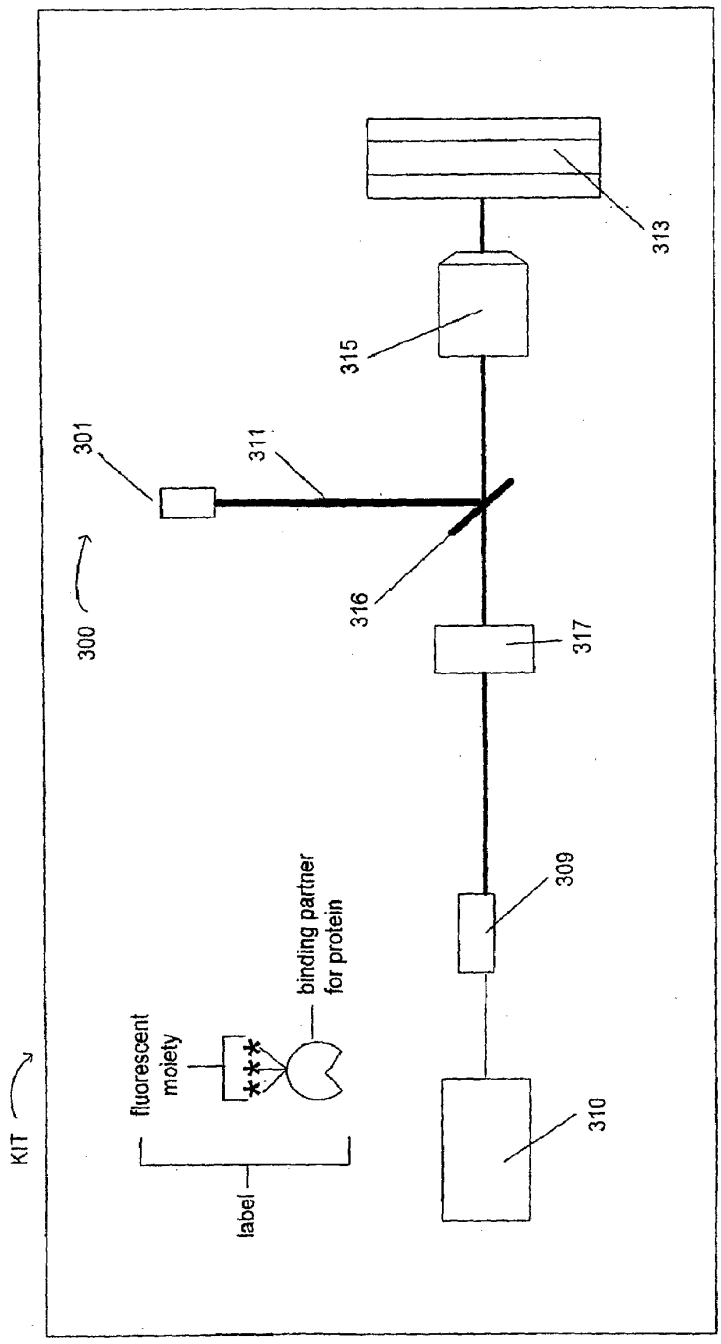


Figure 12

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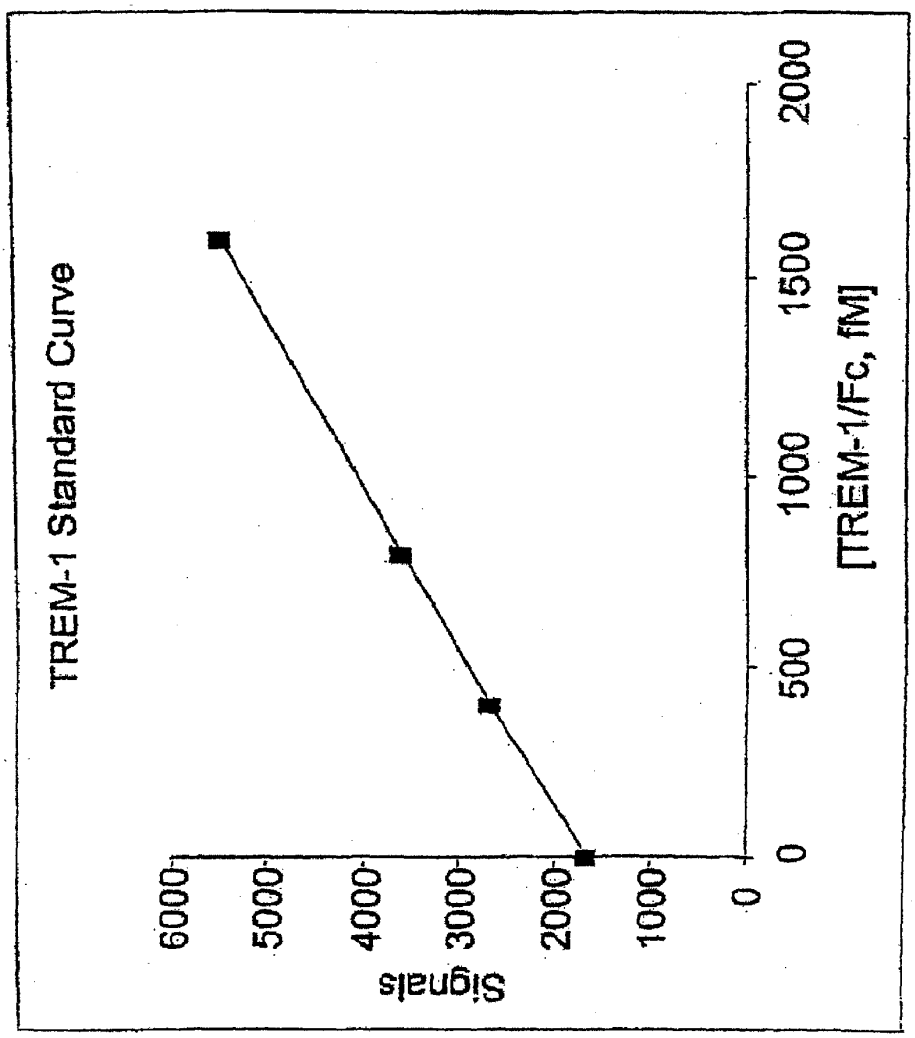


Figure 13

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2168

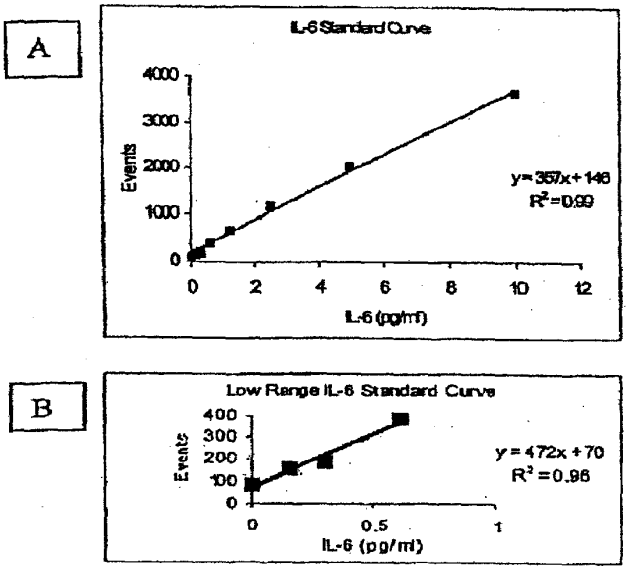
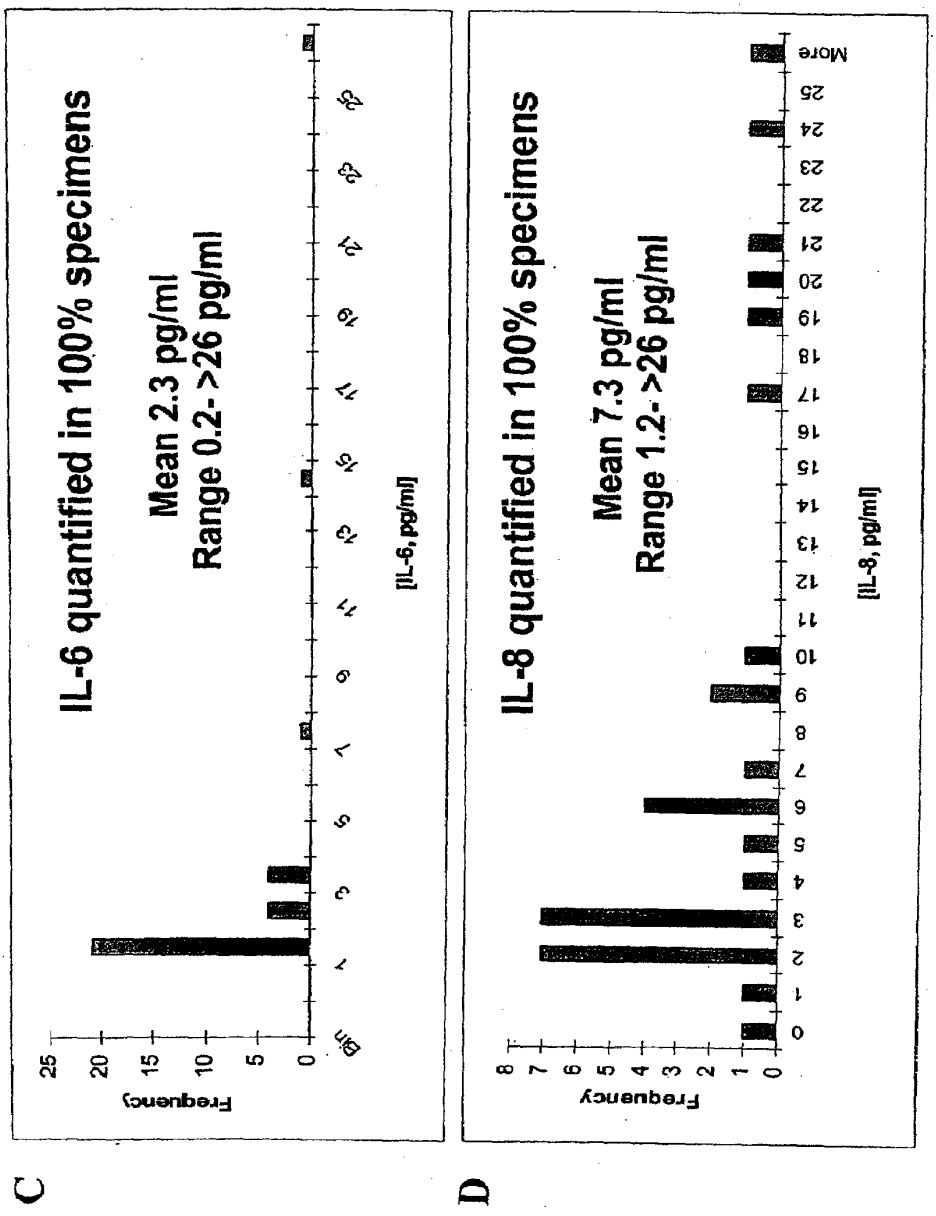


Figure 14 A-B

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C D

Figure 14 C-D

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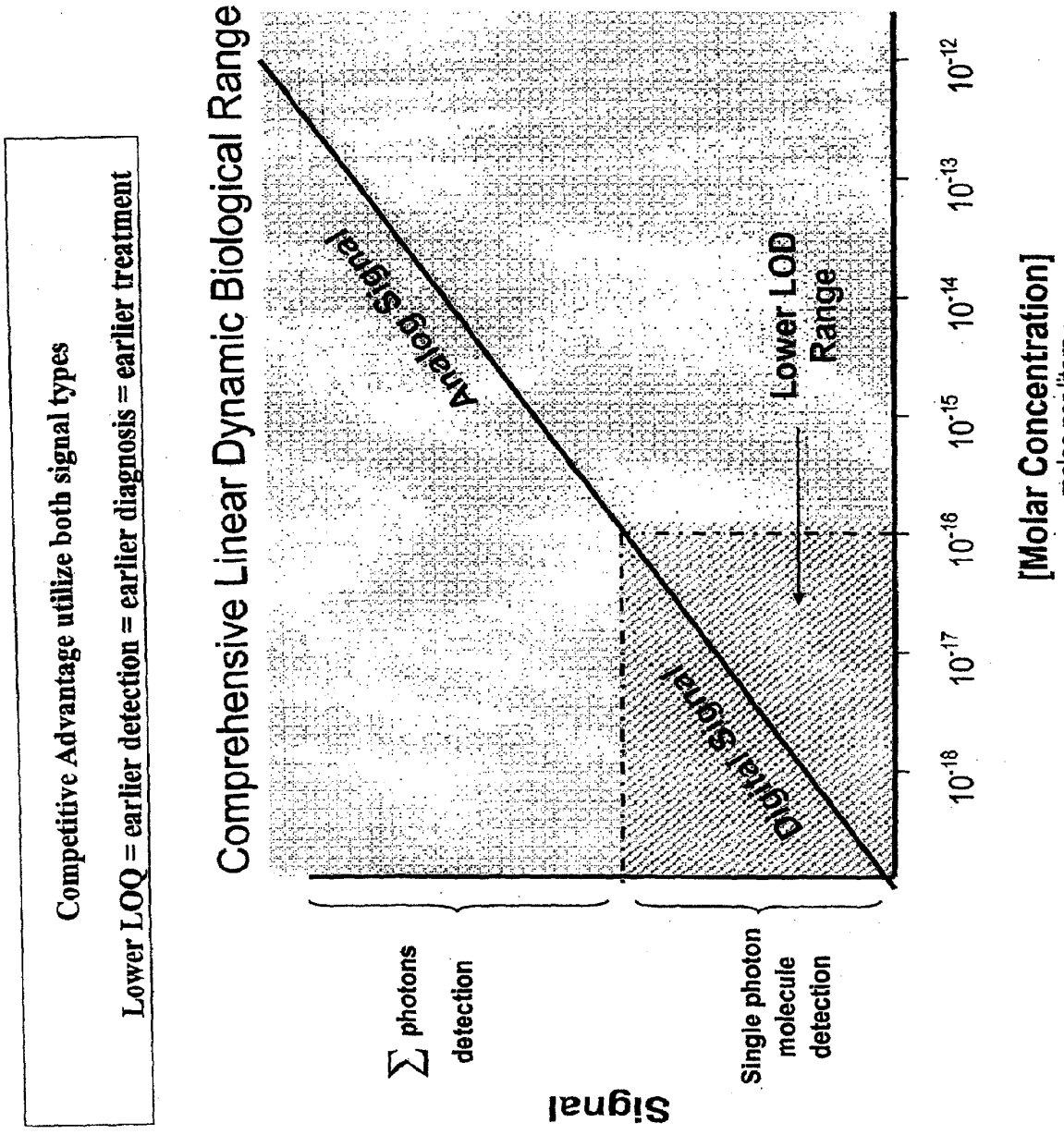


Figure 14 E

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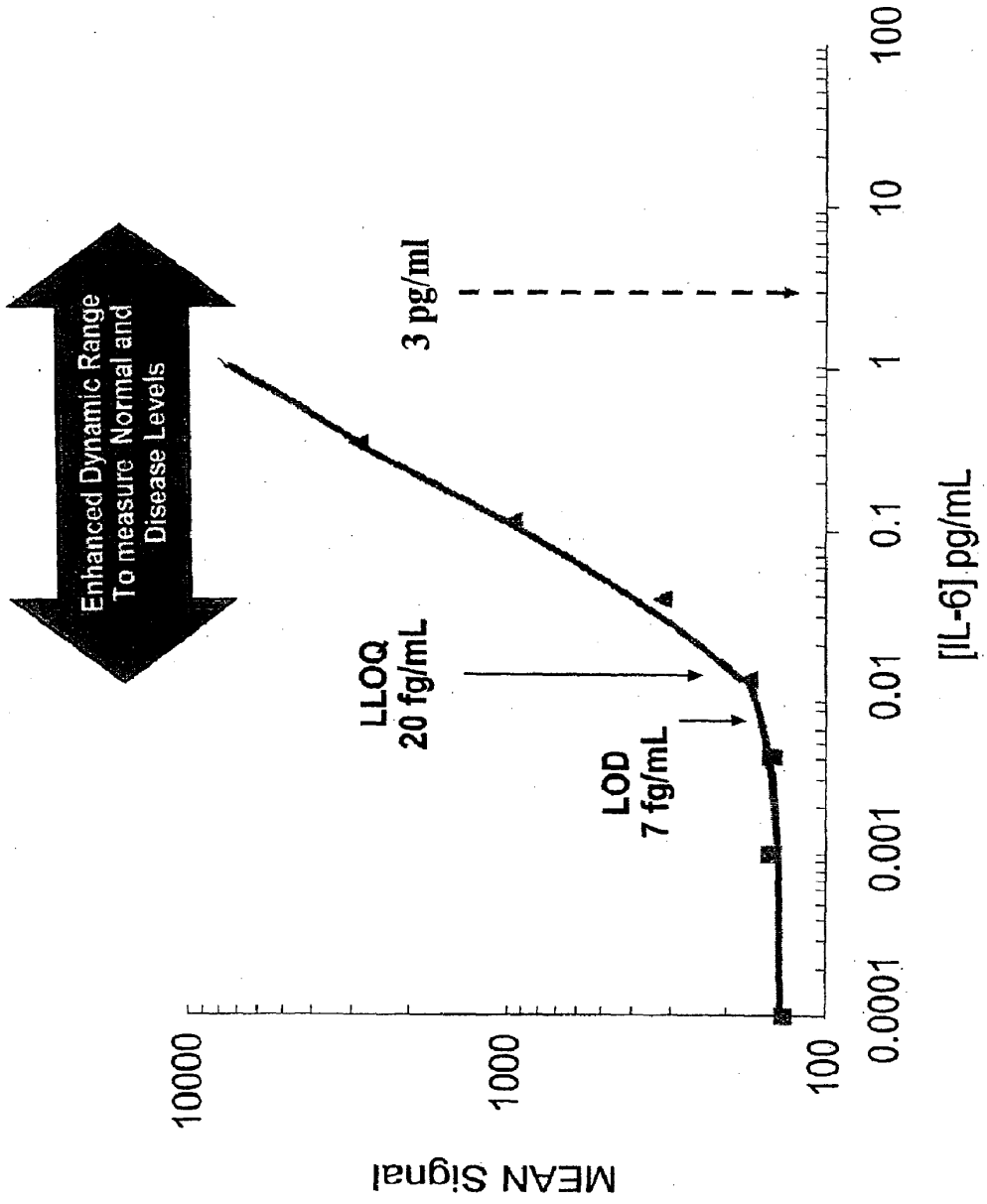
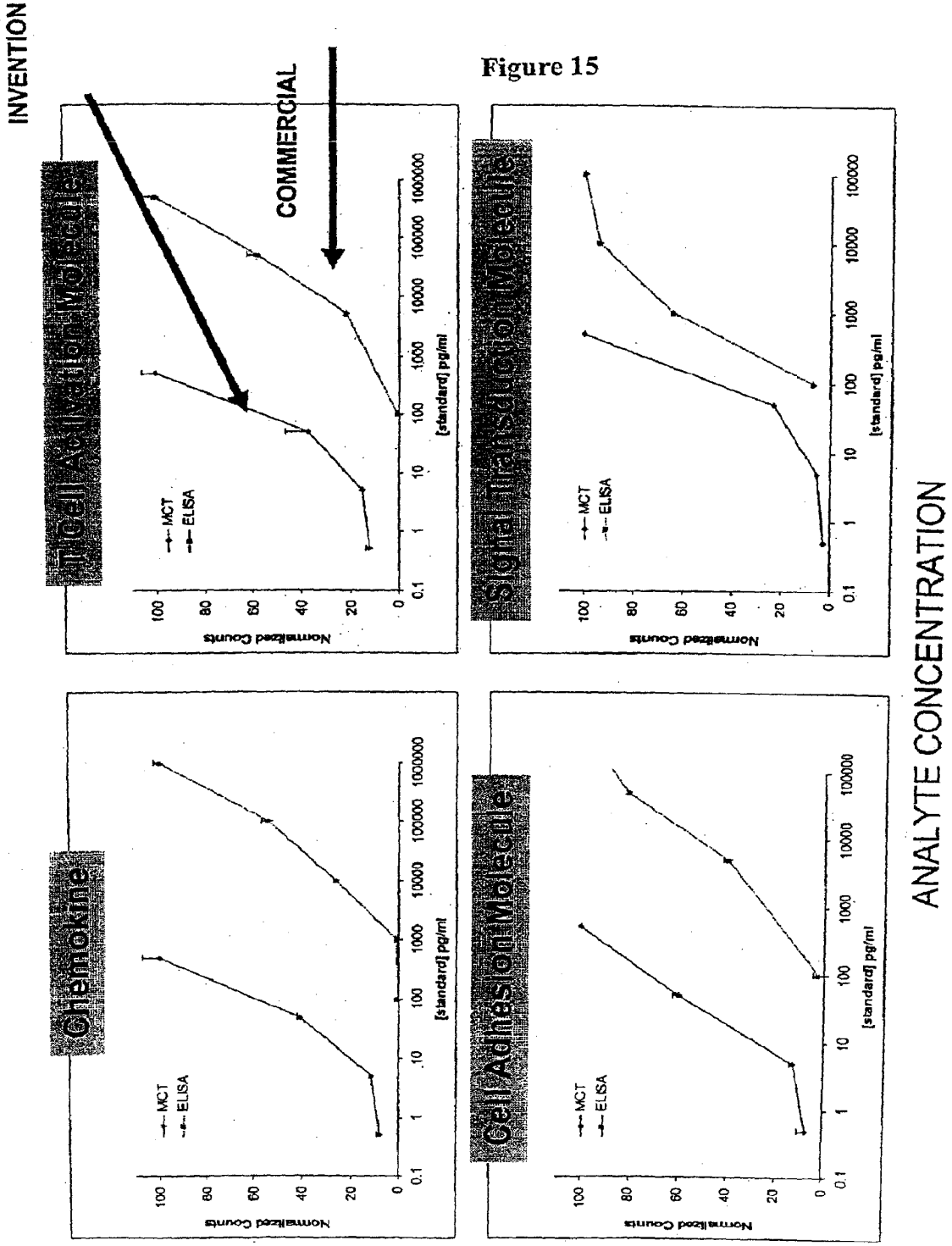


Figure 14 F

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Figure 15



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Published:
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(54) Title: METHODS FOR DETERMINING THE CONCENTRATION OF AN ANALYTE IN SOLUTION.

(57) Abstract: Disclosed is a method for measuring the concentration of an analyte or analytes in a solution. Although the methods can be conducted using a number of different assay formats, in one embodiment, the assays are conducted in reaction vessels defined, at least in part, by the distal ends of fiber optic strands.

WO 2009/029073 A1

Methods for Determining the Concentration of an Analyte in Solution

Government Rights

[001] The United States government may have certain rights in this invention pursuant to Contract No. N00014-01-1 awarded by the Department of Defense, Defense Advanced Research Projects Agency (DARPA) Office of Naval Research.

Background

[002] Methods that implement high-sensitivity and low-level analyte detection in conjunction with rapid and reproducible experimental protocols are the cornerstone of modern analytical measurements. Currently, most known techniques for quantifying low levels of analyte in a sample matrix use amplification procedures to increase the number of reporter molecules and thereby provide a measurable signal. These known processes include enzyme-linked immunosorbent assays (ELISA) for amplifying the signal in antibody-based assays, as well as the polymerase chain reaction (PCR) for amplifying target DNA strands in DNA-based assays. A more sensitive but indirect protein target amplification technique, called immuno-PCR (*see* Sano, T.; Smith, C. L.; Cantor, C. R. *Science* 1992, *258*, 120-122), makes use of oligonucleotide markers, which can subsequently be amplified using PCR and detected using a DNA assay (*see* Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884-1886; Niemeyer, C. M.; Adler, M.; Pignataro, B.; Lenhert, S.; Gao, S.; Chi, L. F.; Fuchs, H.; Blohm, D. *Nucleic Acids Research* **1999**, *27*, 4553-4561; and Zhou, H.; Fisher, R. J.; Papas, T. S. *Nucleic Acids Research* **1993**, *21*, 6038-6039). While the immuno-PCR method permits ultra low-level protein detection, it is a complex assay procedure, and can be prone to false-positive signal generation (*see* Niemeyer, C. M.; Adler, M.; Wacker, R. *Trends in Biotechnology* **2005**, *23*, 208-216).

[003] One disadvantage of known methods for accurately quantifying the concentration of a particular analyte in solution is that they are all based on ensemble responses in which many analyte molecules give rise to the measured signal. Most detection schemes require that a large number of molecules are present in the ensemble for the aggregate signal to be above the detection threshold. This disadvantage limits the sensitivity of most detection techniques and the dynamic range (i.e., the range of concentrations that can be detected).

[004] Therefore, there is a need in the art for an improved method and system of analyte detection. Specifically methods that detect and measure individual molecules rather than an ensemble of molecules would improve the sensitivity, dynamic range and accuracy of analyte

detection. The invention described here divides the sample being analyzed into small samples in which only one or zero analyte molecules are present statistically. These analyte molecules are then detected and counted in a digital fashion to determine the concentration of analyte.

Brief Summary of the Invention

[005] The invention relates to methods, systems and devices for measuring the concentration of an analyte or analytes in a fluid sample. The methods can be performed using a variety of assay platforms, reagents, detectable labels, reaction conditions, and detection systems which are described in detail herein.

[006] In one aspect, the invention provides a method of determining the concentration of an analyte in a fluid sample. The method comprises the steps of: (a) partitioning at least a portion of the analyte molecules in the fluid sample across a plurality of reaction vessels so that a statistically significant fraction of the reaction vessels contain analyte and a statistically significant fraction of the reaction vessels contain no analyte; (b) determining the presence or absence of the analyte in each reaction vessel to identify the number of reaction vessels that contain analyte and/or to identify the number of reaction vessels that contain no analyte; and (c) determining the concentration of the analyte in the fluid sample from the number of reaction vessels that contain the analyte.

[007] In this method, at least 95%, optionally 90%, optionally 80%, optionally 40%, optionally 5%, of the reaction vessels contain no analyte. Depending upon the reaction conditions chosen and the number of wells that contain one or more molecules of analyte, the concentration of the analyte in the fluid sample can be determined by either Poisson distribution analysis or Gaussian distribution analysis of the number of reaction vessels that contain the analyte.

[008] The reaction vessels can be adapted to capture the analyte of interest. For example, depending upon the assay format chosen, the reaction vessel can comprise a microwell and an optional sealing component that connects and seals fluid within the microwell. Accordingly, a capture component for capturing the analyte can be immobilized on a sample contacting surface of the microwell or a sample contacting surface of the sealing component. Alternatively, the capture component can be immobilized on the surface of a particle disposed within the reaction vessel.

[009] The methods preferably are performed using at least 1000 reaction vessels. More specifically, under certain circumstances the methods are performed simultaneously using from about 10,000 to about 200,000 different reaction vessels, more preferably from about 50,000 to about 100,000 different reaction vessels. In addition, in certain embodiments, at least a portion of the reaction vessel is defined by a distal end of an optical fiber. As a result, light from a light source, for example, a laser or lamp, can be transmitted along the optical fiber to the reaction vessel located at the distal end of the optical fiber. Thereupon, depending up the label chosen for a given assay, photoexcitation of the label produces a signal, which then can be detected by a suitable detector.

[010] While multiple embodiments are disclosed, still other embodiments of the present invention will become apparent to those skilled in the art from the following detailed description, which shows and describes illustrative embodiments of the invention. As will be realized, the invention is capable of modifications in various obvious aspects, all without departing from the spirit and scope of the present invention. Accordingly, the drawings and detailed description are to be regarded as illustrative in nature and not restrictive.

Brief Description of the Drawings

[011] FIGS. 1a, 1b, and 1c are side view cross-section schematics representing etched fiber optica bundle that form an array of microwells, according to one embodiment of the present invention. FIGS. 1e, 1f, and 1g are side view cross-section schematics depicting the localization of a capture component within a microwell.

[012] FIGS. 2a, 2b, and 2c are side view cross-section schematics representing a sandwich assay, according to one embodiment of the present invention.

[013] FIGS. 3a and 3b are photographs depicting Streptavidin Alexa Fluor 568® binding to (a) an unpolished biotin-modified array of microwells at the distal end of a fiber optic bundle; and (b) a polished biotinmodified array of microwells, according to one embodiment of the present invention.

[014] FIGS. 4a, 4b, 4c, 4d, 4e, and 4f are photographs depicting experiments according to one embodiment of the present invention in which β-galactosidase hydrolyzes RDG to form resorufin. More specifically, each of these figures depicts a different sample having a different concentration of SβG. The concentrations were: (a) 128 amol, (b) 51 amol, (c) 25 amol, (d) 7.5 amol, and (e) 2.6 amol, and (f) was the control.

[015] FIG. 5 is a chart depicting a log-log plot of the moles of analyte present in a sample with the resulting percentage of active reaction vessels, according to one embodiment of the present invention.

[016] FIG. 6a is a microscopic photograph of an entire of microwells formed at the distal end of a fiber optic bundle and an inset close-up of the array of microwells, according to one embodiment of the present invention.

[017] FIG. 6b is an AFM image of a portion of an etched array of microwells, according to one embodiment of the present invention.

[018] FIGS. 7a, 7b, and 7c depict enclosure of the reaction vessels and evaluation of the sealing component, according to one embodiment. FIG. 7a is a microscopic photograph of a solution of $Ru(bpy)_3Cl_2$ enclosed in the array of reaction vessels. FIG. 7b is a microscopic photograph of a small octagonal portion of the reaction vessels that have been photobleached with UV light. FIG. 7c is a microscopic photograph of FIG. 7b taken 60 minutes later.

[019] FIGS. 8a, 8b, and 8c are microscopic photographs depicting detection of the activity of single molecules of β-galactosidase, according to various embodiments of the present invention. FIG. 8a is a microscopic photograph of a background image of a portion of an array of reaction vessels. FIG. 8b is a microscopic photograph of an image taken of a portion of a 1:5 ratio of enzyme-to-reaction vessel assay. FIG. 8c is a microscopic photograph of a 1:80 ratio of enzyme-to-reaction vessel assay.

[020] FIG. 9 is a schematic representation of an assay format used in Example 3.

[021] FIG. 10 depicts a plot of the concentration of inducible protein 10 (IP-10) present in a test sample with the resulting percentage of active reaction vessels. The plot indicates that the assay was conducted under conditions that permit binary readout detection.

Detailed Description

[022] The present invention relates to methods, systems, and devices for the detection and quantification of an analyte or analytes in a sample. It is contemplated that the methods described herein can be performed in a number of different formats using a variety of different detectable labels, reagents, reaction conditions, and detection systems.

[023] In one aspect, the invention provides a method of determining the concentration of an analyte in a fluid sample to be tested. The method comprises the steps of: (a) partitioning at

least a portion of the analyte molecules in the fluid sample across a plurality of reaction vessels so that a statistically significant fraction of the reaction vessels contain analyte and a statistically significant fraction of the reaction vessels contain no analyte; (b) determining the presence or absence of the analyte in each reaction vessel to identify the number of reaction vessels that contain analyte and/or to identify the number of reaction vessels that contain no analyte; and (c) determining the concentration of the analyte in the fluid sample from the number of reaction vessels that contain the analyte.

[024] In another aspect, the invention provides a method of determining the concentration of an analyte in a fluid sample to be tested. The method comprises the steps of: (a) exposing the fluid sample to a plurality of reaction vessels under conditions so that analyte is captured in a statistically significant fraction of the reaction vessels, wherein each reaction vessel comprises a microwell and an optional sealing component and each reaction vessel defines a binding surface that has a capture component immobilized thereon; (b) determining the presence or absence of the analyte in each reaction vessel so as to identify the number of reaction vessels that contain captured analyte and/or the number of reaction vessels that do not contain captured analyte; and (c) determining the concentration of the analyte in the fluid sample to be tested from the number of reaction vessels that contain and/or do not contain the analyte.

[025] In another aspect, the invention provides a method of determining the concentration of an analyte in a fluid sample to be tested. The method comprises the steps of: (a) partitioning at least a portion of the analyte molecules in the fluid sample into a plurality of reaction vessels, so that, for substantially all of the reaction vessels, each reaction vessel contains either no analyte or a single molecule of the analyte; (b) determining the presence or absence of the analyte in each reaction vessel to provide a number of the reaction vessels that contain the analyte; and (c) determining the concentration of the analyte in the fluid sample from the number of reaction vessels that contain the analyte.

[026] In another aspect, the invention provides a method of determining the concentration of an analyte in a fluid sample to be tested. The method comprises the steps of: (a) partitioning the sample to be tested into a plurality of second, smaller fluid samples of equal volume so that a statistically significant fraction of the second, smaller fluid samples contain either a single molecule of the analyte or no analyte; (b) determining the presence or absence of the analyte in each of the second samples so as to identify the number of second samples that contain the

analyte; and (c) determining the concentration of the analyte in the sample to be tested from the number of second samples that contain the analyte.

[027] It is understood that the foregoing methods can be performed using a number of different assay formats, different reaction conditions, detectable labels and detection systems. In one embodiment, for example, the invention permits the detection and/or quantification of analytes using arrays of micron- to nanoscale-sized reaction vessels. In one approach, the reaction vessels contain capture components that capture the analyte within the reaction vessel. In such an approach, an array of reaction vessels containing an immobilized capture component is contacted with a sample containing at least one analyte. Following capture of the analyte, depending upon what label is used, the presence of analyte can be detected directly or indirectly.

[028] The direct approach can include, for example, the situation where the analyte is an enzyme. In this case, a chromogenic, fluorogenic, or chemoluminescent enzymatic substrate is included in the reaction mixture and the enzymatic activity of the analyte produces a detectable product. The indirect approach can include, for example, the situation where the analyte does not have intrinsic enzymatic activity and a label is permitted to bind, for example, via a secondary binding ligand, to the analyte. For example, a detectable label conjugated to a secondary binding ligand (for example, a capture component different from the one used to capture the analyte) is added to the captured analyte under conditions to permit the labeled secondary binding ligand to bind to the immobilized analyte. Thereafter, the presence of the label can then be determined to provide an indication of whether the analyte is present in the reaction vessel. It is understood that a variety of different labels, as will be discussed in more detail below, may be used in the practice of the invention. To the extent that an enzymatic label is employed, an enzymatic substrate can be added to the reaction vessel whereupon the enzyme converts the substrate into a chromogenic, fluorogenic, or chemoluminescent detectable product to permit the detection of the analyte. The number or percentage of reaction vessels with captured analytes can be used to calculate the amount of analyte in the sample using a binary readout method.

[029] Similarly, it is understood that in certain assay formats, some or all of the reaction vessels can be defined on a planar support rather than within the recesses of an array of microwells. For example, the sample containing analyte is divided into a plurality of second, smaller fluid samples of equal volume. The second fluid samples then are applied as droplets

to the surface of a planar binding surface to create an array of droplets. Each droplet can be applied to a region of the planar binding surface having a capture component immobilized thereon. The samples then are incubated to permit the analyte to bind to the capture component immobilized on the planar binding surface. Afterwards, depending upon the label being employed, the presence of the analyte can be determined directly or indirectly so as to permit the identification of the number of fluid samples that contain the analyte and the number of fluid samples that do not contain the analyte. In certain embodiments, for example, when the label is an enzymatic label, an enzymatic substrate can be added to the surface of the planar binding surface, and the various regions containing the capture component (in the presence or absence of analyte). The planar binding substrate then is sealed against an array of microwells to form an array of reaction vessels to permit the development of a detectable product in each reaction vessel. From the number of reaction vessels containing the analyte in the array of reaction vessels it is possible to determine the concentration of analyte in the test solution.

[030] In one embodiment, the present invention uses an array of micron- to nanoscale-sized reaction vessels specifically functionalized with capture components and capable of capturing analyte molecules. The ability to capture the analyte allows the use of washing steps and indirect assays, as outlined below. In one embodiment, the label used to detect the captured analyte is an enzyme. Once captured in individual reaction vessels, the enzyme catalyzes the production of a sufficient number of chromogenic, fluorogenic, or chemoluminescent product molecules to generate a detectable signal. In accordance with one embodiment relating to samples having low analyte concentrations, only a portion of the reaction vessels capture an analyte molecule, thereby enabling a binary readout of analyte concentration from the array of reaction vessels. The direct enzymatic amplification in the method and system of the present invention allows for direct amplification of a detectable signal.

[031] The present invention allows for detection of low concentrations of analytes, such as proteins. At low concentrations, a statistically significant number of reaction vessels contain zero, one, or several molecules of the target analyte. By observing the presence or absence of a detectable label present in each reaction vessel, a binary readout method can be used to identify the number of reaction vessels that contain one or more molecules of the analyte. As a result, the percentage of reaction vessels occupied by analyte can be used to calculate the original bulk concentration of the analyte in the sample of interest. The following sections discuss exemplary arrays of reaction vessels, capture components, analytes that can be detected by the

invention, detectable labels and signal detection protocols, assay methods, and exemplary uses of the invention.

I. Arrays of Reaction Vessels

[032] The present invention utilizes an array of reaction vessels to carry out steps in an assay utilized to determine the concentration of an analyte of interest. The purpose of the array of reaction vessels is to allow a reaction volume to be partitioned into a plurality of discrete reaction volumes during one or more steps in an assay. By "array" herein is meant a plurality of similar reaction vessels.

[033] The reaction vessels may all have the same volume or may be of differing, but known volumes. The volume of each individual reaction vessel can range from attoliters to nanoliters depending upon the analyte of interest and the expected concentration of that analyte in a solution. In one embodiment, the size of the reaction vessel may be selected such that at the concentration of interest between zero and ten molecules of the analyte of interest would be expected to be found in each reaction vessel. In accordance with one embodiment of the present invention, the reaction vessels have a volume ranging from about 10 attoliters to about 50 picoliters. Alternatively, the reaction vessels range in size from about 1 picoliter to about 50 picoliters. In a further alternative, the reaction vessels range in size from about 1 femtoliter to about 1 picoliter. In a further alternative, the reaction vessels range from about 30 femtoliters to about 60 femtoliters.

[034] The number of reaction vessels in the array will depend on the composition and end use of the array. Arrays containing from about 2 to many billions of reaction vessels can be made by utilizing a variety of techniques and materials. Increasing the number of reaction vessels in the array can be used to increase the dynamic range of an assay or to allow multiple samples or multiple analytes to be assayed in parallel. Generally, the array will comprise between one thousand and one million reaction vessels per sample to be analyzed. Arrays used to simultaneously analyze multiple samples will generally contain between 10 thousand and 10 billion reaction vessels.

[035] The array of reaction vessels may be arranged on a planar structure or in some 3 dimensional arrangement. They may be in a regular design or randomly distributed. A preferred embodiment utilizes a regular pattern of sites on a planar structure such that the sites may be addressed in the X-Y coordinate plane.

[036] The reaction vessels can be formed in a solid material. As will be appreciated by those in the art, the number of possible materials are very large, and include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon™, etc.), polysaccharides, nylon or nitrocellulose, composite materials, ceramics, and plastic resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not appreciably fluoresce.

[037] The reaction vessels can also be formed in a liquid provided that the confining liquid does not mix with the liquid defining the reaction vessel, and that the reaction vessel is stable. Examples of suitable liquids for confining aqueous reactions include, but are not limited to, water-in-oil emulsions, extruded lipid aggregates, stable suspensions of lipids, liquid-crystal aggregates, micelles in water, inverted micelles in oil, and suspensions of cells, bacteria and viruses.

[038] Individual reaction vessels may contain a binding surface. The binding surface may be all or a portion of the interior surface of the reaction vessel or may be on the surface of something that is confined within the reaction vessel such as a bead, or a particle (for example, a micro-particle or a nanoparticle).

[039] In one embodiment, the array of reaction vessels is formed by mating an array of microwells with a sealing component. Microwells are small depressions in a first surface of a supporting material. The microwells may be formed as is generally known in the art using a variety of techniques, including, but not limited to, photolithography, stamping techniques, molding techniques and microetching techniques. As will be appreciated by those in the art, the technique used will depend on the composition and shape of the supporting material. The sealing component has a second surface with the same topology as the first surface that when brought into contact with the first surface creates an array of sealed reaction vessels. Either the first surface or the second surface may be fabricated from a compliant material to aid in sealing. Either or both surfaces may be hydrophobic or contain hydrophobic regions to minimize leakage from the microreactors.

[040] In another embodiment, the array of reaction vessels is formed utilizing a microfluidic device that contains channels and valves that can be closed to isolate discrete reaction volumes.

[041] In one preferred embodiment, an array of reaction vessels is formed creating microwells on the distal end of a fiber optic bundle and utilizing a planar compliant surface as a sealing component. Such an array of reaction vessels can be made as follows. First, an array of microwells are etched into the distal end of a polished fiber optic bundle. The diameter of the optical fibers and the depth of the etch can be varied to create microwells of the desired volume. The etching process creates the microwells in the core material of the individual glass fibers in the bundle such that each well is aligned with a single fiber and isolated from adjacent wells by the slower etching cladding material. One advantage of the fiber optic array format is that it can produce thousands to millions of reaction vessels without the need for complicated microfabrication procedures and provides the ability to observe many reaction vessels simultaneously.

[042] Each microwell is aligned with an optical fiber in the bundle so that the fiber optic bundle can carry both excitation and emission light to and from the wells, enabling remote interrogation of the well contents. Further, an array of optical fibers provides the capability for simultaneous excitation of molecules in adjacent vessels, without signal "cross-talk" between fibers. That is, excitation light transmitted in one fiber does not escape to a neighboring fiber. In one aspect of the present invention, the physical alterations can be made as taught in U.S. Patents 6,023,540, 6,327,410, and 6,858,394, which are each incorporated by reference herein in their entirety. Either the surface of the glass microwells, the surface of the sealing component, or particles can be functionalized to create a binding surface.

[043] Alternatively, the equivalent structures can be fabricated using other methods that do not comprise the ends of an optical fiber bundle. For example, the array may be a spotted, printed or photolithographic substrate known in the art; see for example WO 95/25116; WO 95/35505; PCT US98/09163; U.S. Patent Nos. 5,700,637; 5,807,522 and 5,445,934; and U.S.S.N.s 08/851,203 and 09/187,289, all of which are expressly incorporated by reference.

II. Capture Components

[044] During practice of the invention, the binding surfaces within the reaction vessels (including microwells defined in fiber optical bundles) incorporate at least one capture

component. A capture component (also commonly referred to as a "capture binding ligand," "binding ligand," "capture binding species," or "capture probe") is any molecule, compound, or solid support modification that can be used to probe for, attach, bind or otherwise capture an analyte disposed upon a solid support, such that the analyte is immobilized during the assay. Generally, the capture component allows the attachment of an analyte to a solid support (that is, the surface of the microwells, the sealing component or nanoparticle) for the purposes of detection, quantification, or other analysis.

[045] As will be appreciated by those in the art, the composition of the capture component will depend on the composition of the analyte. Capture components for a wide variety of analytes are known or can be readily found using known techniques. For example, when the analyte is a protein, the capture components include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)) or small molecules. Preferred capture component proteins include peptides. For example, when the analyte is an enzyme, suitable capture components include substrates and inhibitors. Antigen-antibody pairs, receptor-ligands, and carbohydrates and their binding partners are also suitable analyte-capture component pairs. In addition, when the analyte is a single-stranded nucleic acid, the capture component may be a complementary nucleic acid. Similarly, the analyte may be a nucleic acid binding protein and the capture component is either single-stranded or double-stranded nucleic acid; alternatively, the capture component may be a nucleic acid-binding protein when the analyte is a single or double-stranded nucleic acid. Alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptamers" can be developed for capturing virtually any analyte. As will be appreciated by those in the art, any two molecules that will associate may be used, either as an analyte or as the capture component. Similarly, when the analyte is a carbohydrate, suitable capture components include antibodies and lectins. It is understood that there is a wide body of literature relating to the development of capture components based on combinatorial chemistry methods.

[046] Suitable analyte/capture component pairs include, but are not limited to, antibodies/antigens, receptors/ligands, proteins/nucleic acid, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids)/lectins, proteins/proteins, proteins/small molecules; and carbohydrates and their binding partners are also suitable analyte-capture component pairs. These may be wild-type or derivative sequences. According

to one embodiment, the capture components are portions (particularly the extracellular portions) of cell surface receptors that are known to multimerize, such as the growth hormone receptor, glucose transporters (particularly GLUT 4 receptor), and T-cell receptors.

[047] In a preferred embodiment, the capture component is attached to a binding surface (for example, the surface of a microwell) via an "attachment component" (also referred to herein as an "attachment linker"). An "attachment component," as used herein, is defined as any component, functionalization, or modification of the binding surface that results in the attachment of the capture component, and can include bonds and/or linkers. Alternatively, the capture component may utilize a capture extender component. In this embodiment, the capture component comprises a first portion that will bind the analyte and a second portion that can be used for attachment to the binding surface.

[048] This attachment component can also provide a protective layer that screens the binding surface, such as the glass of a fiber optic bundle, from the assay solution. As a result, the attachment component minimizes non-specific attachment of non-target molecules to the binding surface during the assay and detection that could lead to undesired fluorescent wells and false positive signals.

[049] The method of attachment of the capture component to the attachment component will generally be done as is known in the art, and will depend on the composition of the attachment component and the capture component. In general, the capture components are attached to the attachment component through the use of functional groups on each that can then be used for attachment. According to one embodiment, the functional group is a chemical functionality. That is, the binding surface is derivatized such that a chemical functionality is presented at the binding surface. Preferred functional groups for attachment are amino groups, carboxy groups, epoxide groups, maleimide groups, oxo groups and thiol groups. These functional groups can be attached, either directly or through the use of a linker, the combination of which is sometimes referred to herein as a "cross-linker." Linkers are known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical. Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). Preferred linkers include, but are not limited to, alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), with short alkyl groups, esters, amide, amine, epoxy groups and ethylene glycol and derivatives being preferred. Linkers may also be a sulfone group, forming sulfonamide.

[050] According to one embodiment, the functional group is a light-activated functional group. That is, the functional group can be activated by light to attach to the capture component or to the attachment component. One example is the PhotoLink™ technology available from SurModics, Inc. in Eden Prairie, MN.

[051] Alternatively, the attachment component is added without covalently modifying the binding surface. That is, the attachment component can be added to the binding surface by using a molecule that has both a functional group and a group that has binding affinity for the binding surface. Alternatively, the attachment component is any protein capable of binding or sticking to the binding surface. In a further alternative, the attachment component is any molecule capable of binding or sticking to the vessel surface. In one example, the attachment component is serum albumin with free amine groups on its surface. A crosslinker can then be added to attach the amine groups of the albumin to the capture component.

[052] According to one embodiment in which the capture component is a chemical crosslinker, the analyte is captured on the binding surface using chemical crosslinking in the following manner. First, the binding surface is derivatized with a functional group, such as, an amine group. Next, a crosslinker and the analyte are placed in contact with the binding surface such that one end of the crosslinker attaches to the amine group and the analyte attaches to the other end of the crosslinker. In an alternative embodiment described in further detail below in which the analyte is not an enzyme, a label having an enzymatic component can also be attached to the analyte.

[053] In this way, capture components comprising proteins, lectins, nucleic acids, small organic molecules, carbohydrates can be added.

[054] One embodiment utilizes proteinaceous capture components. As is known in the art, any number of techniques may be used to attach a proteinaceous capture component. "Protein" in this context includes proteins, polypeptides, peptides, including, for example, enzymes and antibodies. A wide variety of techniques are known to add moieties to proteins. One preferred method is outlined in U.S. Patent No. 5,620,850, hereby incorporated by reference in its entirety. The attachment of proteins to surfaces is known; see also Heller, Acc. Chem. Res. 23:128 (1990), and related work.

[055] An alternative embodiment utilizes nucleic acids as the capture component, for example for when the analyte is a nucleic acid or a nucleic acid binding protein, or when the nucleic acid serves as an aptamer for binding a protein, as is well known in the art.

[056] According to one embodiment, each binding surface presents a plurality of capture component molecules. The plurality of capture component molecules, in one aspect of the invention, are distributed on the binding surface like a "lawn." Alternatively, the capture components are distributed in any known fashion.

[057] The binding between the capture component and the analyte, in accordance with one embodiment, is specific and the capture component is part of a binding pair. That is, the capture component is a target-specific capture component that specifically binds with or has specificity for the analyte. More specifically, the capture component binds specifically and directly to the analyte. By "specifically bind" or "binding specificity" herein is meant that the capture component binds the analyte with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. For example, the capture component according to one embodiment is an antibody that binds specifically to some portion of the analyte. The antibody, according to one embodiment, can be any antibody capable of binding specifically to an analyte. For example, appropriate antibodies include, but are not limited to, monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies, synthetic antibodies (sometimes referred to as "antibody mimetics"), chimeric antibodies, humanized antibodies, antibody fusions (sometimes referred to as "antibody conjugates"), and fragments of each, respectively.

[058] However, as will be appreciated by those in the art, it is possible to detect analytes using binding that is not highly specific. For example, the systems may use different capture components such as, for example, a panel of different binding ligands, and detection of any particular analyte is via its "signature" of binding to this panel of binding ligands, similar to the manner in which "electronic noses" work. This finds particular utility in the detection of chemical analytes. The binding affinity between analyte and capture component should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding. In some embodiments, for example in the detection of certain biomolecules, the binding constants of the analyte to the capture component will be at least about 10^4-10^6 M^{-1}, with at least about 10^5 to 10^9 M^{-1} being preferred and at least about 10^7 -10^9 M^{-1} being particularly preferred.

[059] According to one embodiment in which the analyte is a cell, including, for example, bacterial cells, the capture component can be an adhesin receptor molecule. In use, the adhesin receptor molecule binds with a surface protein called an adhesin on the extracellular surface of the target cell, thereby immobilizing or capturing the cell. Alternatively, in embodiments in which the analyte is another type of cell (a non-bacterial cell), the capture component is an appropriate cell surface receptor that binds the analyte cell. In a further embodiment in which the analyte is a cell, the capture component is fibronectin. For example, fibronectin can be used when the analyte is a nerve cell.

[060] Alternatively, the capture component is a non-specific capture component. That is, the capture component does not bind specifically to an analyte, but rather binds to a corresponding binding partner associated with or attached to the analyte. For example, the non-specific capture component according to one embodiment is a chemical cross-linker as described above. According to one embodiment, every peptide molecule in a sample of analyte can attach to the chemical cross-linker. In one example of a non-specific capture component according to one embodiment, the capture component is streptavidin, which binds with high affinity to biotin, and thus captures any molecule to which biotin has been attached. Alternatively, the capture component is biotin, and streptavidin is attached to or associated with the analyte such that the analyte can be captured by the biotin.

[061] According to one embodiment, the capture component is added to the binding surface in the following manner. First, an array of microwells on the distal end of a fiber optic bundle are prepared for attachment of the capture component(s). That is, the binding surface of the microwells are modified or an attachment component is added to the binding surface of the microwells such that the capture component(s) attach to the binding surface of the microwells. In one embodiment, the binding surface of the microwells are derivatized with a chemical functionality as described above. Next, the capture component is added.

[062] One example of capture component attachment is depicted in FIG. 1, in which binding surface of an array of microwells of the present invention are functionalized with biotin. As shown in FIG. 1a, the array of microwells of the present invention in this example is formed at the distal end of a fiber optic bundle 26. To attach the capture component, the binding surface of the microwells are first modified with an attachment component 28, which in this example is an aminopropyl silane 28 that is bound to both the core 30 and cladding 32 surfaces of the distal end of the fiber bundle 26, as shown in FIG. 1b. The modification with

aminopropyl silane is effective in this example because NHS-biotin attaches to an amino-silanized binding surface 28. However, since the capture component should be present only within the microwells, the external surfaces of the fiber optic bundle, such as the external surfaces of the cladding 32, should not be silanized. That is, the silanization must be removed from the external cladding surface 32 to avoid biotin attachment. In this example as shown in FIG. 1c, the silanization 28 was removed from the external cladding layer 32 by polishing the amino-silanized fiber optical bundle for 10 seconds with 0.3 μm lapping film, thereby removing the top amino-silanized cladding layer.

[063] After the attachment component 28 has been added to the binding surface of the microwells, the capture component can be attached. The capture component can be biotin. For example, with respect to FIG. 1d, biotin succinimidyl ester is attached to the amino groups 28 on the binding surfaces of the microwells 10.

[064] Examples of capture components within the microwell 10 are depicted in FIGS. 1e-g. A capture component 16 may be localized on the surface of the microwell 10, which may contain an optional seal 38 (FIG. 1e), on a microparticle 34 contained within the microwell 10, which may contain an optional seal 38 (FIG. 1f), and/or on the seal 36 of the microwell 10 (FIG. 1g).

[065] Another example of a capture component attachment is depicted in FIG. 9, in which the binding surface of the microwells of the present invention are functionalized with an antibody to the analyte of interest. As shown in FIG. 9, which is discussed in more detail in Example 3, an array of microwells is formed at the distal end of a fiber optic bundle 10. To attach the antibody capture component 16, the binding surface of the microwells are first modified with an attachment component, which in Example 3 is aminopropyl silane that has then been reacted with the homobifunctional cross-linker N,N′-disuccinimidyl carbonate. This surface then reacts with lysine groups on the antibody to present the capture component at the binding surface of the microwells.

III. Analytes

[066] As discussed herein, the array of reaction vessels in the present invention provides for detection, quantification, and further analysis of analytes. By "analyte" or grammatical equivalents herein is meant any atom, molecule, ion, molecular ion, compound, particle, cell, or virus to be either detected or evaluated.

[067] According to one embodiment, the analyte is an enzyme. For example, the enzyme can be an enzyme from any of the following enzyme classifications: oxidoreductases, transferases, kinases, hydrolases, lyases, isomerases, and ligases. Thus, appropriate enzymes include, but are not limited to, polymerases, cathepsins, calpains, amino-transferases such as, for example, AST and ALT, proteases such as, for example, caspases, nucleotide cyclases, transferases, lipases, enzymes associated with heart attacks, and the like. When the system of the present invention is used to detect viral or bacterial targets, appropriate enzymes include viral or bacterial polymerases and other such enzymes, including viral or bacterial proteases.

[068] Alternatively, the analyte has an enzymatic component. For example, the analyte can be a cell having an enzyme or enzymatic component present on its extracellular surface. Alternatively, the analyte is a cell having no enzymatic component on its surface. Such a cell is typically identified using an indirect assaying method described below such as a "sandwich" assay.

[069] In accordance with another embodiment, the analyte is not an enzyme. As will be appreciated by those in the art, a large number of analytes may be used in the present invention; basically, any analyte can be used which binds a capture component and/or a secondary binding ligand. As will be explained in further detail below, these analytes are typically identified using an indirect assay such as a "sandwich" assay. As mentioned above, one suitable analyte is a cell. In addition, suitable analytes include organic and inorganic molecules, including biomolecules. In a preferred embodiment, the analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous analytes that may be detected or evaluated for binding partners using the present invention. In addition to enzymes as discussed above, suitable protein analytes include, but are not limited to, (1) immunoglobulins; (2) hormones and cytokines (many of which serve as ligands for cellular receptors); (3) cancer markers; and (4) other proteins.

[070] According to one embodiment in which the analyte is not an enzyme and a sandwich assay is performed as described in further detail below, the enzymatic label as described in further detail below can be beta-galactosidase. Alternatively, the enzyme label can be, but is not limited to, alkaline phosphatase or horseradish peroxidase.

[071] Further suitable analytes include, but are not limited to, an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers,

organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc.

[072] In certain embodiments, the analyte may be a post-translationally modified protein (e.g., phosphorylation, methylation, glycosylation) and the capture component may be an antibody specific to a post-translational modification. Modified proteins may be captured with a multiplicity of specific antibodies and then visualized with use of a specific-secondary antibody to a post-translational modification. Alternatively, modified proteins may be captured with an antibody specific for a post-translational modification and then visualized with specific antibodies to each modified protein.

[073] In another embodiment, the analyte is a nucleic acid. A nucleic acid may be captured with a capture nucleic acid (e.g., an oligonucleotide) and then visualized with a different specifically labeled oligonucleotide.

IV. Detectable Labels and Signal Detection

[074] It is understood that a variety of detectable labels can be used in the practice of the methods described herein. For example, it is understood that a variety of colored labels (for example, metallic nanoparticles (for example, gold nanoparticles), semiconductor nanoparticles, semiconductor nanocrystals (for example, quantum dots), spectroscopic labels (for example, fluorescent labels), radiolabels, and enzymatic labels may be used in the practice of the invention.

[075] Depending upon the particular assay format, the detectable labels are indirectly attached to the analyte after it has been captured by the capture component, for example, via a secondary binding ligand that is conjugated to a detectable label and binds to the captured analyte. Once the label has become attached to the analyte, the presence of the label can be detected using suitable detection systems, for example, optical detectors (for example, intensified CCD cameras), fluorescence detectors known in the art.

[076] In one embodiment, the labels are enzymatic labels. In this case, a chromogenic, fluorogenic, or chemiluminescent enzyme substrate is contacted with the enzyme to produce a

detectable product. It is understood in the art that chromogenic, fluorogenic, or chemiluminescent enzyme substrates are known or can be made for many different enzymes. Thus, any known chromogenic, fluorogenic, or chemiluminescent enzyme substrate capable of producing a detectable product in a reaction with a particular enzyme can be used in the present invention, including any of the chromogenic, fluorogenic, or chemiluminescent enzyme substrates disclosed in *The Handbook — A Guide to Fluorescent Probes and Labeling Technologies, Tenth Ed.*, Chapter 10, http://probes.invitrogen.com/handbook/sections/1000.html, which is incorporated herein by reference in its entirety.

[077] According to one embodiment in which the analyte is detected using a sandwich assay as described further herein in which the enzyme label is β-galactosidase, the enzyme substrate added to the array is a β-galactosidase substrate such as resorufin-β-D-galactopyranoside.

V. Assay Methods

[078] The arrays of reaction vessels of the present invention can be used for several different assay methods. More specifically, the present invention provides for both (a) analyte detection and (b) quantification of analyte concentration in a sample.

[079] Generally, the array of microwells of the present invention is exposed to an analyte of interest (or contacted with a sample containing an analyte of interest) and the analyte is captured by a capture component on the binding surface within each microwell under conditions suitable for capture of the analyte by at least one of the capture components, i.e. generally physiological conditions. For purposes of the present application, the term "immobilized" means captured, attached, bound, or affixed to a capture component on the binding surface. For example, in the context of using microwells, the interaction between any analyte molecule and the capture component on the binding surface of a microwell results in immobilization of the analyte molecule within that microwell.

[080] According to one aspect of the invention, the sample of interest is placed in contact with the array of microwells of the present invention (or the array of the microwells is incubated in the sample) for a period of from about 30 minutes to about 12 hours depending upon the concentration of the analyte and the sample volume. In one embodiment, the array of

microwells and sample are contacted for a period of from about 50 minutes to about 70 minutes. In a further embodiment, the incubation period is about 1 hour.

[081] According to one embodiment, a wash step is performed after contacting the array of microwells with the sample. The wash step is intended to wash away any analytes or non-analyte molecules that are not bound to a capture component. Alternatively, no wash step is needed.

[082] In one aspect of the invention, a secondary binding ligand then is added to the array of microwells. Generally, the secondary binding ligand is added if the method of detecting the analyte is an indirect assay such as a "sandwich assay" (when the analyte is not an enzyme), as described in further detail herein. The secondary binding ligand, as discussed above, will associate with or bind to the captured analyte and comprises detectable label, for example, an enzymatic component. The secondary binding ligand is added in an amount sufficient to ensure that a secondary binding ligand comes into contact with every bound analyte on the binding surface of the array of microwells. Alternatively, no secondary binding ligand is added, such as, for example, when the analyte is going to be detected directly.

[083] When colored labels and spectroscopic labels are used, the labels can be detected using an appropriate detection system. In contrast, when the label is an enzymatic label, the enzymatic label is contacted with chromogenic fluorogenic, or chemiluminescent enzyme substrate to produce a detectable product.

[084] In the case of enzymatic labels, a chromogenic, fluorogenic, or chemiluminescent enzymatic substrate as described above then is introduced or added to the array of microwells. The chromogenic, fluorogenic, or chemiluminescent enzymatic substrate is provided in an amount sufficient to contact any captured analyte that is either an enzyme itself or is coupled to an enzyme, for example, via an enzyme-labeled antibody. The chosen enzyme substrate reacts with or is modified by the enzymatic component such that the reaction produces a chromogenic ,fluorogenic, or chemiluminescent product and thus an optical signal. The presence of the chromogenic, fluorogenic, or chemiluminescent product in the array of reaction vessels (as described below) can provide information about the identity and/or concentration of an analyte based on the interaction of the analyte with the capture component and the enzymatic substrate (and the secondary binding ligand, in some cases).

[085] In one embodiment of the present invention, the array of microwells are sealed after the enzymatic substrate is added to form an array of reaction vessels. That is, a sealing component is placed in contact with the face of the array of microwells, thereby fluidly isolating each microwell and sealing its contents therein. A "sealing component," as used herein, is defined as any material or device large enough to cover the entire array of microwells and capable of contacting the exterior surface of the array of microwells (for example, the cladding of a fiber optic bundle) such that each reaction vessel thus formed is sealed or isolated such that the contents of each reaction vessel cannot escape the reaction vessel. According to one embodiment, the sealing component is a silicone elastomer gasket that is placed against the array of microwells with a uniform pressure across the entire substrate. By sealing the contents in each microwell, the enzymatic reaction can proceed within the reaction vessel thus formed, thereby producing a detectable amount of the chromogenic, fluorogenic, or chemiluminescent product that is retained in the reaction vessel for detection purposes. That is, the enzyme converts the substrate into a chromogenic, fluorogenic, or chemiluminescent product that builds up to a locally high concentration in each sealed reaction vessel, generating a detectable chromogenic, fluorogenic, or chemiluminescent signal.

[086] According to one embodiment, the present invention provides for a microscope system equipped with a mechanical platform that applies the sealing component. The platform is positioned beneath the microscope stage on the microscopy system. After the enzymatic substrate have been added to the array of microwells, the sealing component is sandwiched between a flat surface (such as, for example, a microscope slide) and the array of microwells using uniform pressure applied by the mechanical platform.

[087] The assays for detecting analytes may be run under a variety of experimental conditions, as will be appreciated by those in the art. A variety of other reagents may be included in the screening assays. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc, which may be used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used. The mixture of components may be added in any order that provides for the requisite binding. Various blocking and washing steps may be utilized as is known in the art.

[088] The reaction vessels exhibiting enzymatic activity or changes in their optical signature may be identified by a conventional optical train and optical detection system.

Depending on the particular enzymatic substrates used and the operative wavelengths of their products, optical filters designed for a particular wavelengths may be employed for optical interrogation of the reaction vessels. In a preferred embodiment, the array of reaction vessels of the present invention are formed directly as part of a fiber optic bundle.

[089] According to one embodiment, the array of reaction vessels of the present invention can be used in conjunction with an optical detection system such as the system described in U.S. Application No. 09/816,651, which is incorporated herein by reference in its entirety. For example, according to one embodiment, the array of reaction vessels of the present invention is the distal end of a fiber optic assembly comprising a fiber optic bundle constructed of clad fibers so that light does not mix between fibers. As depicted in the 09/816,651 Application, the proximal end of the bundle is received by a z-translation stage and x-y micropositioner.

[090] The optical detection system of U.S. Application No. 09/816,651 operates as follows. Light returning from the distal end of the fiber optic bundle is passed by the attachment to a magnification changer which enables adjustment of the image size of the fiber's proximal or distal end. Light passing through the magnification changer is then shuttered and filtered by a second wheel. The light then is imaged on a charge coupled device (CCD) camera. A computer executes imaging processing software to process the information from the CCD camera and also possibly control the first and second shutter and filter wheels.

[091] The array of reaction vessels of the present invention may be integral or attached to the distal end of the fiber optic bundle using a variety of compatible processes. Microwells are formed at the center of each individual fiber of the fiber optic bundle and reaction vessels are formed by sealing this array of microwells with a sealing component. Thus, each optical fiber of the fiber optic bundle conveys light from the single microwell formed at the center of the fiber's distal end. This feature is necessary to enable the interrogation of the optical signature of individual reaction vessels to identify reactions in each microwell. Consequently, by imaging the end of the bundle onto the CCD array, the optical signatures of the reaction vessels are individually interrogatable.

A. Detection

[092] As discussed previously, the detection system will depend upon the label being detected. For example, when the label is an optical label, spectroscopic label or radiolabel, the

presence of the label can be determined using commercially available detectors known in the art.

[093] When the label is an enzymatic label, the label can be detected directly (for example, when the analyte has inherent enzyme activity) or indirectly (for example, when the analyte does not exhibit enzyme activity) via techniques known in the art. In other words, if the analyte is an enzyme, the analyte can be identified by a direct method of detection. Alternatively, if the target analyte is not an enzyme and thus cannot produce a chromogenic, fluorogenic, or chemiluminescent product in the presence of an enzymatic substrate, the analyte is identified by an indirect method of detection.

[094] The direct method of detection, which involves a analyte that is an enzyme, proceeds as follows. First, the sample of interest and the array of microwells are placed in contact as described in further detail above under suitable conditions. The enzymatic substrate is added either simultaneously with the sample or after addition of the sample.

[095] After sealing the microwells, the presence or absence of the analyte in any given reaction vessel then is detected by optical interrogation. That is, any change in the optical signal caused by production of a chromogenic, fluorogenic, or chemiluminescent product is detected. In any reaction vessel containing the analyte, the analyte modifies or acts upon the enzymatic substrate in some way, thereby resulting in the release of a chromogenic, fluorogenic, or chemiluminescent product, resulting in a change in the optical signal from the reaction vessel. The chromogenic, fluorogenic, or chemiluminescent product is then optically detected.

[096] In one embodiment of the present invention, the array of microwells are sealed with the sealing component to form reaction vessels after the enzymatic substrate is added, as described above.

[097] The indirect methods of detection involve an analyte that does not have enzymatic properties. Two indirect methods that can be used with the present invention are the "sandwich" assay and the "competitive" assay. It is contemplated that similar assay formats can be employed when the labels are optical labels, spectroscopic labels and radiolabels.

[098] A sandwich assay can be performed as depicted in FIG. 2. First, the sample of interest and the array of microwells 10 are placed in contact as shown in FIG. 2a and as described in further detail above. Under suitable conditions, analyte 12 present in the sample is

captured by the capture components 16 presented on the binding surfaces of the microwells 14, as shown in FIG. 2b. According to one embodiment, a wash step then is performed.

[099] Next, a "secondary binding ligand" 18 is added to the array of microwells 10, as shown in FIG. 2c. Secondary binding ligands 18 are similar to capture components 16 in that they bind to the analyte 12. The secondary binding ligand 18 may be the same or different from the capture component 16. The binding of the secondary binding ligand 18 to a captured analyte 12 forms a "sandwich" of sorts. In the absence of the analyte, the secondary binding ligand 18 is washed away.

[0100] A secondary binding ligand 18 has two components, a binding component 22 and an enzymatic label or a label that can capture an enzyme 24. The binding component 22 is the portion of the secondary binding ligand 18 that binds to the analyte 12. Typically, the secondary binding ligand 18 binds to a different portion of the analyte 12 than the capture component 16, because if both the capture component 16 and secondary binding ligand 18 were to bind to the same portion, the secondary binding ligand 18 would not be capable of binding to a captured analyte 12. Thus, the chosen secondary binding ligand 18 can bind to the analyte 12 while the analyte 12 is bound to the binding surface 14 via a capture component 16.

[0101] The label 24 is the portion of the secondary binding ligand 18 that exhibits enzymatic activity or can be used to subsequently to capture an enzyme. According to one embodiment, the label 24 is an enzyme attached to the secondary binding ligand 18.

[0102] Subsequently, the enzymatic substrate is added. In one embodiment of the present invention, the array of microwells are sealed after the enzymatic substrate is added to form reaction vessels, as described above. The presence or absence of the analyte in any given reaction vessel then is detected by optical interrogation. That is, any change in the optical signal caused by production of a chromogenic, fluorogenic, or chemiluminescent product is detected. In any reaction vessel containing the analyte and the secondary binding ligand, the enzyme associated with the secondary binding ligand modifies or acts upon the enzymatic substrate in some way, thereby producing a chromogenic, fluorogenic, or chemiluminescent product, resulting in a change in the optical signal from the reaction vessel. The product then is optically detected.

[0103] The competitive assay operates as follows. First, a labelled molecule is added to the array of microwells of the present invention, wherein the label is a enzyme or enzymatic

component. In this embodiment, the chosen labelled molecule binds with the capture component such that the addition of the labelled molecule to the array results in labelled molecules being bound to capture components on the binding surface of the microwells.

[0104] Next, the sample of interest and the array of microwells are placed in contact as described in further detail above. The presence of the analyte in the array of microwells causes the displacement of the labelled molecule and binding of the analyte to the capture components. The displacement occurs for the following reason: in this embodiment, the chosen capture component is capable of binding to either of the labeled molecule or the analyte, thus resulting in a competitive binding situation. As a result, if a labelled molecule is bound to a capture component on the binding surface of a microwell and an analyte is added, the analyte will displace the labeled molecule under suitable conditions.

[0105] According to one embodiment, a wash step is then performed to remove any non-bound labeled molecules from the binding surface of the array of microwells.

[0106] Subsequently, the enzymatic substrate is added. As discussed above, according to one aspect of the invention, the array of microwells are sealed after the enzymatic substrate is added to form reaction vessels. Alternatively, the array of microwells are not sealed. The presence or absence of the analyte in any given reaction vessel then is detected by optical interrogation. But unlike the optical interrogations that are described above, in this interrogation it is the lack of a chromogenic, fluorogenic, or chemiluminescent product that indicates the presence of the analyte in the reaction vessel. In any reaction vessel containing the analyte, no enzymatic action occurs and no change occurs in the optical signal from the reaction vessel. In contrast, in any reaction vessel in which the labelled molecule is still present, an optical signal is detected.

[0107] In an alternative version of the competitive assay embodiment, both the labelled molecule and sample of interest are added to the array of microwells at the same time in fixed volumes. In this version, the analyte and labelled molecule compete directly for the binding sites on the capture components.

[0108] In addition, it is understood that the methods and compositions described herein can be used in a variety of multiplexed assays to detect the presence of one or more analytes in a sample. Exemplary multiplex assays are described in the following sections.

1. Multiple Different Capture Components to Same Target Analyte

[0109] The reaction vessel array of the present invention according to one embodiment utilizes a plurality of capture components that are directed to a single target analyte but are not identical. In other words, the binding components bind to different binding sites on an analyte. This embodiment thus provides for more than one different capture component on each binding surface or different capture components on different binding surfaces. In one example, a single analyte is interrogated by two or more capture components, each of which is capable of binding to different sites on the analyte. This adds a level of confidence to the assay as non-specific binding interactions can be statistically minimized. In this embodiment, when proteinaceous analytes are evaluated, preferred embodiments utilize capture components that bind to different parts of the target. For example, when two or more antibodies (or antibody fragments) to different portions of the same analyte protein are used as capture components, preferred embodiments utilize antibodies to different epitopes. Similarly, when nucleic acid analytes are to be evaluated, the redundant nucleic acid probes may be overlapping, adjacent, or spatially separated. However, it is preferred that two probes do not compete for a single binding site, so adjacent or separated probes are preferred.

[0110] In this embodiment, a plurality of different capture components may be used, with from about 2 to about 20 being preferred, and from about 2 to about 10 being more preferred, and from 2 to about 5 being particularly preferred, including 2, 3, 4, or 5. However, as above, more may also be used, depending on the application.

2. Multiple Different Capture Components To Multiple Target Analytes

[0111] In another embodiment, the reaction vessel array of the present invention uses a plurality of different capture components that are directed to a plurality of target analytes, which can be the same or different. This embodiment includes more than one different capture component on each binding surface or different capture components on different binding surfaces. In one example, two or more analytes may be provided to which two or more capture components on the same binding surfaces or on different binding surfaces are capable of binding.

[0112] In this embodiment, more than one target analyte can be identified. For example, two or more target analytes can be identified so long as, in the case of direct assays, each different analyte is a different enzyme or, in the case of indirect assays, each target is coupled to binding

moieties conjugated with different labels. In one embodiment, the analytes are identified using multiple enzymatic substrates wherein each substrate produces a different color upon interaction with the appropriate enzyme. Thus, each target analyte can be distinguished based on the color produced by reaction with the enzymatic substrate. When labels other than enzymes are used, the different labels should have different characteristics so that each different label can be identified with the appropriate detector.

[0113] In an alternative approach, referred to as a sequential approach, the analytes are identified using the same or different labels. In this approach, the one or more analytes of interest are captured on one or more binding surfaces. Then a first labeled binding moiety that binds the analyte is permitted to bind to a first immobilized analyte. The presence of the label can be detected with an appropriate detection system. Thereafter, the first labeled binding moiety is removed, for example, by washing. Then a second labeled binding moiety that binds to a second, different analyte is permitted to bind to the second analyte. The presence of the label can be detected with an appropriate detection system. Thereafter, the second labeled binding moiety can be removed by washing and the process repeated as desired. It is understood that, in this approach, the same or different labels can be present in the first and second labeled binding moieties. It is understood that all the labels described herein can be used in this approach. When the label is a enzyme, it is understood that the sequential reactions can be performed using substrates, each of which produce the same or different colors. .

[0114] In this embodiment, a plurality of different capture components can be used, with from about 2 to about 100 being preferred, and from about 2 to about 20 being especially preferred, and from 2 to about 10 being particularly preferred, including 2, 3, 4 or 5, 6, 7, 8, 9. However, as above, more may also be used, depending on the application.

[0115] In an another approach, referred to as a spatial approach, it is possible to use either (i) two different capture components disposed within separate regions of a binding surface, (ii) two different labeled binding moieties that are applied to an separate regions of a binding surface, or (iii) a combination or (i) and (ii).

[0116] In the first example of the spatial approach, different capture components are applied to different regions of the binding surface. For example, this can be achieved by placing micro-droplets on different regions of a fiber microwell array if it is used as a binding surface or to the sealing component if it is used as the binding surface. Each micro-droplet would contain a

different capture component that would functionalize the surface it contacts with the different capture component. In this way , depending on the size of the droplets, it is possible to make a array of different capture components on the binding surface that are spatially discrete. The number of multiplexed analytes that could be detected in this manner would depend on the size of the different capture component regions and the size of the array of reaction vessels.

[0117] In the second example of the spatial approach, it is understood that the analytes of interest can be captured uniformly across a binding surface or within discrete regions of the binding surface. In this approach, however a first labeled binding moiety is applied to a first region of the binding surface and the presence of a first analyte is detected. Simultaneously or sequentially, a second labeled binding moiety is applied to a second region of the binding surface and the presence of a second analyte is detected.

[0118] It is understood that that each of the different assay configurations above, including the capture components directed to different analytes and the plurality of capture components directed to the same analyte, can also be utilized for quantification as described below.

[0119] In addition, under certain circumstances it is understood that the array of microwells may be reused during a subsequent assay. In this approach, a log of the wells that contained analyte in a first assay is recorded and then in a subsequent assay the wells that previously contained analyte are mathematically subtracted from the array of microwells used in the subsequent assay.

B. Quantification

[0120] According to one embodiment of the present invention, the methods, systems, and devices of the invention can be used to both detect the presence and concentration of an analyte in the sample. That is, there is a correlation between the percentage of reaction vessels containing one or more analyte molecules and the concentration of the analyte in the sample. Thus, the quantification method of the present invention allows for calculation of the amount of a analyte in a sample based on the percentage of reaction vessels that contain an analyte molecule.

[0121] The present invention is distinguished by its ability to quantify low numbers of individual molecules and is, therefore, well suited to measuring very low concentrations of target analyte. This ability is achieved by spatially isolating single analyte molecules, for example, in an array of reaction vessels, and then optionally generating and confining a

chromogenic product, for example, a fluorescent label, in the reaction vessels. The presence of an analyte molecule can easily be counted in a binary fashion (zero when a molecule is absent; one when a molecule is present) due to the high concentration of chromogenic product in any reaction vessel that contains at least one analyte molecule.

[0122] Without being limited by theory, the quantification method is driven in part by the fact that the number and volume of reaction vessels employed govern the dynamic range of concentrations that can be determined by this technique. That is, based on the number and volume of the reaction vessels in an array of the present invention, an estimate can be made of the range of concentrations of target analyte in solution that allow for the concentration to be determined using the method of the present invention.

[0123] For example, for an array as disclosed in Example 2 with reaction vessels each having a volume of 46 fL, a solution having a concentration of 3.6×10^{-11} M β-galactosidase will yield, on average, one enzyme molecule per vessel. However, it is important to note that distributing a solution having a target analyte concentration within the appropriate range into an array of reaction vessels will not result in the distribution of exactly one enzyme molecule per vessel; statistically, some vessels will have multiple molecules while others will have zero. In the case where the number of vessels containing one or more enzyme molecules to the number of vessels containing no enzyme molecule is high, the data can be fit to a Gaussian distribution. As the ratio of reaction vessels containing an enzyme molecule to the number of vessels containing no enzyme molecule approaches zero, the Poisson distribution applies. This limiting distribution is used to calculate the probability of rare events occurring in a large number of trials. For example, based on Poisson statistics, for a concentration of 3.6×10^{-11} M, a distribution between zero and five enzyme molecules per container is predicted, with the most probable values being zero and one.

[0124] Equation 1 can be used to determine the probability of observing v events based on the expected average number of events per trial, μ.

Equation 1: $P_\mu(v) = e^{-\mu} (\mu^v/v!)$

[0125] If the concentrations used are much less than 3.6×10^{-11} M, the expected average becomes exceptionally low, the distribution is narrowed, and the probability of observing anything other than 0 or 1 events per trial is improbable in all experimental cases. At these low concentrations, the relationship between the percentage of active reaction vessels and the bulk

enzyme concentration is approximately linear. Thus, based on this knowledge, the array of the present invention can be used to determine the concentration of a target analyte in a sample by a simple digital readout system, i.e., "counting" of active wells, as described herein.

[0126] According to one embodiment, the quantification method of the present invention can be performed as follows. The method is a digital readout system (also referred to as a "binary readout system") that includes first detecting the analytes in the array of reaction vessels by any detection method described above. The number of reaction vessels is then counted and a percentage of the total number of reaction vessels is calculated. That is, utilization of a yes or no response, in conjunction with the high-density array of reaction vessels, permits the digital readout of bulk concentrations of analyte molecules labeled with a label of interest, for example, the enzyme, β-galactosidase. This readout is accomplished by counting the vessels containing an active enzyme molecule across the array, with the resulting "active well" percentage correlating to the concentration of analyte assuming one enzyme label per target molecule. Given the large number of vessels simultaneously interrogated in the array of the present invention, the ratio of enzyme molecules to reaction vessels could be 1:1000 or less, as the large number of wells provides a statistically significant signal even at this low ratio.

[0127] Without being limited by theory, it is believed that the quantification method of the present invention is only limited by the number of individual reaction vessels that can be fabricated and interrogated. Thus, expanding the number of reaction vessels will increase both the dynamic range and the sensitivity of the assay.

[0128] The limitations of this technique are realized above and below the thresholds of the dynamic range. As the concentration goes below the lower limit of the dynamic range, the number of enzyme molecules is too low to observe sufficient occupied wells and, therefore, the number of wells must be increased in order to make sure that a statistically significant number of them are occupied by enzyme molecules. Results for extremely dilute concentrations have large relative errors associated with them, due to the very small number of reaction vessels that are expected to show activity. Slight deviation from the expected Poisson value, in this case, will result in a large error. The ultimate upper limit to this technique occurs when 100% of the reaction vessels contain at least one enzyme molecule. At this limit, discrimination between two solutions of high enzyme concentrations is not feasible.

[0129] In the range where the fraction of reaction vessels containing at least one molecule is less than about 20%, the probability that any well contains two or more analyte molecules is very small and the number of analyte molecules closely matches the number of occupied reaction vessels. Between 20% occupied and 100% occupied, an increasing number of wells will contain more than one molecule, however Gaussian statistics can still be used to correlate occupancy fraction with concentration until the occupancy fraction reaches 100%.

[0130] In practice, non-specific signal creates a lower "noise floor" and faulty reaction vessels create an upper "noise ceiling." Furthermore, with reference to the number of wells occupied or not occupied by analyte, statistically significant occupancy can be determined using standard statistical methods known in the art. For example, a statistically significant measurement can be one that is three times the standard deviation from the mean.

[0131] If desired, the practical dynamic range of the method can be increased in several ways. In one approach, the sample is diluted by a factor of 10 or more. Both the sample and the diluted sample can be assayed concurrently using the method of this invention. The dynamic ranges of the two assays will overlap, but be offset by the dilution factor, hence extending the dynamic range.

[0132] In a second approach, the amount of signal in each reaction vessel is measured at multiple time points prior to the time when all of the reaction vessels are positive for analyte. The fraction of wells that are positive at an early timepoint can be calibrated against a known reference standard to determine the concentration of the sample being analyzed. In the embodiment of the invention that utilizes a fiber optic bundle and a CCD camera, it is straightforward to image all of the wells in near real-time.

[0133] In a third approach, multiple arrays of reaction vessels can be used with differing volumes, differing areas of binding surface, or differing density of concentration of capture components on the binding surface. These arrays can be constructed as either distinct arrays or as one large array with sub-arrays with varying characteristics. Since the probability of an analyte molecule being detected in a given reaction vessel is related to volume, binding surface, and capture component density, the sub-arrays may be designed to cover different sensititivy ranges. If those ranges overlap, the effective range of the combined array can be extended.

[0134] In another aspect, the invention provides a method of determining the concentration of an analyte in a fluid sample to be tested. The method comprises the steps of (a) dividing the fluid sample into an array of wells, so that, for substantially all of the wells, each well contains either no analyte or a single molecule of the analyte; (b) determining the presence or absence of the analyte in each well to provide a number of wells in the array that contain the analyte; and (c) determining the concentration of the analyte in the fluid sample to be tested from the number of wells in the array that contain the analyte.

[0135] In one embodiment, less than about 20% of the total number of wells contain at least one molecule. Under these circumstances, the number of wells containing at least one molecule falls within the linear range of a Poisson distribution. In another embodiment, less than about 20% of the total number of wells contain about one molecule. In another embodiment, more than about 20% but less than about 60% of the total number of wells contain at least one molecule. Under these circumstances, the number of wells containing at least one molecule falls within the non-linear range of a Poisson distribution. In another embodiment, more than about 20% but less than about 60% of the total number of wells contain about one molecule. In another embodiment, more than about 60% but less than about 95% of the total number of wells contain at least one molecule. Under these circumstances, the number of wells containing at least one molecule falls within the highly non-linear range of a Poisson distribution. In another embodiment, more than about 60% but less than about 95% of the total number of wells contain about one molecule.

[0136] In another aspect, the invention provides a method of determining the concentration of an analyte in a fluid sample to be tested. The method comprises the steps of: (a) dividing the sample to be tested into a plurality of second, smaller fluid samples of equal volume so that a statistically significant fraction of the second, smaller fluid samples contain either no analyte or a single molecule of the analyte; (b) determining the presence or absence of the analyte in each of the second, smaller fluid samples so as to identify the number of second, smaller fluid samples that contain the analyte; and (c) determining the concentration of the analyte in the sample to be tested from the number of second, smaller samples that contain the analyte.

[0137] In this approach, the statistically significant fraction of the second, smaller fluid samples that contain either no analyte or a single molecule of the analyte is defined as 50%, optionally 75%, optionally 95% of the total number of second fluid samples that contain either no analyte or a single molecule of analyte.

[0138] In one aspect of the present invention, the array can also be used to analyze enzyme kinetics. "Enzyme kinetics" as used herein refers to the study of the rates of enzyme-controlled reactions. It is understood in the art of enzyme kinetics that the rate of an enzymatic reaction at low substrate concentrations is proportional to the substrate concentration (is "substrate dependent"). This is referred to as first order. It is further understood that the rate of the reaction at high substrate concentrations reaches a maximum rate and is independent of substrate concentration because the reaction becomes saturated. Thus, if reaction velocity is plotted as a function of substrate concentration, the line initially increases linearly with an increase in substrate and then begins to level off as substrate concentration approaches saturation.

[0139] Thus, according to one embodiment, the kinetics of any particular enzyme can be studied using the present system and array. Reaction velocity varies across enzymes for various reasons, including, for example, reaction inhibition caused by allosteric inhibition. The array of the present invention allows for study of these varied kinetic characteristics.

[0140] According to one embodiment, kinetics are examined in the following fashion. The target analyte is allowed to bind to the capture component, the substrate is added, and the reaction vessel is sealed. Given that a finite amount of substrate is present in the reaction vessel and that no further substrate can be added due to the sealing of the vessel, the reaction velocity can be determined based on the amount of chromogenic product detected over time.

VI. Exemplary Uses of the Present Invention

[0141] The system and array of the present invention has many uses. For example, the array has application to fundamental enzymology studies, as well as digital concentration measurements. Further, the array permits studies with multiple different enzymes and extends the limits of ultra-low detection for protein and DNA targets. With the ability to simultaneously monitor a large array of reaction vessels, single molecule enzymology can be used to resolve individual enzyme molecule behavior from bulk kinetic signal.

[0142] Another use, for example, is environmental monitoring of bacteria or viruses or both. An environmental sample potentially containing certain bacteria can be placed in contact with an array of the present invention. To detect the bacteria, the bacteria cells are lysed and a bacterial enzyme (or more than one enzyme) is targeted for detection. According to one

embodiment, the cells are lysed prior to being added to the array. Alternatively, the cells are captured and a lysing step occurs on the array prior to detection. In a further alternative, no lysis may be necessary if a cell surface marker is targeted. For example, the bacteria or virus of interest can be captured with an antibody that is specific to a surface marker on the target, and then the capture can be detected with a sandwich-type assay by adding an enzyme-labelled antibody that binds to the target in another location.

[0143] Another use, for example, involves measuring gene expression. In one embodiment, the target analytes are cells and these are introduced into the reaction vessels of the array under conditions such that some reaction vessels contain single cells. This is the first step in measuring gene expression, which can be done if desired at the whole cell level by detecting surface proteins and secreted proteins. Alternatively, the cells can be lysed and transcripts (or other components) from the cells can be interrogated. In one embodiment, each site contains one or no transcripts. In one embodiment, normal and cancer cells are compared. The cell to cell variation in gene expression can be addressed by examining gene expression at the single cell level.

[0144] Although the present invention has been described herein with reference to preferred embodiments, persons skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the invention.

EXAMPLES

Example 1

[0145] In this example, a proof-of-concept binding assay is performed using enzymatic signal amplification in an array of femtoliter sized reaction vessels. More specifically, various assays are performed to detect varying amounts of streptavidin-β-galactosidase (SβG) in solution using a biotinylated array of the present invention and then the correlation between the number of wells with captured SβG molecules and the concentration of the SβG in the sample is examined.

[0146] In this example, an etched fiber optic array is used to create a collection of femtoliter sized reaction vessels, each specifically functionalized and capable of capturing enzyme-labeled target molecules. Single enzyme molecules are confined to individual reaction vessels

and catalyze the production of a sufficient number of fluorescent product molecules to generate a positive signal. At low target molecule concentrations, only a percentage of the capture sites bind a target molecule, enabling a binary readout of target concentration from the high-density array.

Materials

[0147] The reactor vessel arrays in this example are generated using an acid etch of the distal face of a polished 1mm fiber optic array, consisting of 24,000 individual 4.5 μm optical fibers. The core fiber material is silica, and the cladding around each fiber is germania-doped silica, which etches at a slower rate. The 4.5 μm fibers are etched to a depth of 2.9 μm, creating an array of reactor vessels, each with a 46 fL volume (*see* Figure 1a).

[0148] The fibers were first modified with an aminopropyl silane bound to both the core and cladding surfaces (*see* Figure 1b). To avoid biotin attachment to the cladding, the amino-silanized fibers were polished for 10 seconds with 0.3 μm lapping film, which removed the top amino-silanized cladding layer from the fiber array (*see* Figure 1c). After polishing, NHS-biotin was attached to the amino groups on the well surfaces (*see* Figure 1d).

Methods

[0149] First, the effectiveness of the capture component was tested. To test the effectiveness of the biotinylation of the substrate, streptavidin Alexa Fluor 568® was attached directly to the biotin groups on the surfaces of both a polished and an unpolished fiber, followed by image acquisition of the modified surface (*see* FIG. 3). FIG. 3 shows Streptavidin Alexa Fluor 568® binding to (a) an unpolished biotin modified fiber optic array, and (b) a polished biotin modified fiber optic array. As seen in image (a), streptavidin binding occurred on all surfaces, in comparison to image (b), where binding occurred only on the surfaces of the microwell reactors. Thus, the unpolished fiber shows dye over the entire array including the cladding surface, while the polished fiber shows dye localized only on the well surfaces.

[0150] Subsequent to array modification, the biotinylated fiber arrays were incubated for 1 hour at room temperature in 150 μL PBS buffer containing varying amounts of SβG. The concentration of the SβG was chosen so that during the incubation time, statistically either one molecule or no molecules would bind to each well. The arrays were then washed repeatedly in PBS buffer, to ensure that unbound target was removed.

[0151] For a binary readout of SβG binding, the fiber array was loaded and secured on an upright microscope system equipped with a mechanical platform. A solution of β-galactosidase substrate, resorufin-β-D-galactopyranoside (RDG), was introduced to the distal end of the fiber containing the reaction vessels, and subsequently sealed. The substrate was sealed using a 0.01-inch thick silicone elastomer gasket sandwiched between a microscope slide and the fiber array by means of a mechanical platform located beneath the microscope stage. This platform applied a uniform pressure to the gasket material, across the entire bundle, sealing off each reaction chamber and enabling well to well interrogation of enzyme activity. β-galactosidase hydrolyzes RDG to form resorufin, which builds up to a locally high concentration in each sealed reaction vessel, generating a detectable fluorescent signal (Figure 4).

[0152] FIG. 4 depicts a portion of the fiber array for each experiment. Each of the experiments tested a different sample having a different concentration of SβG. The concentrations for each experiment were as follows: (a) 128 amol, (b) 51 amol, (c) 25 amol, (d) 7.5 amol, and (e) 2.6 amol. FIG. 4(f) depicts the control.

[0153] Analysis of over 5000 reaction vessels for each experiment allowed for a correlation between the percentage of reaction vessels that captured an enzyme molecule and the amount of enzyme present in the interrogated sample. The variation seen in the intensity differences from active well to active well is most likely a result of molecule-to-molecule variation in catalytic activity, in combination with surface effects, which may modulate the relative activities of enzyme molecules based on their orientation to the reaction chamber surface.

[0154] Two control experiments were also conducted to ensure that the binding of enzyme to the surface of the reactors was based exclusively on the biotin-streptavidin interaction, and not on non-specific binding to the glass surface. One control experiment consisted of an etched, unmodified fiber incubated with the most concentrated SβG target solution (128 amol in 150 μL). The second control experiment was performed using the modified fiber incubated in a solution of β-galactosidase lacking streptavidin (128 amol in 150 μL). Both control experiments generated a negligible active well percentage (less than 0.06%, versus 0.2% for the 2.6 amol experiment discussed below).

Results

[0155] FIG. 5 depicts a log-log plot of the moles of target present in a sample with the resulting percentage of active reaction vessels. The linear relationship between the percentage

of active reaction vessels and the moles of target in the log-log plot shown in FIG. 5 suggests that a binary readout detection method can be used for the detection of real targets such as DNA and antigens. This method permits rapid analysis and accurate concentration information via digital readout, while maintaining a straightforward assay procedure.

[0156] It is also interesting to note that the lowest limit of detection (LOD) for binding streptavidin-β-galactosidase (SβG) to a biotinylated femtoliter array in this example was 2.6 amoles (150 μL of 17 fM solution) using a target incubation time of 1 hour.

Example 2

[0157] In this example, single molecules of β-galactosidase were monitored using a 1 mm diameter fiber optic bundle with over 2.0×10^5 individually sealed, femtoliter microwell reactors. By observing the buildup of fluorescent products from single enzyme molecule catalysis over the array of reaction vessels and by applying a Poisson statistical analysis, a digital concentration readout was obtained.

Materials

[0158] 1mm bundled 4.5 μm optical fibers were purchased from Illumina (San Diego, CA). β-galactosidase and $Ru(bpy)_3Cl_2$ was obtained from Sigma-Aldrich (St. Louis, MO). Resorufin-D-β-galactopyranoside was purchased from Molecular Probes (Eugene, OR). 0.01-inch non-reinforced gloss silicone sheeting material was purchased from Specialty Manufacturing Inc. (Saginaw, MI). All other chemicals used were of reagent grade and obtained from Sigma-Aldrich (St. Louis, MO).

[0159] A custom-built, upright epifluorescence imaging system acquired all fluorescence images using a mercury light source, excitation and emission filter wheels, microscope objectives, and a CCD camera (QE, Sensicam). Filter wheels and shutters were computer controlled and analysis was performed with IPlab software (Scanalytics, Fairfax, VA). The system was equipped with a fastening device to fix the fiber optic array onto the system through the entire experiment. A mechanical platform beneath the stage was used to house the silicone-sealing layer, which was subsequently brought into contact with the distal end of the fiber array, sealing off each reaction vessel. All measurements were performed with femtowell arrays at the distal end of the optical fiber bundle.

[0160] Optical fiber bundles containing approximately 2.4 x 10^5 individual 4.5 μm diameter optical fibers were used as the substrate for fabricating femtoliter reaction vessel arrays. The well volume can be precisely controlled, as etch depth varies with etch time and etchant concentration. The optical fibers used in these experiments were etched to a depth of approximately 2.9 μm, yielding a 46 fL well volume. FIG. 6 depicts images of the etched surface of the fiber optic bundles. More specifically, FIG. 6a depicts the entire fiber array and close-up microscope images of the fiber bundle, emphasizing the regularity of both the array and each individual optical fiber. Further, FIG. 6b is an AFM image of a portion of the etched surface, showing wells created from the etching process.

Methods

[0161] *Assay*. For the β-galactosidase assay, the substrate used was resorufin-β-D-galactopyranoside. After the individual wells in the array were sealed in the presence of enzyme and substrate, the fluorescence intensity was monitored across the array of vessels for the enzymatic product, resorufin (ex 558 nm / em 573 nm). A 100 μM solution of resorufin-D-β-galactopyranoside (RDG) was prepared in 100mM Tris buffer pH 8.0 containing 2.0 mM KCl and 0.1mM $MgCl_2$. All enzyme solutions were prepared from previously aliquoted and frozen stock samples in the same reaction buffer. Just prior to experimentation, the two samples were centrifuged for 2 min at 7000 RPM to remove any particulate material that could interfere with the mechanics of the silicone seal. Approximately 1 cm^2 of silicone and a microscope slide were cleaned with absolute ethanol. The silicone sheeting was placed on the surface of the glass, to which it adhered. Subsequently, 75 μL volumes of enzyme and RDG solutions were mixed on the silicone gasket using a pipette. The gasket was mechanically raised towards the distal end of the fiber bundle until it experienced resistance, suggesting that a seal was formed. An initial fluorescence image was acquired, followed by periodic image acquisition for approximately 2 hr.

[0162] *Sealing component*. To seal the femtoliter array, a 0.01-inch thick silicone elastomer gasket was sandwiched between a microscope slide and the fiber array using a mechanical platform. This platform applied uniform pressure to the gasket material, across the entire bundle, sealing off each microwell to create the reaction vessels.

[0163] The silicone/glass seal used to create and isolate the femtoliter containers was inspected for its sealing ability by performing a photobleaching experiment (*see* Fig. 7). FIG. 7

depicts enclosure of a solution into the microchambers and evaluation of the silicone seal for integrity. FIG. 7a depicts a solution of $Ru(bpy)_3Cl_2$ enclosed into the array of chambers as observed by the red fluorescence across the array. FIG. 7b depicts a small octagonal portion of the fiber bundle that was photobleached via UV light. FIG. 7c depicts the array 60 minutes later. As shown in the figure, diffusion of $Ru(bpy)_3Cl_2$ from one well to another as a result of an imperfect silicone seal would display increased fluorescence intensity in photobleached wells and was not observed. This experiment substantiated the integrity of the seal for its ability to successfully isolate the array of vessels. Enzyme molecule denaturation on the glass surface was prevented by blocking with a BSA blocking buffer. Enzyme to vessel ratios used ranged from 1:5, down to 1:500, achieving accurate detection over two orders of magnitude.

[0164] *Photobleaching Experiment.* A solution of 1mM $Ru(bpy)_3Cl_2$ in DI water was used for the photobleaching experiments. A piece of silicone, approximately 1 cm^2, and a microscope slide were cleaned with absolute ethanol using lint-free swabs. The silicone sheeting was placed on the surface of the glass, to which it adhered. 50 μL of the $Ru(bpy)_3Cl_2$ solution was placed on the silicone, and subsequently brought into contact with the fiber bundle, to enclose the solution in the individual vessels. Using a field stop on the imaging system, UV light was used to illuminate a small portion of the array for 10 minutes, photobleaching the $Ru(bpy)_3Cl_2$. The field stop was then opened, and an image was acquired, displaying the difference in fluorescence. The array was then allowed to rest with the seal maintained. A final image was taken after 60 minutes, confirming the integrity of the seal.

[0165] As discussed above, the number and volume of reaction vessels employed govern the dynamic range of concentrations that can be determined by this technique. The reaction vessel volumes employed in this example were 46 fL (vide infra); therefore, it was calculated that a solution of 3.6×10^{-11} M β-galactosidase will yield, on average, one enzyme molecule per vessel. As also discussed above, if the concentrations used are much less than 3.6×10^{-11} M, the expected average becomes exceptionally low, the distribution is narrowed, and the probability of observing anything other than 0 or 1 events per trial is improbable in all experimental cases. At these low concentrations, the relationship between the percentage of active reaction vessels and the bulk enzyme concentration is approximately linear. After waiting for sufficient time to allow enzyme catalysis to occur, individual vessels were interrogated for an on/off response, correlating to each vessel either possessing or lacking enzymatic activity.

[0166] The substrate resorufin-D-β-galactopyranoside (RDG) was used as the substrate for experiments, which was sealed into all the vessels, along with the trapped enzyme molecules, using a silicone gasket material and mechanical arm. The expected percentages of active wells were calculated for each concentration used by applying the Poisson distribution statistics.

Results

[0167] As shown in FIG. 8, for the β-galactosidase assay, different bulk solution enzyme concentrations correspond to different ratios of enzyme to vessel volume, resulting in variation in the percentage of vessels that contain an enzyme molecule. Figure 8 depicts the detection of the activity of single molecules of β-galactosidase. FIG. 8a is a background image of a portion of the array, while FIG. 8b depicts an image taken of a portion of a 1:5 enzyme to vessel assay, and FIG. 8c shows a 1:80 enzyme to vessel assay.

[0168] Table 1 is a comparison of each experimental result with the percentage of occupied vessels calculated from the Poisson distribution. As shown by the data in the table, the array measurements successfully correlated with the number of single enzyme β-galactosidase molecules over the entire range of interrogated concentrations. There is minor disparity in the observed signals as a result of molecule-to-molecule variation in catalytic activity. This result is most likely due to the inherent stochastic nature of enzymes, in addition to surface effects, resulting in modulation of enzyme activity.

TABLE 1 – Digital Readout of Enzyme Concentrations

Enzyme to well ratio	Concentration (M)	Poisson % of active wells	Actual % active
1:5	7.20E-12	18.2	**14.9**
1:10	3.60E-12	9.5	**11.5**
1:20	1.80E-12	4.9	**5.6**
1:40	9.00E-13	2.5	**3.5**
1:80	4.50E-13	1.2	**1.5**
1:100	3.60E-13	1.0	**1.1**
1:200	1.80E-13	0.5	**0.3**
1:500	7.20E-14	0.2	**0.1**

Table 1. Digital readout from the arrays. The actual percentage of chambers exhibiting activity, in comparison to the expected percentage calculated from the Poisson distribution, are listed for the various concentrations analyzed.

[0169] The variation between the calculated and experimental results can be attributed to the intrinsic variability associated with the probability distribution, as well as experimental error in the preparation of enzyme solutions.

Example 3

[0170] In this example, a proof-of-concept sandwich assay is performed to detect a non-enzymatic target protein using an array of femtoliter sized reaction vessels (see, Figure 9). More specifically, various assays were performed to detect varying amounts of interferon-inducible protein 10 (IP-10) in solution using an array with the capture component, an anti-IP-10 antibody, disposed upon the surface of the reaction vessels. The captured target IP-10 then was labeled with a secondary binding ligand (biotin-labeled anti-IP-10) and strepatavidin-conjugated β-galactosidase (SβG). The correlation between the number of wells with captured SβG molecules and the concentration of IP-10 in the sample was examined.

Materials

[0171] The reactor vessel arrays in this example were generated using an acid etch of the distal face of a polished 1mm fiber optic array, consisting of 24,000 individual 4.5 μm optical fibers. The core fiber material was silica, and the cladding around each fiber was germania-doped silica, which etches at a slower rate. The 4.5 μm fibers were etched to a depth of 2.9 μm, creating an array of reactor vessels, each with a 46 fL volume (*see* Figure 1a).

[0172] The fibers were first modified with an aminopropyl silane (*see* Figure 1b). These amine-presenting fibers then were reacted with 100 mM N,N′-disuccinimidyl carbonate (DSC) to activate them with an ester of succinimide. The resulting substrates then were reacted with 0.1 mg/mL of anti-IP-10 capture antibody and washed.

Methods

[0173] Subsequent to array modification, the fiber arrays having anti-IP-10 antibody disposed thereon were incubated for 1 hour at room temperature in 150 μL PBS buffer containing varying amounts of IP-10. The captured IP-10 arrays then were incubated with 2 μg/mL of a secondary binding ligand, i.e., the detection anti-IP-10 antibody labeled with biotin.

These arrays then were incubated with 300 pM of SβG to label the bound IP-10 molecules with enzyme. The arrays then were washed repeatedly after each incubation in PBS buffer, to ensure that unbound target, antibody and SβG was removed.

[0174] For a binary readout of SβG bound to IP-10, the fiber array was loaded and secured on an upright microscope system equipped with a mechanical platform. A solution of β-galactosidase substrate, resorufin-β-D-galactopyranoside (RDG), was introduced to the distal end of the fiber containing the reaction vessels, and subsequently sealed. The substrate was sealed using a 0.01-inch thick silicone elastomer gasket sandwiched between a microscope slide and the fiber array by means of a mechanical platform located beneath the microscope stage. This platform applied a uniform pressure to the gasket material, across the entire bundle, sealing off each reaction chamber and enabling well to well interrogation of enzyme activity. β-galactosidase hydrolyzes RDG to form resorufin, which builds up to a locally high concentration in each sealed reaction vessel, generating a detectable fluorescent signal.

[0175] Analysis of over 5000 reaction vessels for each experiment allowed for a correlation between the percentage of reaction vessels that captured an enzyme molecule and the amount of IP-10 present in the interrogated sample.

Results

[0176] FIG. 10 depicts a plot of the concentration of IP-10 present in a sample with the resulting percentage of active reaction vessels. The linear relationship between the percentage of active reaction vessels and the concentration of target in the plot shown in FIG. 10 suggesting that a binary readout detection method can be used for the detection of non-enzyme protein targets using a sandwich detection approach.

Bibliography

[0177] Each of the following references is incorporated by reference in their entirety, for all purposes.

Sano, T.; Smith, C. L.; Cantor, C. R. *Science* **1992**, *258*, 120-122.

Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884-1886.

Niemeyer, C. M.; Adler, M.; Pignataro, B.; Lenhert, S.; Gao, S.; Chi, L. F.; Fuchs, H.; Blohm, D. *Nucleic Acids Research* **1999**, *27*, 4553-4561.

Zhou, H.; Fisher, R. J.; Papas, T. S. *Nucleic Acids Research* **1993**, *21*, 6038-6039.

Niemeyer, C. M.; Adler, M.; Wacker, R. *Trends in Biotechnology* **2005**, *23*, 208-216.

Whitesides, G. M. *Nature Biotechnology* **2003**, *21*, 1161-1165.

Rondelez, Y.; Tresset, G.; Tabata, K. V.; Arata, H.; Fujita, H.; Takeuchi, S.; Noji, H. *Nature Biotechnology* **2005**, *23*, 361-365.

Nakano, M.; Komatsu, J.; Matsuura, S.; Takashima, K.; Katsura, S.; Mizuno, A. *Journal of Biotechnology* **2003**, *102*, 117-124.

Nagai, H.; Murakami, Y.; Yokoyama, K.; Tamiya, E. *Biosensors and Bioelectronics* **2001**, *16*, 1015-1019.

Lipman, A. E.; Shuler, B.; Bakajin, O.; Eaton, W. A. *Science* **2003**, *301*, 1233-1235.

Chiu, D. T.; Wilson, C. F.; Ryttsen, F.; Stromberg, A.; Farre, C.; Karlsson, A.; Nordholm, S.; Gaggar, A.; Modi, B. P.; Moscho, A.; Garza-Lopez, R. A.; Orwar, O.; Zare, R. N. *Science* **1999**, *283*, 1892-1895.

Rissin, D. M.; Walt, D. R. *Journal of the American Chemical Society* **2007**, 128(19) 6286-6287.

Pantano, P.; Walt, D. R. *Chemistry of Materials* **1996**, *8*, 2832-2835.

Monk, D. J.; Ueberfeld, J.; Walt, D. R. *Journal of Materials Chemistry* **2005**, *15*, 4361-4366.

Song, L. N.; Ahn, S.; Walt, D. R. *Emerging Infectious Diseases* **2005**, *11*, 1629-1632.

Lee, J. Y.; Li, H. W.; Yeung, E. S. *Journal of Chromatography A* **2004**, *1053*, 173-179.

Xue, Q. F.; Yeung, E. S. *Nature* **1995**, *373*, 681-683.

Foquet, M.; Korlach, J.; Zipfel, W. R.; Webb, W. W.; Craighead, H. G. *Analytical Chemistry* **2004**, *76*, 1618-1626.

Gratzl, M.; Lu, H.; Matsimoto, T.; Yi, C.; Bright, G. R. *Analytical Chemistry* **1999**, *71*, 2751-2756.

Stamou, D.; Duschl, C.; Delamarche, E.; Vogel, H. *Angewandte Chemie-International Edition* **2003**, *42*, 5580-5583.

Gosalia, D. N.; Diamond, S. L. *Proceedings of the National Academy of Sciences USA* **2003**, *100*, 8721-8726.

Lu, H. P.; Xun, L. Y.; Xie, X. S. *Science* **1998**, *282*, 1877-1882.

Taylor, J. R. *An Introduction to Error Analysis*; Second Addition ed.; University Science Books: Sausalito, CA, 1997.

Wheeler, A. R.; Throndset, W. R.; Whelan, R. J.; Leach, A. M.; Zare, R. N.; Liao, Y. H.; Farrell, K.; Manger, I. D.; Daridon, A. *Analytical Chemistry* **2003**, *75*, 3581-3586.

CLAIMS

1. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) partitioning at least a portion of the analyte molecules in the fluid sample across a plurality of reaction vessels so that a statistically significant fraction of the reaction vessels contain analyte and a statistically significant fraction of the reaction vessels contain no analyte;

(b) determining the presence or absence of the analyte in each reaction vessel to identify the number of reaction vessels that contain analyte and/or to identify the number of reaction vessels that contain no analyte; and

(c) determining the concentration of the analyte in the fluid sample from the number of reaction vessels that contain the analyte.

2. The method of claim 1, wherein, in step (a), at least 95% of the reaction vessels contain no analyte.

3. The method of claim 1, wherein, in step (a), at least 90% of the reaction vessels contain no analyte.

4. The method of claim 1, wherein, in step (a), at least 80% of the reaction vessels contain no analyte.

5. The method of claim 1, wherein, in step (a), at least 40% of the reaction vessels contain no analyte.

6. The method of claim 1, wherein, in step (a), at least 5% of the reaction vessels contain no analyte.

7. The method of any one of claims 1-4, wherein, in step (c), the concentration of the analyte in the fluid sample is determined by a Poisson distribution analysis of the number of reaction vessels that contain the analyte.

8. The method of any one of claims 1, 5 or 6, wherein, in step (c), the concentration of the analyte in the fluid sample is determined by a Gaussian distribution analysis of the number of reaction vessels that contain the analyte.

9. The method of any one of claims 1-8, wherein the analyte is captured in the reaction vessel by a capture component immobilized on a binding surface defined by a microwell.

10. The method of any one of claims 1-8, wherein the analyte is captured in the reaction vessel by a capture component immobilized on a particle disposed within a microwell.

11. The method of any one of claims 1-8, wherein the analyte is captured in the reaction vessel by a capture component immobilized on a sealing component that seals a microwell.

12. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) exposing the fluid sample to a plurality of reaction vessels under conditions so that analyte is captured in a statistically significant fraction of the reaction vessels, wherein each reaction vessel comprises a microwell and an optional sealing component and each reaction vessel defines a binding surface that has a capture component immobilized thereon;

(b) determining the presence or absence of the analyte in each reaction vessel so as to identify the number of reaction vessels that contain captured analyte and/or the number of reaction vessels that do not contain captured analyte; and

(c) determining the concentration of the analyte in the fluid sample to be tested from the number of reaction vessels that contain and/or do not contain the analyte.

13. The method of claim 12, wherein less than about 20% of the total number of reaction vessels contain about one molecule of analyte.

14. The method of claim 12, wherein less than about 10% of the total number of reaction vessels contain about one molecule of analyte.

15. The method of claim 12, wherein less than about 5% of the total number of reaction vessels contain about one molecule of analyte.

16. The method of any one of claims 12-15, wherein, in step (c), the concentration of the analyte in the fluid sample is determined by a Poisson distribution analysis of the number of reaction vessels that contain the analyte.

17. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) partitioning at least a portion of the analyte molecules in the fluid sample into a plurality of reaction vessels, so that, for substantially all of the reaction vessels, each reaction vessel contains either no analyte or a single molecule of the analyte;

(b) determining the presence or absence of the analyte in each reaction vessel to provide a number of the reaction vessels that contain the analyte; and

(c) determining the concentration of the analyte in the fluid sample from the number of reaction vessels that contain the analyte.

18. The method of claim 17, wherein less than about 20% of the total number of reaction vessels contain about one molecule of analyte.

19. The method of claim 17, wherein less than about 10% of the total number of reaction vessel contain about one molecule of the analyte.

20. The method of claim 17, wherein less than about 5% of the total number of reaction vessels contain about one molecule of the analyte.

21. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) partitioning the sample to be tested into a plurality of second, smaller fluid samples of equal volume so that a statistically significant fraction of the second, smaller fluid samples contain either a single molecule of the analyte or no analyte;

(b) determining the presence or absence of the analyte in each of the second samples so as to identify the number of second samples that contain the analyte; and

(c) determining the concentration of the analyte in the sample to be tested from the number of second samples that contain the analyte.

22. The method of claim 21, wherein 60% of the total number of second samples contain either no analyte or a single molecule of analyte.

23. The method of claim 21, wherein 80% of the total number of second fluid samples contain either no analyte or a single molecule of analyte.

24. The method of claim 21, wherein 90% of the total number of second fluid samples contain either no analyte or a single molecule of analyte.

25. The method of any one of claims 1-20, wherein, in step (a), the sample to be tested is partitioned into at least 1,000 reaction vessels.

26. The method of any one of claims 1-20, wherein, in step (a), the sample to be tested is partitioned into from about 10,000 to about 200,000 reaction vessels.

27. The method of claim 26, wherein the sample to be tested is partitioned into from about 50,000 to about 100,000 reaction vessels.

28. The method of any one of claims 21-24, wherein, in step (a), the sample to be tested is partitioned into at least 1,000 second, smaller samples.

29. The method of any one of claims 21-25, wherein, in step (a), the sample to be tested is partitioned into from about 10,000 to about 200,000 second, smaller samples.

30. The method of claim 29, wherein the sample to be tested is partitioned into from about 50,000 to about 100,000 second, smaller samples.

31. The method of any one of claims 1-30, wherein the analyte is a biomolecule.

32. The method of claim 31, wherein the biomolecule is selected from the group consisting of a protein, a nucleic acid, a lipid, and a carbohydrate.

33. The method of any one of claims 1-20, wherein the reaction vessels have a volume of from about 10 attoliters to about 50 picoliters.

34. The method of any one of claims 21-24, wherein the second, smaller samples have a volume of from about 10 attoliters to about 50 picoliters.

35. The method of any one of claims 1-20, wherein at least a portion of the reaction vessel is defined by a distal end of an optical fiber.

AMENDED CLAIMS
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CLAIMS

1. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) partitioning at least a portion of the analyte molecules in the fluid sample across a plurality of reaction vessels so that a statistically significant fraction of the reaction vessels contain analyte and a statistically significant fraction of the reaction vessels contain no analyte;

(b) determining the presence or absence of the analyte in each reaction vessel to identify the number of reaction vessels that contain analyte and/or to identify the number of reaction vessels that contain no analyte; and

(c) determining the concentration of the analyte in the fluid sample from the number of reaction vessels that contain the analyte.

2. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) exposing the fluid sample to a plurality of reaction vessels under conditions so that analyte is captured in a statistically significant fraction of the reaction vessels, wherein each reaction vessel comprises a microwell and each reaction vessel defines a binding surface that has a capture component immobilized thereon;

(b) determining the presence or absence of the analyte in each reaction vessel so as to identify the number of reaction vessels that contain captured analyte and/or the number of reaction vessels that do not contain captured analyte; and

(c) determining the concentration of the analyte in the fluid sample to be tested from the number of reaction vessels that contain and/or do not contain the analyte.

3. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) partitioning at least a portion of the analyte molecules in the fluid sample into a plurality of reaction vessels, so that, for substantially all of the reaction vessels, each reaction vessel contains either no analyte or a single molecule of the analyte;

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(b) determining the presence or absence of the analyte in each reaction vessel to provide a number of the reaction vessels that contain the analyte; and

(c) determining the concentration of the analyte in the fluid sample from the number of reaction vessels that contain the analyte.

4. A method of determining the concentration of an analyte in a fluid sample to be tested, the method comprising the steps of:

(a) partitioning the sample to be tested into a plurality of second, smaller fluid samples of equal volume so that a statistically significant fraction of the second, smaller fluid samples contain either a single molecule of the analyte or no analyte;

(b) determining the presence or absence of the analyte in each of the second samples so as to identify the number of second samples that contain the analyte; and

(c) determining the concentration of the analyte in the sample to be tested from the number of second samples that contain the analyte.

5. A method of detecting analyte molecules or particles in a fluid sample, comprising:

partitioning a fluid sample containing or suspected of containing analyte molecules or particles across an array comprising a plurality of reaction vessels, so that at least some of the reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle;

immobilizing within each reaction vessel containing at least one analyte molecule or particle, a first binding ligand comprising a label;

applying an enzyme to the array and capturing the enzyme with the label;

contacting the enzyme with an enzymatic substrate, wherein the enzymatic substrate produces a detectable product upon contact with the enzyme;

detecting the detectable product; and

determining from detecting the presence of the detectable product the number of reaction vessels which contain an analyte molecule or particle.

6. A method of detecting analyte molecules or particles in a fluid, comprising:

partitioning a fluid containing or suspected of containing analyte molecules or particles across an array comprising a plurality of reaction vessels, so that at least some of the

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reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle;

exposing the reaction vessels to an enzymatic substrate, wherein the enzymatic substrate produces a detectable product in reaction vessels containing at least one analyte molecule or particle;

detecting the detectable product; and

determining from detecting the presence of the detectable product the number of reaction vessels which contain at least one analyte molecule or particle.

7. A method of detecting analyte molecules or particles in a fluid, comprising:

immobilizing with respect to each analyte molecules or particles, an enzyme molecule;

partitioning the enzyme molecules across an array comprising a plurality of reaction vessels, so that at least some of the reaction vessels contain no enzyme molecules and at least some of the reaction vessels contain at least one enzyme molecule;

exposing the reaction vessels to an enzymatic substrate, wherein the enzymatic substrate produces a detectable product in reaction vessels containing at least one enzyme molecule; and

determining from detecting the presence of the detectable product the number of reaction vessels which contain at least one enzyme molecule.

8. The method of any one of claims 1-3, wherein the analyte molecules are exposed to a plurality of secondary binding ligands after the analyte molecules have been partitioned across the plurality of reaction vessels, wherein at least one secondary binding ligand becomes associated with each analyte molecule.

9. The method of claim 8, wherein the presence or absence of the analyte in each reaction vessel is determined by determining the presence or absence of a secondary binding ligand in each reaction vessel.

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10. The method of claim 9, wherein the number of reaction vessels that contain or do not contain the analyte is determined by determining the number of reaction vessels that contain or do not contain the secondary binding ligand.

11. The method of any one of claims 1-3 or 5-7, wherein at least one wash step is performed.

12. The method of any one of claims 1-3, wherein the reaction vessels are sealed during at least step (b).

13. The method of any one of claims 1-3, wherein each reaction vessel is exposed to a plurality of reaction components, wherein at least one of a chromogenic, fluorogenic, or chemiluminescent product is produced upon exposure of plurality of reaction components to the secondary binding ligand.

14. The method of claim 13, wherein the presence or absence of the analyte in each reaction vessel is determined by determining the presence or absence of at least one chromogenic, fluorogenic, or chemiluminescent product in each reaction vessel.

15. The method of claim 14, wherein the concentration of the analyte in the fluid sample is determined from determining the number of reaction vessels that contain or do not contain at least one chromogenic, fluorogenic, or chemiluminescent product.

16. The method of claim 1 or 3, wherein the partitioning of at least a portion of the analyte molecules in the fluid sample across a plurality of reaction vessels comprises partitioning the fluid sample across a plurality of wells of an array comprising the plurality of wells.

19. The method of claim 1 or 3, wherein at least one analyte of a first type and at least one analyte of a second type are partitioned across the plurality of reaction vessels.

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20. A method of claim 19, wherein the reaction vessels comprises at least one capture component per reaction vessel, wherein the capture component is able to immobilize at least one analyte molecule.

21. A method of claim 20, wherein the first analyte or the second analyte is able to be immobilized with respect to a first capture component.

22. The method of claim 19, wherein the reaction vessels comprise at least one capture component of a first type per reaction vessel and at least one capture component of a second type per reaction vessel.

23. The method of claim 23, wherein the at least one analyte of the first type associates with the at least one capture component of the first type and the at least one analyte of the second type associates with the at least one capture component of the second type.

24. The method of claim 19, wherein the plurality of reaction vessels is exposed to a plurality of secondary binding ligands of a first type and a plurality of secondary binding ligands of a second type, and wherein at least one secondary binding ligand of the first type forms a complex with the at least one analyte of the first type and at least one secondary binding ligand of the second type forms a complex with the at least one analyte of the second type.

25. The method of claim 24, wherein the presence or absence of the at least one analyte of the first type and/or the at least one analyte of the second type in each reaction vessel is determined by detecting the presence or absence of a secondary binding ligand of the first type and/or a secondary binding ligand of the second type in each reaction vessel.

26. The method of claim 19, wherein both the concentration of the analyte of the first type in the fluid sample and the concentration of the analyte of the second type in the fluid sample are determined.

27. The method of claim 1, wherein the plurality of reaction vessels is reused.

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28. The method of claim 27, wherein the plurality of reaction vessels is reused comprising the additional steps of:

determining the log of the reaction vessels that comprise an analyte from the first fluid sample;

partitioning a second analyte in a second fluid sample across the reaction vessels;

determining the presence or absence of the first analyte or the second analyte in each reaction vessel to identify the number of reaction vessels that contain the first or the second analyte and/or identify the number of reaction vessels that do not contain the first or the second analyte;

determining the number of reaction vessels that contain the second analyte by mathematically subtracting the log of the reaction vessels that contain the first analyte from the number of reactions vessels that contain the first or the second analyte.

29. The method of determining the concentration of an analyte in the fluid sample using the method of claim 1 or 3, wherein a portion of the analyte molecules in a fluid sample is partitioned across a plurality of first reaction vessels and second plurality of reaction vessels, wherein the volume of the plurality of second reaction vessels differs from the volume of the plurality of first reaction vessels.

30. The method of claim 29, wherein the volume of a first reaction vessel is approximately 10 times greater that the volume of a second reaction vessels.

31. The method of claim 30, wherein the volume of a first reaction vessel is approximately 100 times greater that the volume of a second reaction vessel.

32. The method of any one of claims 1-3 or 5-7, wherein at least 95% of the reaction vessels contain no analyte.

33. The method of any one of claims 1-3 or 5-7, wherein at least 90% of the reaction vessels contain no analyte.

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34. The method of any one of claims 1-3 or 5-7, wherein at least 80% of the reaction vessels contain no analyte.

35. The method of any one of claims 1-3 or 5-7, wherein at least 40% of the reaction vessels contain no analyte.

36. The method of any one of claims 1-3 or 5-7, wherein at least 5% of the reaction vessels contain no analyte.

37. The method of any one of claim 1-3 or 5-7, wherein less than about 20% of the total number of reaction vessels contain about one molecule of analyte.

38. The method of any one of claims 1-4, wherein, in step (c), the concentration of the analyte in the fluid sample is determined by a Poisson distribution analysis of the number of reaction vessels that contain the analyte.

39. The method of any one of claim 1-4, wherein, in step (c), wherein, in step (c), the concentration of the analyte in the fluid sample is determined by a Gaussian distribution analysis of the number of reaction vessels that contain the analyte.

40. The method of claim 1 or 3, wherein the analyte is captured in the reaction vessel by a capture component immobilized on a binding surface defined by a microwell.

41. The method of claim 1 or 3, wherein the analyte is captured in the reaction vessel by a capture component immobilized on a particle disposed within a microwell.

42. The method of claim 1 or 3, wherein the analyte is captured in the reaction vessel by a capture component immobilized on a sealing component that seals a microwell.

43. The method of any one of claims 1-3 or 5-7, wherein less than about 20% of the total number of reaction vessels contain about one molecule of analyte.

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44. The method of any one of claims 1-3 or 5-7, wherein less than about 10% of the total number of reaction vessels contain about one molecule of analyte.

45. The method of any one of claims 1-3 or 5-7, wherein less than about 5% of the total number of reaction vessels contain about one molecule of analyte.

46. The method of any one of claims 1-3 or 5-7, wherein less than about 5% of the total number of reaction vessels contain about one molecule of the analyte.

47. The method of claim 4, wherein the plurality of second, smaller fluid samples are exposed to a substrate, wherein a plurality of chromogenic, fluorogenic, or chemiluminescent products are produced upon exposure of the substrate to the second, smaller fluid samples.

48. The method of claim 47, wherein the presence or absence of the analyte in the second samples is determined by determining the presence of a plurality of chromogenic, fluorogenic, or chemiluminescent products in each second, smaller fluid sample.

49. The method of claim 48, wherein the concentration of the analyte in the fluid sample is determined by determining the number of second, smaller fluid that contain a plurality of chromogenic, fluorogenic, or chemiluminescent products.

50. The method of claim 4, wherein 60% of the total number of second samples contain either no analyte or a single molecule of analyte.

51. The method of claim 4, wherein 80% of the total number of second fluid samples contain either no analyte or a single molecule of analyte.

52. The method of claim 4, wherein 90% of the total number of second fluid samples contain either no analyte or a single molecule of analyte.

53. The method of any one of claims 1-3 or 5-7, wherein the sample to be tested is partitioned into at least 1,000 reaction vessels.

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54. The method of any one of claims 1-3 or 5-7, wherein, the sample to be tested is partitioned into from about 10,000 to about 200,000 reaction vessels.

56. The method of claim 54, wherein the sample to be tested is partitioned into from about 50,000 to about 100,000 reaction vessels.

57. The method of claim 4, wherein, in step (a), the sample to be tested is partitioned into at least 1,000 second, smaller samples.

58. The method of claim 57, wherein, in step (a), the sample to be tested is partitioned into from about 10,000 to about 200,000 second, smaller samples.

59. The method of claim 58, wherein the sample to be tested is partitioned into from about 50,000 to about 100,000 second, smaller samples.

60. The method of any preceding claim, wherein the analyte is a biomolecule.

61. The method of claim 60, wherein the biomolecule is selected from the group consisting of a protein, a nucleic acid, a lipid, a carbohydrate, a hormone, a cytokine, an antibodies, and a cellular antigen.

62. The method of any one of claims 1-3, or 5-7, wherein the reaction vessels have a volume of from about 10 attoliters to about 50 picoliters.

63. The method of claim 4, wherein the second, smaller samples have a volume of from about 10 attoliters to about 50 picoliters.

64. The method of any preceding claim, wherein at least a portion of the reaction vessel comprises a plurality of microwells formed distal end of a fiber optic bundle.

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65. The method of any one of claims 1-3 or 5-7, wherein the analyte is selected from the group consisting of environmental pollutants, pesticides, insecticides, drugs, and toxins.

66. The method of any preceding claim, wherein the analyte is an enzyme.

67. The method of any one of claim 2 or 9-11, wherein the capture component is an antibody or DNA probe.

68. The method of claim 6, wherein each analyte molecule or particle comprises an enzyme.

69. The method of claim 68, wherein the enzyme is linked to the analyte molecule or particle via a binding ligand.

70. The method of claim 6, further comprising immobilizing with respect to each analyte molecule or particle, a first binding ligand.

71. The method of claim 70 wherein the binding ligand is immobilized within each reaction vessel containing at least one analyte molecule or particle.

72. The method of claim 70, wherein the first binding ligand comprises an enzymatic component.

73. The method of claim 70, wherein the first binding ligand comprises a label.

74. The method of claim 73, further comprising the step of applying an enzyme to the array and capturing the enzyme with the label.

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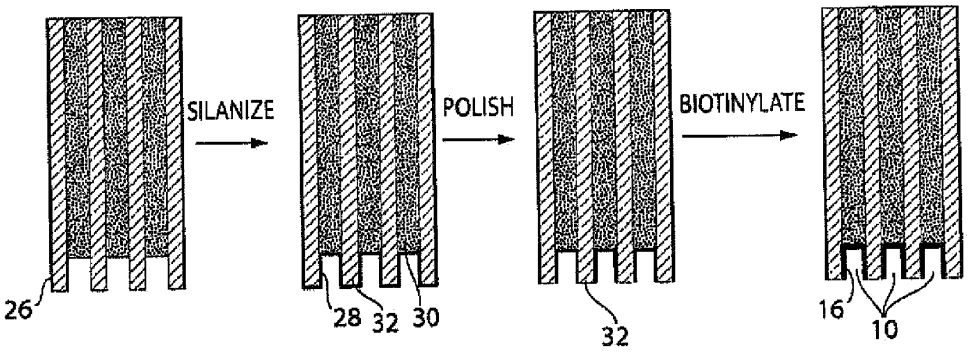


Fig. 1A Fig. 1B Fig. 1C Fig. 1D

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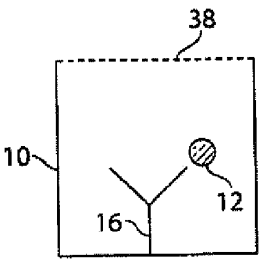


Fig. 1E

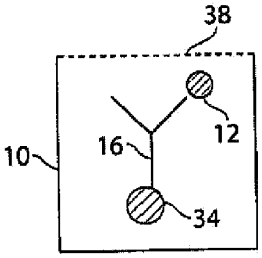


Fig. 1F

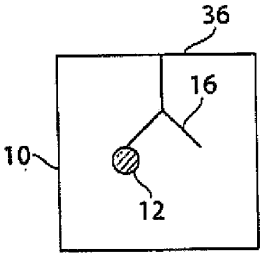


Fig. 1G

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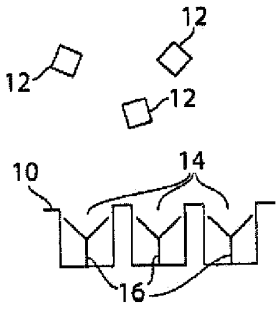


Fig. 2A

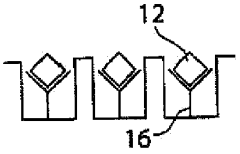


Fig. 2B

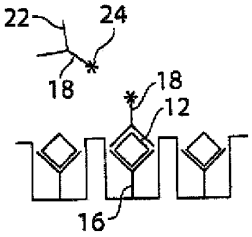


Fig. 2C

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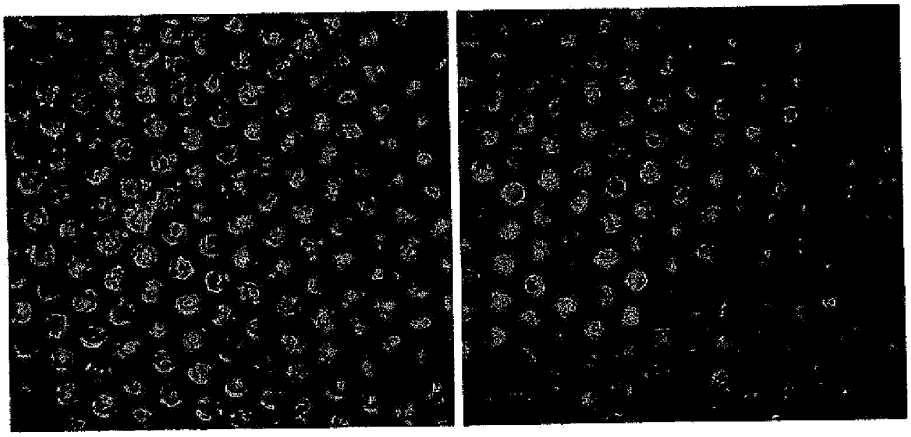


Fig. 3A Fig. 3B

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Fig. 4A Fig. 4B Fig. 4C

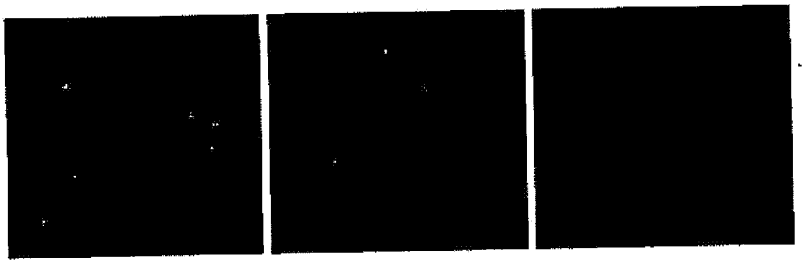


Fig. 4D Fig. 4E Fig. 4F

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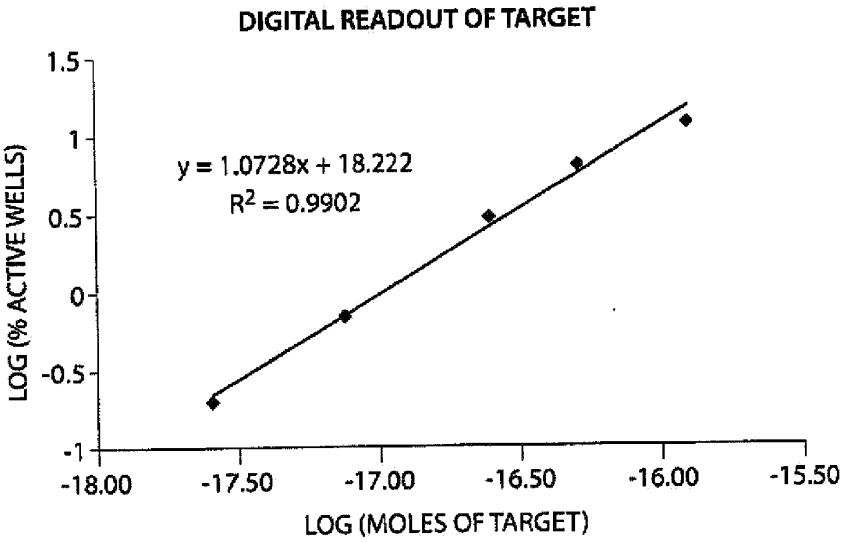


Fig. 5

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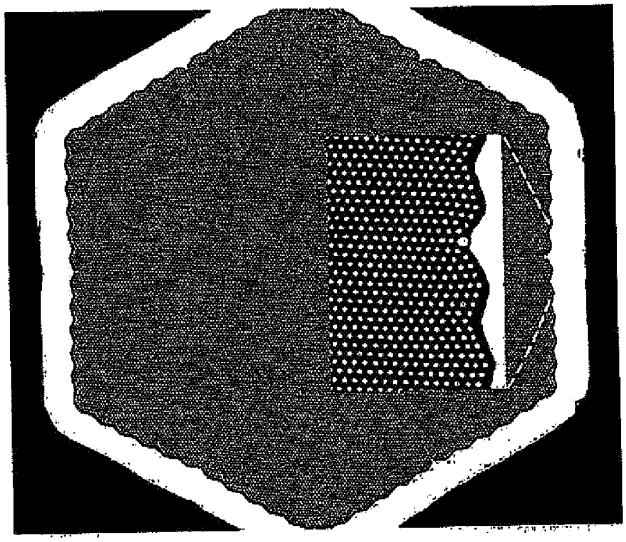


Fig. 6A

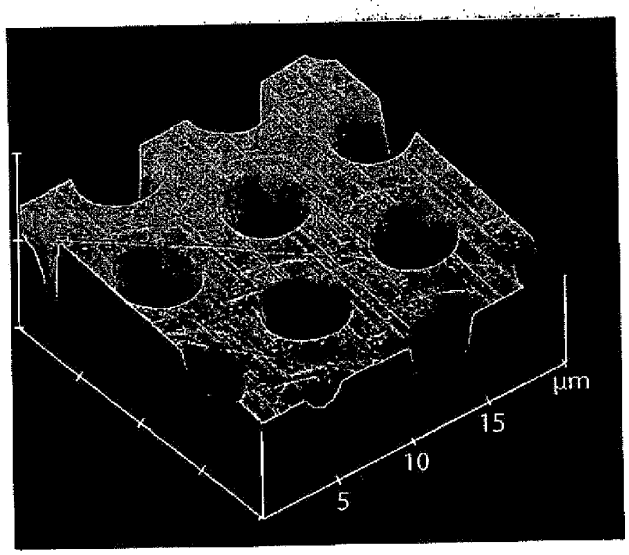


Fig. 6B

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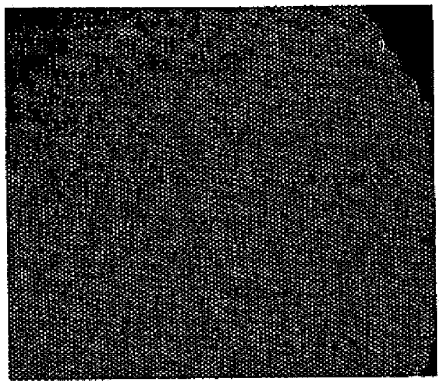


Fig. 7A

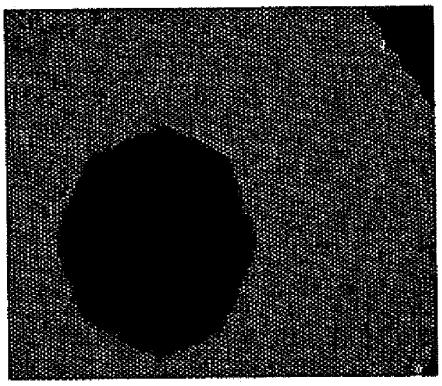


Fig. 7B

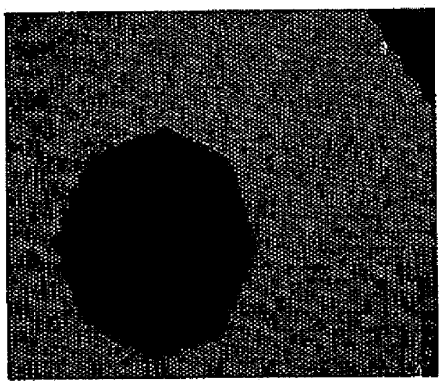


Fig. 7C

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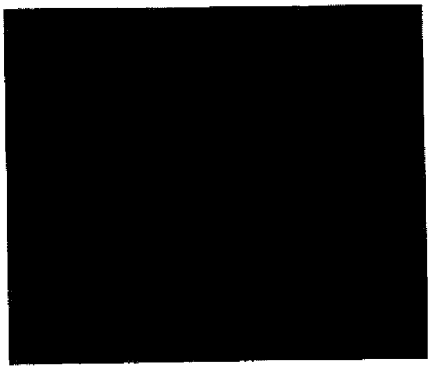


Fig. 8A

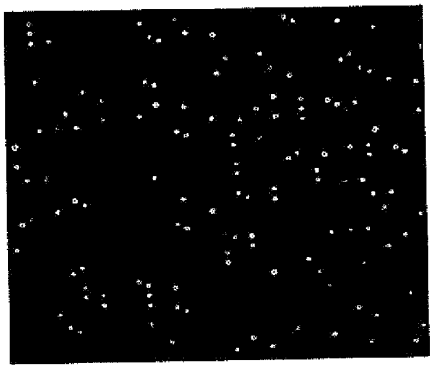


Fig. 8B



Fig. 8C

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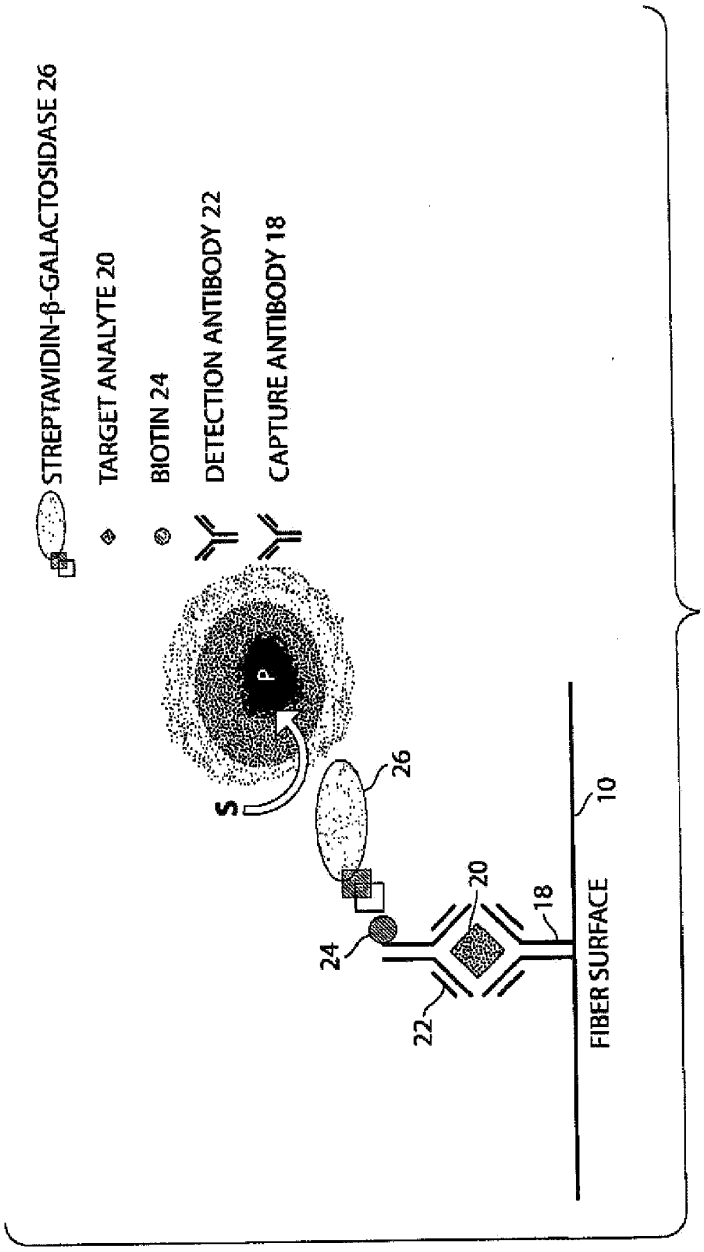


Fig. 9

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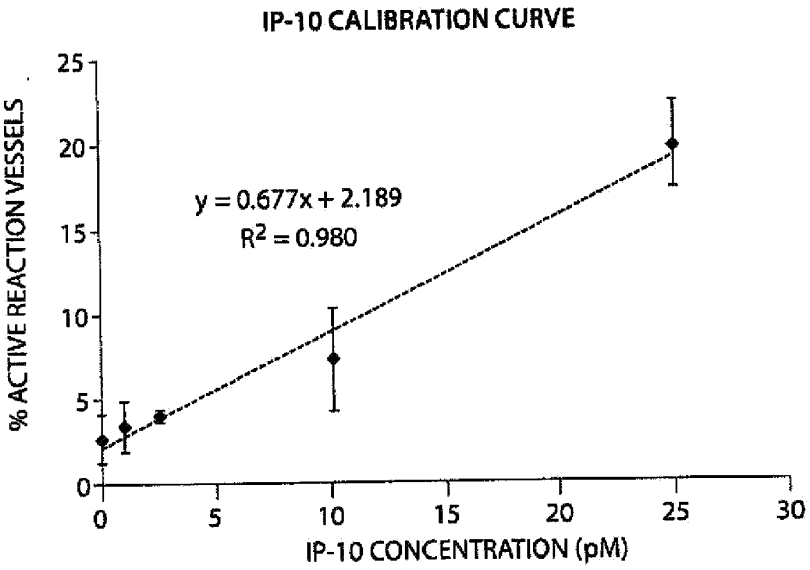


Fig. 10

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/019184

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/543 G01N33/50 G01N33/53

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RISSIN DAVID M ET AL: "Digital concentration readout of single enzyme molecules using femtoliter arrays and Poisson statistics." NANO LETTERS MAR 2006, vol. 6, no. 3, March 2006 (2006-03), pages 520-523, XP002483266 ISSN: 1530-6984 the whole document ----- -/--	1-35

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
6 June 2008	19/06/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Petri, Bernhard

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/019184

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RISSIN DAVID M ET AL: "Digital readout of target binding with attomole detection limits via enzyme amplification in femtoliter arrays." JOURNAL OF THE AMERICAN CHEMICAL SOCIETY 17 MAY 2006, vol. 128, no. 19, 17 May 2006 (2006-05-17), pages 6286-6287, XP002483267 ISSN: 0002-7863 the whole document	1-35
A	WO 99/58948 A (DDX INC [US]; STARZL TIMOTHY W [US]; CLARK SCOTT [US]; ROBINSON MARYBE) 18 November 1999 (1999-11-18) the whole document	1-35

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/019184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9958948 A	18-11-1999	AU 4190099 A	29-11-1999
		CA 2331787 A1	18-11-1999
		EP 1188059 A2	20-03-2002
		JP 2002526743 T	20-08-2002
		NZ 508062 A	26-04-2002

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(74) Agent: **ELSY, David**; WITHERS & ROGERS LLP, Goldings House, 2 Hays Lane, London SE1 2HW (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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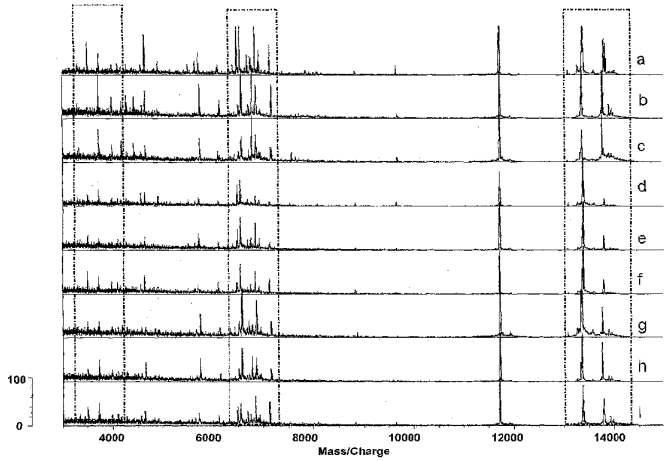
— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(88) Date of publication of the international search report:
25 February 2010

(54) Title: ASSAY



(57) Abstract: The invention relates to a mass spectroscopy dementia brain condition fingerprint for identifying the presence of a dementia brain condition and/or identifying the progression of a dementia brain condition comprising peaks derived from the following ions: ions derived from a proteolytically digested peptide the ions having (*m/z*) values of : 2048, 1434 and 1336 (+/- 0.02 %) and optionally one or more of the following additional ions, the additional ions having (*m/z*) values of : 2734, 1018, 3225 and/or 3121 (+/- 0.02 %). Methods of identifying a dementia brain disorder are also provided.

WO 2009/156747 A3

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/001624

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 G06F19/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N G06F

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

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

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABDI FADI ET AL: "Detection of biomarkers with a multiplex quantitative proteomic platform in cerebrospinal fluid of patients with neurodegenerative disorders" JOURNAL OF ALZHEIMER'S DISEASE, IOS PRESS, AMSTERDAM, NL, vol. 9, no. 3, 1 August 2006 (2006-08-01), pages 293-348, XP009090146 ISSN: 1387-2877 appendix I ----- -/--	5-10, 16-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 2009	22/12/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Rosin, Oliver

Form PCT/ISA/210 (second sheet) (April 2005)

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/001624

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Matharoo-Ball: "Computational Methods for the diagnosis of Alzheimer's disease from cerebro-spinal fluid by matrix assisted laser desorption-ionisation mass spectrometry (MALDI-MS)"[Online] 10 July 2007 (2007-07-10), XP002550116 Retrieved from the Internet: URL:http://www.tech.plym.ac.uk/spmc/pdf/CIMED2007/S3P7.pdf> [retrieved on 2009-10-13] figure 1	5-10, 16-22
A	Ball G. et al.: "Diagnosis of Alzheimer's disease from cerebro-spinal fluid"[Online] 24 July 2007 (2007-07-24), XP002550115 Retrieved from the Internet: URL:http://www.alzheimers-research.org.uk/assets/docs/20070718143555CIMED_programme.pdf> [retrieved on 2009-10-13] page 9	5
A	Matharoo-Ball B. et al.: Alzheimer's and Dementia, vol. 2, no. 3 Suppl 1, 2007, page S283, XP002550127 DOI: 10.1016/j.jalz.2006.05.1005 abstract	5

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-4

Rule 39.1(v) PCT - Presentation of information

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2009/001624

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-4
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5-6, 16-17(completely); 7-10, 18-22(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5-6, 16-17(completely); 7-10, 18-22(partially)

   Methods and entities in dementia brain conditions involving all of the markers SPARC-like 1, Fibrinogen alpha, Contactin-1 and Serum albumin at once.

   ---

2. claims: 7-10, 18-22(partially)

   Methods and kits for the detection of dementia brain disorder involving SPARC-like 1.

   ---

3. claims: 7-10, 18-22(partially)

   Methods and kits for the detection of dementia brain disorder involving Fibrinogen alpha.

   ---

4. claims: 7-10, 18-22(partially)

   Methods and kits for the detection of dementia brain disorder involving Contactin-1.

   ---

5. claims: 7-10, 18-22(partially)

   Methods and kits for the detection of dementia brain disorder involving Serum albumin.

   ---

6. claims: 11-15

   Kits and methods in dementia brain disease involving samples.

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DUFFY, David, C.** [GB/US]; 8 Aldersey Street, Unit 1, Somerville, MA 02143 (US). **RISSIN, David, M.** [US/US]; 568 Main Street, Medford, MA 02155 (US). **SONG, Linan** [CN/US]; 53 Cedar Street, Apt. 3206, Woburn, MA 01801 (US). **WALT, David, R.** [US/US]; 233 Marlborough Street, Boston, MA 02116 (US).

(74) Agent: **POMIANEK, Michael, J.**; Wolf, Greenfield & Sacks, P.C., Federal Reserve Plaza, 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: HIGH SENSITIVITY DETERMINATION OF THE CONCENTRATION OF ANALYTE MOLECULES OR PARTICLES IN A FLUID SAMPLE

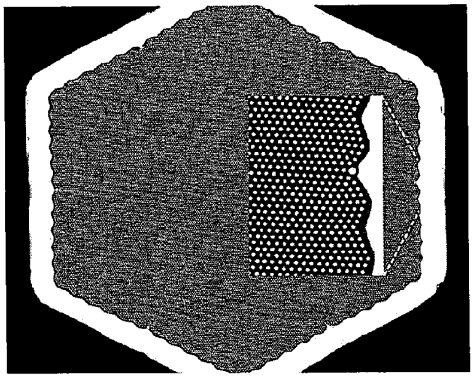


Fig. 6A

(57) Abstract: The present invention relates to methods, systems, and kits for detecting, quantifying and/or analyzing a fluid sample comprising molecules or particles at low concentration. In certain embodiments, the methods for detection and/or quantifying analyte molecules in a sample comprise capturing a plurality of analyte molecules on a substrate (e.g., an array comprising a plurality of reaction vessels). The substrate may then be exposed to additional reaction components such as at least one binding ligand. The substrate may additionally be exposed to a precursor labeling agent molecule, wherein the precursor labeling agent molecule, in some cases, is converted to a labeling agent molecule, which may be detected, either directly or indirectly, which determination may be related to the presence of and/or may be employed to quantify the analyte molecules. Although the various aspects of the present invention may use a number of different assay formats, in one embodiment, the assays are conducted in a plurality of reaction vessels defined, at least in part, by the distal ends of fiber optic strands.

WO 2010/039180 A2

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

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HIGH SENSITIVITY DETERMINATION OF THE CONCENTRATION OF ANALYTE MOLECULES OR PARTICLES IN A FLUID SAMPLE

Field of the Invention

The present invention relates to systems and methods for detecting analyte molecules or particles in a fluid sample and in some cases, determining a measure of the concentration of the molecules or particles in the fluid sample.

Related Applications

This application claims the benefit of U.S. Patent Application Serial No. 12/236,484, filed September 23, 2008, entitled "High Sensitivity Determination of the Concentration of Analyte Molecules or Particles in a Fluid Sample," by Duffy, *et al.*, herein incorporated by reference.

Background of the Invention

Methods and systems that are able to quickly and accurately detect and, in certain cases, quantify a target analyte molecule or particle in a sample are the cornerstones of modern analytical measurements. Such systems and methods are employed in many areas such as academic and industrial research, environmental assessment, food safety, medical diagnosis, and detection of chemical, biological and/or radiological warfare agents. Advantageous features of such techniques may include specificity, speed, and sensitivity.

Most current techniques for quantifying low levels of analyte molecules in a sample use amplification procedures to increase the number of reporter molecules in order to be able to provide a measurable signal. For example, these known processes include enzyme-linked immunosorbent assays (ELISA) for amplifying the signal in antibody-based assays, as well as the polymerase chain reaction (PCR) for amplifying target DNA strands in DNA-based assays. A more sensitive but indirect protein target amplification technique, called immunoPCR *(see* Sano, T.; Smith, C. L.; Cantor, *C. R. Science* 1992, *258,* 120-122), makes use of oligonucleotide markers, which can subsequently be amplified using PCR and detected using a DNA hybridization assay *(see* Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* 2003; *301,* 1884-1886; Niemeyer, C.

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M.; Adler, M.; Pignataro, B.; Lenhert, S.; Gao, S.; Chi, L. F.; Fuchs, H.; Blohm, *D. Nucleic Acids Research* 1999, *27,*4553-4561; and Zhou, H.; Fisher, R. J.; Papas, T. S. *Nucleic Acids Research* 1993, *21,* 6038-6039). While the immuno-PCR method permits low-level protein detection, it is a complex assay procedure, and can be prone to false-positive signal generation *(see* Niemeyer, C. M.; Adler, M.; Wacker, R. *Trends in Biotechnology* 2005, 23,208-216).

One disadvantage of many known methods and systems for accurately detecting and quantifying low concentrations of a particular analyte in solution is that they are based on ensemble responses in which many analyte molecules give rise to the measured signal. Most detection schemes require that a large number of molecules are present in the ensemble for the aggregate signal to be above the detection threshold. This disadvantage limits the sensitivity of most detection techniques and the dynamic range (i.e., the range of concentrations that can be detected). Many of the known methods and techniques are further plagued with problems of non-specific binding, which is the binding of analyte molecules/particles to be detected or reporter species non-specifically to sites other than those expected. This leads to an increase in the background signal, and therefore limits the lowest concentration that may be accurately or reproducibly detected.

Accordingly, improved methods for detecting and, optionally, quantifying analyte molecules or particles, especially in samples where such molecules or particles are present at very low concentration are needed.

Summary of the Invention

The present invention relates to systems and methods for detecting analyte molecules or particles in a fluid sample and in some cases, determining a measure of the concentration of the molecules or particles in the fluid sample. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In one aspect, the invention is directed towards a method. According to one set of embodiments, a method of detecting analyte molecules or particles in a fluid sample containing or suspected of containing analyte molecules or particles, comprises partitioning the fluid sample across an array comprising a plurality of reaction vessels, so

that at least some of the reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle, immobilizing at least one binding ligand with respect to an analyte molecule or particle within each reaction vessel containing at least one analyte molecule or particle, exposing the at least one binding ligand to a liquid in which is solubilized or suspended precursor labeling agent molecules, wherein exposure of the precursor labeling agent molecules to the at least one binding ligand converts at least some of the precursor labeling agent molecules into a labeling agent molecules which are insoluble in the liquid and/or which become immobilized within the reaction vessel, and determining from detecting the presence of the labeling agent molecules the number of reaction vessels which contain an analyte molecule or particle.

In another aspect, the invention is directed towards a system. According to one set of embodiments, a system for detecting analyte molecules or particles, comprises an array comprising a plurality of reaction vessels, wherein at least some of the reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle, at least one binding ligand immobilized with respect an analyte molecule or particle within each reaction vessel containing an analyte molecule or particle, and precursor labeling agent molecules solubilized or suspended in a liquid contained within the reaction vessels, wherein the precursor labeling agent molecules are able to convert upon exposure to a binding ligand to labeling agent molecules that are insoluble within the liquid and/or that become immobilized within reaction vessels containing a binding ligand.

In another embodiment, a method of detecting analyte molecules or particles in a fluid sample containing or suspected of containing analyte molecules or particles, comprises providing a fluid sample containing or suspected of containing analyte molecules or particles, immobilizing at least one binding ligand with respect to at least some of the analyte molecules or particles, exposing the at least one binding ligand to a liquid in which is solubilized or suspended precursor labeling agent molecules, wherein exposing the precursor labeling agent molecules to the at least one binding ligand converts at least some of the precursor labeling agent molecules into labeling agent molecules which are insoluble in the liquid and/or which become immobilized within the reaction vessel, and determining a measure of the concentration of the analyte molecules or particles in the fluid sample based on the detection of labeling agent molecules

wherein the true concentration of the analyte molecules or particles in the fluid sample is less than about 100×10^{-15} molar, and wherein the measure of the concentration determined in the determining act differs from the true concentration by no greater than 10%.

In another aspect, the invention is directed towards a kit. According to one set of embodiments, a kit for detecting analyte molecules or particles comprises an array comprising a plurality of reaction vessels, each reaction vessel having a volume not exceeding about 100 femtoliters and each reaction vessel containing at least one analyte capture component immobilized or able to become immobilized within the reaction vessels having binding specificity for the analyte molecules or particles, at least one binding ligand having binding specificity for the analyte molecules or particles, and precursor labeling agent molecules able to be solubilized or suspended in a liquid, wherein the precursor labeling agent molecules are able to convert upon exposure to the at least one binding ligand to labeling agent molecules that are insoluble within the liquid and/or that become immobilized within the reaction vessels.

In another set of embodiments, a method of detecting analyte molecules or particles in a fluid sample containing or suspected of containing analyte molecules or particles, comprises partitioning the fluid sample across an array comprising a plurality of reaction vessels, so that at least some of the reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle, immobilizing at least one binding ligand comprising a binding site with respect to an analyte molecule or particle within each reaction vessel containing at least one analyte molecule or particle, applying an enzymatic component to the array and capturing the enzymatic component with the binding site, contacting the enzymatic component with precursor labeling agent molecules, wherein the precursor labeling agent molecules are converted to labeling agent molecules upon contact with enzymatic components, detecting the labeling agent molecules, and determining from the detection of the labeling agent molecules the number of reaction vessels which contain an analyte molecule or particle.

Brief Description of the Drawings

FIG. 1 is a schematic flow diagram depicted a sequence of steps (A-G) for performing an assay according to some embodiments of one method of the present invention;

FIG. 2 is a schematic flow diagram depicting a sequence of steps (A-I) according to some embodiments of one method of the present invention;

FIGS. 3A-3D is a schematic flow diagram illustrating the conversion of a precursor labeling agent molecule into a labeling agent molecule and direct or indirect detection of the labeling agent molecule, according to some embodiments;

FIG. 4A is a schematic diagram depicting an experimental set-up for detection using light, according to one embodiment of the present invention;

FIG. 4B is a schematic diagram showing a fiber optic array that has been sealed with a sealing component, according to one embodiment;

FIG. 4C shows a photocopy of a photograph of an entire fiber optic array, according to one embodiment;

FIG. 5 is a schematic flow diagram depicting an embodiment of a method (steps A-D) for the formation of a plurality of reaction vessels through mating of a substrate and a sealing component and depicting examples of the size (panels E, F) of a sealing component relative to a substrate;

FIG. 6A is a photocopy of a microscopic photograph of an entire fiber optic array and an inset close-up of the bundle, according to one embodiment of the present invention;

FIG. 6B is a photocopy of an AFM image of a portion of an etched surface of a fiber optic array, according to one embodiment of the present invention;

FIG. 7 is a schematic diagram showing side view cross-sectional views of an etched fiber optic bundle that forms an array of microwells (panels A-D), according to one embodiment of the present invention, and depicting the localization of a capture component within a reaction vessel (panels E-G), according to some embodiments of the present invention;

FIG. 8 is a schematic flow diagram illustrating a non-limiting example of one assay method of the present invention;

FIGS. 9A and 9B are schematic diagrams showing a system employing and optical detection system of the present invention according to some embodiments;

FIG. 10 is a schematic block diagram showing a system employing a fiber optic assembly with an optical detection system according to an embodiment of the invention;

FIG. 11 shows a graph of a schematic calibration curve of a form that may be used to determine the concentration of an analyte molecule or particle in a fluid sample, according to some embodiments of the present invention;

FIGS. 12A and 12B show images of a fiber optic array analyzed in a non-limiting example of the present invention, with and without sealing of the array, respectively;

FIG. 13 shows a calibration curve determined when using an array that was sealed, according to one embodiment of the present invention;

FIG. 14 shows a calibration curve determined for a non-limiting example of the present invention;

FIG. 15 shows a plot of the number of active wells when using an array of the present invention for the detection of TNF-alpha, according to one embodiment.

Other aspects, embodiments and features of the invention will become apparent from the following detailed description when considered in conjunction with the accompanying drawings. The accompanying figures are schematic and are not intended to be drawn to scale. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. All patent applications and patents incorporated herein by reference are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Detailed Description

Systems and methods for the detection and/or quantification of analyte molecules, particles (such as, for example, cells, cell organelles and other biological or non-biological particulates) and the like, in a sample are described herein. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles. It should be understood, that while much of the discussion below is directed to analyte molecules, this is by way of example only, and other materials may be detected and/or quantified, for example, analyte particles. Particular examples of analytes will be discussed more below.

The systems and methods of the present invention, in certain embodiments, may help reduce the negative effects of non-specific binding on detection sensitivity when compared to typical conventional systems and methods for performing similar assays. Non-specific binding is the binding or association in a non-specific fashion of one component of an assay with another component of the assay with which it is not desirable that it interact. For example, association, binding, or immobilization of a binding ligand with the material forming a reaction vessel wall as opposed to with an analyte molecule or particle to which it has binding specificity. Non-specific binding may lead to false positive signals. Non-specific binding may not only affect the accuracy of the assay measurement, but may also limit the lowest level of detection. Therefore, methods and/or systems of the present invention, which in certain embodiments involve improvements in the level of non-specific binding, may allow for the detection and/or quantification of analyte molecules in a sample at a lower detection limit as compared to typical current technologies.

In certain embodiments, the methods for detection and/or quantifying analyte molecules in a sample comprise capturing a plurality of analyte molecules on a substrate (e.g., an array comprising a plurality of reaction vessels). The substrate may then be exposed to additional reaction components such as at least one binding ligand, as discussed more below. The substrate may additionally be exposed to a precursor labeling agent molecule, wherein the precursor labeling agent molecule, in some cases, is converted to a labeling agent molecule, which may be detected, either directly or indirectly, which determination may be related to the presence of and/or may be employed to quantify the analyte molecules.

An exemplary assay method according to some embodiments of the present invention is illustrated in FIG. 1. In this particular non-limiting example, only a first binding ligand is provided; however, in other embodiments, additional binding ligands could be employed, as described in more detail below. Substrate 200 comprising a capture component 202 is exposed to analyte molecule 204 (step (A)). Analyte molecule 204 associates with capture component 202, as indicated by arrow 201, thereby forming analyte molecule-capture component complex 206 (step (B)). The substrate is then exposed to binding ligand 208 and binding ligand 208 may associate with analyte molecule 204, as indicated by arrow 203, thereby forming analyte molecule-capture component-binding ligand complex 210 (step (C)). Binding ligand 208 may comprise a

component 209 which is capable of converting a precursor labeling agent molecule into a labeling agent molecule. The reaction vessel may then be exposed to precursor labeling agent molecule 212, as indicated by arrow 205, which upon exposure to binding ligand 208, is converted to labeling agent molecule 214, as indicated by arrow 213 (step (D)). The labeling agent molecule may be insoluble and, optionally, forms a precipitate and/or become immobilized with respect to the substrate, as discussed herein. Various assay methods and suitable reaction components (e.g., analyte molecules or particles, binding ligands, precursor labeling agent molecules, labeling agent molecules, etc.) are discussed more herein.

In certain embodiments, precursor labeling agent molecules are converted into labeling agent molecules upon exposure to a binding ligand. A "precursor labeling agent molecule" is any molecule, particle, or the like, that is able to be converted to a labeling agent molecule upon exposure to a suitable binding ligand. In some cases, the binding ligand may comprise a converting agent to promote the conversion. For example, the binding ligand may comprise an enzymatic component, a gold nanoparticle, etc. A "labeling agent molecule" is any molecule, particle, or the like, that facilitates detection, by acting as the detected entity, using a chosen detection technique.

In some embodiments, a precursor labeling agent molecule is converted into a labeling agent molecule that is insoluble. For example, as shown in FIG. 1, labeling agent molecule 214 is insoluble and precipitates (Step (E)), as indicated by arrow 207.

In other embodiments, a precursor labeling agent molecule is converted into a labeling agent molecule that is immobilized with respect to the substrate. In some cases, the labeling agent molecule will be immobilized with respect to the substrate by associating with a labeling agent capture component which is associated with the substrate. For example, the surfaces within the reaction vessels (including microwells defined in fiber optical bundles) may incorporate at least one labeling agent capture component. For example, as shown in FIG. 1, Step (G), substrate 200 comprises at least one labeling agent capture component 216. Labeling agent molecule 214 becomes associated with labeling agent capture component 216, as indicated by arrow 211, and is immobilized with respect to substrate 200.

In certain cases, the labeling agent molecule will be immobilized within the reaction vessel by associating with a binding ligand. For example, the labeling agent molecule may be able to associate or interact with the binding ligand such that the

labeling agent molecule is immobilized within the reaction vessel. For example, as shown in FIG. 1, Step (F), labeling agent molecule 214 becomes associated with binding agent 208, as indicated by arrow 209.

In some embodiments of the present invention, more than one binding ligand may be provided in an assay method. A "binding ligand," as used herein, is any molecule, particle, or the like which specifically binds to or otherwise specifically associates with an analyte molecule or another molecule that binds to or otherwise associates with the analyte molecule (e.g., another binding ligand). In instances where a first and a second binding ligand are be employed in any given assay method, the first binding ligand is able to associate with an analyte molecule and the second binding ligand is able to associate with the first binding ligand. When the substrate is exposed to a plurality of types of binding ligands, at least some of the plurality of immobilized complexes may additionally comprise, in some cases, at least one of each type of binding ligand. In certain embodiments, the binding ligand can be exposed to the substrate after capture of the analyte molecule so that the binding ligand binds to the immobilized complex. In other embodiments, the binding ligand may become associated with the analyte molecule to form a complex followed by capture of the complex by the substrate to form the immobilized complex. The binding ligands may be provided in certain embodiments in an amount sufficient such that at least one of each type of binding ligand comes into contact with every immobilized analyte molecule on the binding surface of the plurality of reaction vessels. At least one type of binding ligand may in certain embodiments comprise a converting agent which promotes conversion of a precursor labeling agent molecule into a labeling agent molecule, as discussed more herein.

In certain embodiments, a first and a second binding ligand may be used. The first binding ligand may associate with the analyte molecule and the second binding ligand may associate with the first binding ligand. For example, as shown in FIG. 2, substrate 80 comprising capture component 82 is exposed to analyte molecule 84 (step (A)). Analyte molecule 84 associates with capture component 82, as indicated by arrow 81 (step (B)). Substrate 80 is then exposed to first binding ligand 86 and first binding ligand 86 associates with analyte molecule 84, as indicated by arrow 83 (step (C)). Substrate 80 is then exposed to second binding ligand 88 which associates with first binding ligand 86, as indicated by arrow 85 (step (D)). At least one of first or second binding ligand may comprise a converting agent. The substrate is additionally exposed

to precursor labeling agent molecule 90, as indicated by arrow 87, which upon exposure to converting agent comprised in either first or second binding ligand, is converted to labeling agent molecule 92, as indicated by arrow 89 (step (E)). The labeling agent molecule may be insoluble and/or immobilized with respect to substrate 80, as discussed more herein. For example, as indicated by arrow 91, labeling agent molecule 92 may be immobilized in the reaction vessel by associating with labeling agent capture component 94 (step (F)). In other instances, labeling agent 92 may associate with a binding ligand, for example, second binding ligand 88, as indicated by arrow 93 (step (G)), or first binding ligand 86, as indicated by arrow 95 (step (H)). In yet other instances, labeling agent molecule 92 may be insoluble and remain in the reaction vessel, as indicated by arrow 97 (step (I)). In some instances, at least two, at least three, at least four, at least five, at least eight, at least ten, or more, types of binding ligands may be provided. In some cases, at least one binding ligand may comprise a binding side, wherein the binding site is capable of capturing an enzymatic component.

In some embodiments, the method comprises partitioning a fluid sample containing or suspected of containing at least one analyte molecule or particle across a plurality of reaction vessels. The plurality of reaction vessels may comprise an array, for example, a fiber optic array. The fluid sample may be partitioned such that at least some of the reaction vessels contain at least one or, in certain cases, one analyte molecule or particle, and at least some of the reaction vessels contain zero analyte molecules or particles, at discussed more herein. In some embodiments, the fluid sample may be partitioned such that a statistically significant fraction of reaction vessels contains about at least one or, in certain cases, one analyte molecule or particle and a statistically significant fraction of reaction vessels contains zero analyte molecules or particles.

It should be understood, that while most of the discussion herein primarily focuses on analyte molecules, this is by way of example only, and other materials may be detector or quantified, for example, analyte particles, such as cells, subcellular organelles and/or non-biological particles. Other example of analyte molecules are discussed more herein.

In some embodiments, the plurality of analyte molecules will be immobilized with respect to a substrate. In particular embodiments, the substrate comprises an array of reaction vessels. The plurality of reaction vessels are exposed to a fluid (e.g. a liquid) sample containing or suspected of containing at least one analyte molecule of interest. In

some cases, the analyte molecules are immobilized in the reaction vessel (or otherwise with respect to the substrate). The analyte molecule may be immobilized in the reaction vessel by association with a capture component on a binding surface of a reaction vessel. For example, in the context of a substrate comprising microwells, the interaction between any analyte molecule and the capture component on the binding surface of a microwell results in immobilization of the analyte molecule within that microwell. For example, the analyte molecule may be immobilized by a capture component on the binding surface within each reaction vessel under conditions suitable for capture of the analyte molecule by at least one of the capture components (e.g., physiological conditions).

A "capture component", as used herein, is any molecule, other chemical/biological entity or solid support modification disposed upon a solid support that can be used to specifically attach, bind or otherwise capture molecules or particles, such as an analyte molecule, labeling agent molecule, etc. such that the molecule becomes immobilized with respect to the capture component and solid substrate. As used herein, "immobilized" means captured, attached, bound, or affixed so as to prevent dissociation or loss of the target molecule/particle, but does not require absolute immobility with respect to either the capture component or the solid substrate. Capture components which are useful or potentially useful for practicing certain aspects and embodiments of the invention are discussed in more detail below. The term "analyte agent capture component" is sometimes used herein, for clarity to refer to a capture component having specific affinity for an analyte molecule or particle. Similarly, the term "labeling agent capture component" is sometimes used to refer to a capture component having specific affinity for a labeling agent. At least some of the analyte molecules, upon exposure to the plurality of reaction vessels comprising a plurality of analyte capture components, can become immobilized with respect to analyte capture components, thereby forming a plurality of immobilized complexes. For example, in certain embodiments, substantially all of the plurality of analyte molecules may become immobilized with respect to capture components such that essentially each of the plurality of immobilized complexes comprises a capture component and an analyte molecule.

In certain embodiments, the plurality of analyte molecules are partitioned across a plurality of reaction sites, such as, for example, a plurality of reaction vessels (e.g., in

an array format). The plurality of reaction vessels may be formed in or of any suitable material, and in some cases, the reaction vessels can be sealed or may be formed upon the mating of a substrate with a sealing component, as discussed in more detail below. In certain embodiments, especially where quantification of the analyte molecules is desired, the partitioning of the analyte molecules is performed such that at least some reaction vessels comprise at least one or, in certain cases, only one analyte molecule and at least some reaction vessel comprise no analyte molecules. The analyte molecules may be detected in certain embodiments, thereby allowing for the detection and quantification of the analyte molecule in the fluid sample by techniques described in more detail below.

Certain methods of the present invention may be useful for characterizing analyte molecules in a sample. In some cases, the methods may be useful for detecting and/or quantifying analyte molecules in a fluid sample which is suspected of containing at least one analyte molecule, since, as explained in more detail below, the number of reaction vessels which contain one or more of the analyte molecules can be correlated to the concentration of analyte molecules in the fluid sample (e.g., the number of reaction vessels which comprise an analyte molecule can be related to a measure of the concentration of the analyte molecules in the sample) under certain conditions. Certain embodiments of present invention thus can provide a measure of the concentration of analyte molecules in a fluid sample based on the proportion of reaction vessels which contain an analyte molecule. Specific methods and calculations of how to quantify analyte molecules in a fluid sample using embodiments of the invention are discussed more below.

In some embodiments, the plurality of reaction vessels (or substrate) may be washed at least once. In one instance, the plurality of reaction vessels may be washed after contacting the array with a solution comprising analyte molecules, binding ligands, precursor labeling agent molecules, or the like. In this instance, the wash step may be used to wash away any molecules that are not immobilized with respect to the plurality of reaction vessels. The wash step may be performed by any method known to those skilled in the art, for example, by placing the plurality of reaction vessels in a wash solution. In some cases, the wash solution may be a solution that does not cause change to the surface of the plurality of reaction vessels or the interaction between at least two components of the assay (e.g., a capture component and an analyte molecule).

The methods of certain embodiments of the present invention may be used to characterize analyte molecules in the fluid sample. In some cases, the methods may be used to detect and/or quantify analyte molecules in a fluid sample which is suspected of containing at least one analyte molecule. That is, there is correlation between the numbers of reaction vessels which containing one or more analyte molecules and the concentration of analyte molecules in the fluid sample. Certain embodiments of present invention allow for the quantification of the amount of analyte molecules in a fluid sample based on the percentage of reaction vessels determined to contain an analyte molecule. Specific methods and calculations of how to quantify analyte molecules in a fluid sample are discussed more herein.

In some embodiments, the number of reaction vessels which contain an analyte molecule is determined by determining the number of reaction vessels which contain at least one labeling agent molecule. That is, the number of reaction vessels which contain a labeling agent molecule is proportional to the number of reaction vessels which contain an analyte molecule. The method of detecting the number of reaction vessels which contain labeling agent molecules will depend on the labeling agent being detected. For example, the labeling agent molecule may be an optical label or a spectroscopic or radiolabel, etc.

A labeling agent molecule may be detected directly or indirectly. A labeling agent molecule is detected directly if the labeling agent molecule itself is detected by direct interrogation of a reaction vessel. For example, the precursor labeling agent molecule may be converted to a labeling agent molecule that is fluorescent, chemiluminescent, forms a precipitate, or the like. A non-limiting example of direct detection is depicted in FIG. 3A. Precursor labeling agent molecule 100 is converted, as indicated by arrow 101, to labeling agent molecule 102 which may be detected directly using a suitable detection system.

In other instances, the labeling agent molecule may be detected indirectly, for example, in instances where the labeling agent molecule itself is not directly detectable. A precursor labeling agent molecule may be converted into a labeling agent molecule and then the labeling agent molecule may be exposed to a labeling agent reactant. In some cases, the labeling agent reactant may be converted to a detectable product (e.g., a fluorogenic product) upon exposure to the labeling agent molecule. For example, as shown in FIG. 3B, a precursor labeling agent molecule 104 is converted to labeling agent

molecule 106, as indicated by arrow 105, which is not directly detectable. Labeling agent molecule 106 is exposed to a labeling agent reactant 108, which is converted to a detectable product 110, as indicated by arrow 109. In some cases, however, the labeling agent reactant may be detectable directly (e.g., without conversion into a detectable product). For example, the labeling agent reactant may be fluorogenic, chromogenic, etc. molecule/particle/compound which is able to associate with the labeling agent molecule. For example, as shown in FIG. 3D, a precursor labeling agent molecule 113 is converted to labeling agent molecule 119, as indicated by arrow 117, which is not directly detectable. Labeling agent molecule 119 is exposed to a labeling agent reactant 122, which associates with labeling agent molecule and comprises a detectable component. In other cases, the labeling agent molecule may be exposed to a first labeling agent reactant and a second labeling agent reactant. The first labeling agent reactant may associate with the binding ligand and the second labeling agent reactant may be converted to a detectable product upon exposure to the first labeling agent reactant. For example, as shown in FIG. 3C, precursor labeling agent molecule 112 is converted to labeling agent molecule 114, as indicated by arrow 111. Labeling agent molecule 114 is exposed to a first labeling agent reactant, 116, as indicated by arrow 115, wherein first labeling agent reactant 116 associates with labeling agent molecule 114. First labeling agent reactant 116 is then exposed to second labeling agent reactant 118, which is converted to detectable product 120 as indicated by arrow 117. In a particular embodiment, the first labeling agent reactant may comprise an enzymatic component and the second labeling agent reactant may comprise an enzymatic substrate. For example, the first labeling agent reactant may become immobilized with respect to the binding ligand. The immobilized first labeling agent reactant may be exposed to a second labeling agent reactant (e.g., an enzymatic substrate) and the enzymatic substrate may be converted to a detectable product.

In certain embodiments, the systems and/or methods of the present invention can be employed in an assay using a first and a second substrate, wherein the first substrate may comprise beads or a microtitre plate and the second substrate is configured as describe herein. In certain such assays, the analyte molecules or particles are contacted with the first substrate, and a dissociated reporter molecule/particle is then released from the first substrate, which is then detected using the second substrate. For example, the systems and/or methods of the present invention may be used in combination with the

systems and/or methods as described in commonly owned U.S. Patent Application Serial No. 12/236,490, filed September 23, 2008, entitled "Ultra-Sensitive Detection of Enzymes by Capture-And-Release Followed by Quantification" by Duffy, *et al.*; U.S. Patent Application Serial No. 12/236,488, filed September 23, 2008, entitled "Ultra-Sensitive Detection of Molecules by Capture-And-Release Using Reducing Agents Followed by Quantification" by Duffy, *et al.*; and U.S. Patent Application Serial No. 12/236,486, filed September 23, 2008, entitled "Ultra-Sensitive Detection of Molecules On Single Molecule Arrays" by Duffy, *et al.*

One of ordinary skill in the art will appreciate that the range of materials that may be employed in the discussed methods is numerous and the following sections provide a broad overview of only a non-limiting list of exemplary materials and techniques.

Arrays of Reaction Vessels

Certain embodiments of the present invention utilize an array of reaction vessels to carry out steps in an assay utilized to determine the concentration of an analyte molecule of interest. An array of reaction vessels allows a fluid sample to be partitioned into a plurality of discrete reaction volumes during one or more steps in an assay. In some embodiments, the reaction vessels may all have approximately the same volume.

In other embodiments, the reaction vessels may be of differing volumes. The volume of each individual reaction vessel can range for different embodiments from attoliters or smaller to nanoliters or larger depending upon the nature of analyte molecules, the detection technique and equipment employed, and the expected concentration of the analyte molecules in the fluid applied to the array for analysis. In one embodiment, the size of the reaction vessel may be selected such that at the concentration of interest, between zero and ten analyte molecules or particles would be statistically expected to be found in each reaction vessel. In a particular embodiment, the volume of the reaction vessel is selected such that at the concentration of interest, either zero or one analyte molecules or particles would be statistically expected to be found in each reaction vessel. In accordance with one embodiment of the present invention, the reaction vessels may have a volume between about 1 femtoliter and about 1 picoliter, between about 10 femtoliters and about 100 femtoliters, between about 10 attoliters and about 50 picoliters, between about 1 picoliter and about 50 picoliters, between about 1 femtoliter and about 1 picoliter, between about 30 femtoliters and about 60 femtoliters,

or the like. In some cases, the reaction vessels have a volume of less than about 1 picoliter, less than about 500 femtoliters, less than about 100 femtoliters, less than about 50 femtoliters, less than about 1 femtoliter, or the like. In some cases, the reaction vessels have a volume of about 10 femtoliters, about 20 femtoliters, about 30 femtoliters, about 40 femtoliters, about 50 femtoliters, about 60 femtoliters, about 70 femtoliters, about 80 femtoliters, about 90 femtoliters, or of about 100 femtoliters.

For embodiments employing an array of reaction vessels, the number of reaction vessels in the array will depend on the composition and end use of the array. Arrays containing from about 2 to many billions of reaction vessels can be made by utilizing a variety of techniques and materials. Increasing the number of reaction vessels in the array can be used to increase the dynamic range of an assay or to allow multiple samples or multiple types of analyte molecules to be assayed in parallel. Generally, the array will comprise between one thousand and one million reaction vessels per sample to be analyzed. In some cases, the array will comprise greater than one million reaction vessels. In some embodiments, the array will comprise between about 1,000 and about 50,000, between about 1,000 and about 1,000,000, between about 1,000 and about 10,000, between about 10,000 and about 100,000, between about 100,000 and about 1,000,000, between about 1,000 and about 100,000, between about 50,000 and about 100,000, between about 20,000 and about 80,000, between about 30,000 and about 70,000, between about 40,000 and about 60,000, or about 50,000, reaction vessels.

The array of reaction vessels may be arranged on a substantially planar surface or in a non-planar three-dimensional arrangement. The reaction vessels may be arrayed in a regular pattern or may be randomly distributed. In a specific embodiment, the array is a regular pattern of sites on a substantially planar surface permitting the sites to be addressed in the X-Y coordinate plane.

In some embodiments, the reaction vessels are formed in a solid material. As will be appreciated by those in the art, the number of potentially suitable materials in which the reaction vessels can be formed is very large, and includes, but is not limited to, glass (including modified and/or functionalized glass), plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon®, polysaccharides, nylon or nitrocellulose, etc.), composite materials, ceramics, silica or silica-based materials (including silicon and modified silicon), carbon, metals, optical fiber bundles, or the like. In general, the

substrate material may be selected to allow for optical detection without appreciable autofluorescence. In certain embodiments, the reaction vessels may be formed in a flexible material. In yet other embodiments, the reaction vessels may be formed in a material that is both flexible and solid.

In some embodiments of the present invention, the plurality of reaction vessels may be formed through the mating of the substrate and a sealing component, wherein at least one of the substrate and the sealing component comprises a plurality of microwells. In some cases, an array comprises a plurality of depressions in a first surface of the substrate (e.g., a support material). The sealing component may comprise a second surface with the same or different topology as the first surface may be brought into contact with the first surface to create a plurality of sealed reaction vessels. Either the first surface or the second surface may be fabricated from a compliant material to aid in sealing. Either or both of the surfaces may be hydrophobic or contain hydrophobic regions to minimize leakage of aqueous samples from the microwells. In some cases, the sealing component may be capable of contacting the exterior surface of an array of microwells (e.g., the cladding of a fiber optic bundle as shown in FIG. 4B) such that each reaction vessel thus formed is sealed or isolated such that the contents of each reaction vessel cannot escape the reaction vessel. According to one embodiment, the sealing component may be a silicone elastomer gasket that may be placed against an array of microwells with application of uniform pressure across the entire substrate. In some cases, the reaction vessels may be sealed after the addition of an analyte molecule and, optionally, a precursor labeling agent molecule to facilitate detection of the analyte molecules. For embodiments employing precursor labeling agent molecules, by sealing the contents in some of each reaction vessel, a reaction to produce the detectable labeling agent molecule can proceed within the sealed reaction vessels, thereby producing a detectable amount of a labeling agent molecule that is retained in the reaction vessel for detection purposes. In some cases, the plurality of reaction vessels formed on a substantially planar substrate upon the mating of at least a portion of a sealing component comprising a plurality of microwells and at least a portion of the substantially planar substrate (e.g., see FIG. 5B below).

Non-limiting embodiments of the formation of a plurality of reaction vessels on a substrate are depicted in FIG. 5. FIG. 5, panel (A) shows a surface comprising a plurality of microwells 139, which have been exposed to a fluid sample 141, and a

sealing component 143. Sealing component 143 in this example comprises a substantially planar bottom surface. Mating of the microwell containing surface 139 with sealing component 143 forms a plurality of sealed reaction vessels 145. The areas between the reaction vessels 148 may be modified to aid in the formation of a tight seal between the reaction vessels.

A second embodiment is shown in FIG. 5, panel (B), in which sealing component 162 comprising a plurality of microwells 163 is mated with a substantially planar surface 158 which has been exposed to fluid sample 162, thereby forming a plurality of reaction vessels 164.

In a third embodiment, as shown in FIG. 5, panel (C), substrate surface 166 comprising a plurality of microwells 167 is mated with sealing component 170 also comprising a plurality of microwells 171. In this embodiment, the microwells in the substrate and the microwells in the sealing components are substantially aligned so each reaction vessel 172 formed comprises a portion of the microwell from the sealing component and a portion of a microwell from the substrate. In FIG. 5, panel (D), the microwells are not aligned such that each reaction vessel comprises either a microwell from the sealing component 173 or a microwell from the substrate 175.

The sealing component may be essentially the same size as the substrate or may be different in size. In some cases, the sealing component is approximately the same size as the substrate and mates with substantially the entire surface of the substrate. In other cases, as depicted in FIG. 5, panel (E), the sealing component 176 is smaller than the substrate 174 and the sealing component only mates with a portion 178 of the substrate. In yet another embodiment, as depicted in FIG. 5, panel (F), the sealing component 182 is larger than the substrate 180, and only a portion 184 of the sealing component mates with the substrate 180.

Individual reaction vessels may contain a binding surface. The binding surface may comprise essentially the entirety or only a portion of the interior surface of the reaction vessel or may be on the surface of another material or object that is confined within the reaction vessel, such as, for example, a bead, or a particle (for example, a micro-particle or a nanoparticle).

A microwell in a surface (e.g., substrate or sealing component) may be formed using a variety of techniques known in the art, including, but not limited to, photolithography, stamping techniques, molding techniques, etching techniques, or the

like. As will be appreciated by those of the ordinary skill in the art, the technique used will depend on the composition and shape of the supporting material and the size and number of reaction vessels.

In a particular embodiment, an array of reaction vessels is formed by creating microwells on the end of a fiber optic bundle and utilizing a substantially planar compliant surface as a sealing component. In certain such embodiments, an array of reaction vessels in the end of a fiber optic bundle may be formed as follows. First, an array of microwells is etched into one end of a polished fiber optic bundle. Techniques and materials for forming and etching a fiber optic bundle are known to those of ordinary skill in the art. For example, the diameter of the optical fibers, the presence, size and composition of core and cladding regions of the fiber, and the depth and specificity of the etch may be varied by the etching technique chosen so that microwells of the desired volume may be formed. In certain embodiments, the etching process creates microwells by preferentially etching the core material of the individual glass fibers in the bundle such that each well is approximately aligned with a single fiber and isolated from adjacent wells by the cladding material. Potential advantages of the fiber optic array format is that it can produce thousands to millions of reaction vessels without complicated microfabrication procedures and that it can provide the ability to observe and optically address many reaction vessels simultaneously. An example of an etched fiber optic array is shown in FIGS. 6A and 6B. FIG. 6A shows a fiber optic array that has been etched to form a plurality or reaction vessels. In this particular example, the wells have a diameter of approximately 4.5 microns and a volume of about 46 fL. FIG. 6B shows an AFM image of a portion of the fiber optic array of FIG. 6A. FIG. 4C also shows another picture of a fiber optic array.

Each microwell may be aligned with an optical fiber in the bundle so that the fiber optic bundle can carry both excitation and emission light to and from the wells, enabling remote interrogation of the well contents. Further, an array of optical fibers may provide the capability for simultaneous or non-simultaneous excitation of molecules in adjacent vessels, without signal "cross-talk" between fibers. That is, excitation light transmitted in one fiber does not escape to a neighboring fiber.

In certain embodiments of the present invention, the physical alterations to a fiber optic may be made as taught in U.S. Patents 6,023,540, 6,327,410, and 6,858,394. Any one or more of the surface of the glass microwells, the surface of the sealing component,

or particles within microwells can be functionalized in certain embodiments to create binding surface(s).

Alternatively, the equivalent structures can be fabricated using other methods that do not utilize the ends of an optical fiber bundle as a substrate. For example, the array may be a spotted, printed or photolithographically fabricated substrate produced by techniques known in the art; see for example WO95/25116; WO95/35505; PCT US98/09163; U.S. Patent Nos. 5,700,637, 5,807,522, 5,445,934, 6,406,845, and 6,482,593.

In certain embodiments, the present invention provides a system equipped with a mechanical platform that applies a sealing component to a substrate. The platform may be positioned beneath a stage on the system. After the chosen reaction components have been added to an array of reaction vessels, the sealing component may be mated with the array. For example, the sealing component may be sandwiched between a flat surface (such as, for example, a microscope slide) and the array of reaction vessels using uniform pressure applied by the mechanical platform.

A non-limiting embodiment of such equipment is illustrated in FIG. 4A. A sealing component 300 is placed on top of mechanical platform 302. The fluid sample 304 is placed on top of the sealing component 300. The mechanical platform is moved upwards towards the array 306 (e.g., fiber optic array) such that uniform pressure is applied. As shown in FIG. 4B, the sealing component 300 forms a tight seal with the array 306. In other instances, varying pressure may be applied to the sealing component to form a tight seal between the sealing component and the array. The system may also comprise additional components 312 that may be utilized to analyze the array (e.g., microscope, computer, etc.) as discussed more herein.

In one embodiment of the present invention, the array of reaction vessels may be sealed. The array may be sealed by contacting a sealing component with the face of an array of reaction vessels, thereby fluidically isolating each reaction vessel and sealing its contents therein. In some cases, an array comprises a plurality of depressions in a first surface of a support material. The sealing component has a second surface with the same or different topology as the first surface may be brought into contact with the first surface to create an array of sealed reaction vessels. Either the first surface or the second surface may be fabricated from a compliant material to aid in sealing. Either or both

surfaces may be hydrophobic or contain hydrophobic regions to minimize leakage from the microreactors of aqueous components.

A "sealing component," as used herein, is defined as any material or device large enough to cover at least a portion, or in certain cases, an entire array of reaction vessels, and which is capable of contacting the exterior surface of the array of reaction vessels (e.g., the cladding of a fiber optic bundle) such that each reaction vessel thus formed is sealed or isolated such that the contents of each reaction vessel cannot escape the reaction vessel under assay conditions employed. According to one embodiment, the sealing component is a silicone elastomer gasket that is placed against the array of reaction vessels with application of uniform pressure across the entire substrate. In some cases, the reaction vessels may be sealed after the addition of precursor labeling agent molecules and/or reactants. By sealing the contents in each reaction vessel, the reaction may proceed within the reaction vessel thus formed, thereby producing a detectable amount of a detectable product that is retained in the reaction vessel for detection purposes. For example, the labeling agent molecule may convert a substrate into a chromogenic, fluorogenic, or chemiluminescent product that builds up to a locally high concentration in each sealed reaction vessel, thereby generating a detectable chromogenic, fluorogenic, or chemiluminescent signal in the reaction vessel.

In some embodiments, the array to which the analyte molecules or particles are immobilized may be subjected to at least one washing step. In one instance, an array may be washed after exposing the substrate to one or more solutions comprising analyte molecules, binding ligands, precursor labeling agent molecules, etc. In some instances, the wash step(s) may be used to wash away any analyte molecules or non-analyte molecules, any binding ligands or non-binding ligands, or any precursor labeling agent molecules or non-precursor labeling agent molecules that are not immobilized with respect to the substrate. The wash step(s) may be performed by any suitable technique known to those of ordinary skill in the art, for example, by submersion of the substrate in a wash solution, flushing the substrate with a wash solution, etc. In certain embodiments, the wash solution is selected so that it does not cause appreciable change to the configuration of the substrate surface and/or does not disrupt an interaction between at least two components of the assay (e.g., a capture component and an analyte molecule). In other cases, the wash solution may be a solution that is selected to chemically interact with one or more components of the substrate. As will be understood

by those of ordinary skill in the art, a wash step may be performed at any appropriate time point during the inventive methods (e.g., after exposure of the array to a reagent or after immobilization of and agent with respect to an array) during a method of the present invention.

Exemplary Target Analytes

As will be appreciated by those in the art, a large number of analyte molecules and particles may be detected and, optionally, quantified using methods and systems of the present invention; basically, any analyte molecule or particle that is able to be made to become immobilized with respect to (e.g., by binding) a capture component can be potentially investigated using the invention. Certain more specific targets of potential interest that may comprise an analyte molecule or particle are mentioned below. The list below is exemplary and non-limiting.

In some embodiments, the analyte molecule may be an enzyme. Non-limiting examples of enzymes include an oxidoreductase, transferase, kinase, hydrolase, lyase, isomerase, ligase, and the like. Additional examples of enzymes include, but are not limited to, polymerases, cathepsins, calpains, amino-transferases such as, for example, AST and ALT, proteases such as, for example, caspases, nucleotide cyclases, transferases, lipases, enzymes associated with heart attacks, and the like. When a system/method of the present invention is used to detect the presence of viral or bacterial agents, appropriate target enzymes include viral or bacterial polymerases and other such enzymes, including viral or bacterial proteases, or the like.

In other embodiments, the analyte molecule or particle may comprise an enzymatic component. For example, the analyte particle can be a cell having an enzyme or enzymatic component present on its extracellular surface. Alternatively, the analyte particle is a cell having no enzymatic component on its surface. Such a cell is typically identified using an indirect assaying method described below. A non-limiting example of an enzymatic component is beta-galactosidase.

In yet other embodiments, the analyte molecule may be a biomolecule. Non-limiting examples of biomolecules include hormones, antibodies, cytokines, proteins, nucleic acids, lipids, carbohydrates, lipids cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, or combinations thereof. Non-limiting embodiments of proteins include peptides, polypeptides, protein

fragments, protein complexes, fusion proteins, recombinant proteins, phosphoproteins, glycoproteins, lipoproteins, or the like. As will be appreciated by those in the art, there are a large number of possible proteinaceous analyte molecules that may be detected or evaluated for binding partners using the present invention. In addition to enzymes as discussed above, suitable protein analyte molecules include, but are not limited to, immunoglobulins, hormones, growth factors, cytokines (many of which serve as ligands for cellular receptors), cancer markers, etc.

In certain embodiments, the analyte molecule may be a host-translationally modified protein (e.g., phosphorylation, methylation, glycosylation) and the capture component may be an antibody specific to a post-translational modification. Modified proteins may be captured with capture components comprising a multiplicity of specific antibodies and then the captured proteins may be further bound to a binding ligand comprising a secondary antibody with specificity to a post-translational modification. Alternatively, modified proteins may be captured with capture components comprising an antibody specific for a post-translational modification and then the captured proteins may be further bound to binding ligands comprising antibodies specific to each modified protein.

In another embodiment, the analyte molecule is a nucleic acid. A nucleic acid may be captured with a complementary nucleic acid fragment (e.g., an oligonucleotide) and then optionally subsequently labeled with a binding ligand comprising a different complementary oligonucleotide.

Suitable analyte molecules and particles include, but are not limited to small molecules (including organic compounds and inorganic compounds), environmental pollutants (including pesticides, insecticides, toxins, etc.), therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.), biomolecules (including hormones, cytokines, proteins, nucleic acids, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc), whole cells (including prokaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells), viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.), spores, etc.

The fluid sample comprising or suspected of comprising and analyte molecule or particle may be derived from any suitable source. In some cases, the sample may comprise a liquid, fluent particulate solid, fluid suspension of solid particles,

supercritical fluid and/or gas. In some cases, the analyte molecule or particle may be separated or purified from its source prior to determination; however, in certain embodiments, an untreated sample containing the analyte molecule or particle may be tested directly. The source of the analyte molecule may be synthetic (e.g., produced in a laboratory), the environment (e.g., air, soil, etc.), a mammal, an animal, a plant, or any combination thereof. In a particular example, the source of an analyte molecule is a human bodily substance (e.g., blood, urine, saliva, tissue, organ, or the like).

According to certain embodiments of the invention, the fluid sample comprising the analyte molecules is placed in contact with an array of a plurality of reaction vessels. The array may be in contact with the fluid sample for at least about 1 second, at least about 1 minutes, at least about 2 minutes, at least about 5 minutes, at least about 10 minutes, at least about 30 minutes, at least about 1 hour, at least about 6 hours, at least about 12 hours, at least about 24 hours, at least about 48 hours, and the like. In a particular embodiment, the plurality of reaction vessels and sample are contacted for a period of from about 50 minutes to about 70 minutes. In another embodiment, the incubation period is about 1 hour. The period of time the sample is in contact with the plurality of reaction vessels may be varied depending on various parameters, for example, the concentration of the analyte molecule in the fluid sample. The period of incubation may be determined by determining the time which is required for an appropriate portion of the analyte molecules in a fluid sample to be immobilized with respect to the array. For example, the fluid sample may be incubated with the array such that about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 97%, about 98%, about 99%, about 99.5%, about 99.9%, about 100%, and the like, of the analyte molecules are immobilized with respect to the array.

Capture Components

In some embodiments of the present invention, the surfaces of the substrate may, as mentioned previously, incorporate at least one type of capture component. As mentioned above, a capture component is any molecule, other chemical/biological entity or solid support modification disposed upon a solid support that can be used to specifically attach, bind or otherwise capture a molecule or particle, such that the molecule or particle becomes immobilized with respect to the capture component and solid substrate. Generally, the capture component allows the attachment of a molecule,

particle or complex to a solid support (that is, a surface of a substrate) for the purposes of immobilization, detection, quantification, and/or other analysis of the molecule, particle or complex. A capture component is used in the present invention, in some cases, to immobilize an analyte molecule with respect to the substrate. Those of ordinary skill in the art will be able to select appropriate capture component in accordance with the analyte molecules or particles to be immobilized.

As will be appreciated by those in the art, the composition of the capture component will depend on the composition of the analyte molecule. Capture components for a wide variety of target molecules are known or can be readily found using known techniques. For example, when the target molecule is a protein, the capture components may comprise proteins, particularly antibodies or fragments thereof (e.g., antigen-binding fragments (Fabs), Fab' fragments, pepsin fragments, $F(ab')_2$ fragments, full-length polyclonal or monoclonal antibodies, antibody-like fragments, etc.), other proteins, such as receptor proteins, Protein A, Protein C, etc., or small molecules. In some cases, capture components for proteins comprise peptides. For example, when the target molecule is an enzyme, suitable capture components may include enzyme substrates and enzyme inhibitors. In some cases, when the target analyte is a phosphorylated species, the capture component may comprise a phosphate-binding agent. For example, the phosphate-binding agent may comprise metal-ion affinity media such as those describe in U.S. Patent No. 7,070,921 and U.S. Patent Application No. 20060121544. In addition, when the target molecule is a single-stranded nucleic acid, the capture component may be a complementary nucleic acid. Similarly, the target molecule may be a nucleic acid binding protein and the capture component may be a single-stranded or double-stranded nucleic acid; alternatively, the capture component may be a nucleic acid-binding protein when the target molecule is a single or double stranded nucleic acid. Alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867, 5,705,337, and related patents, nucleic acid "aptamers" may be developed for capturing virtually any target molecule. As will be appreciated by those or ordinary skill in the art, any molecule that can specifically associate with a target molecule of interest may potentially be used as a capture component. For example, when the target molecule is a carbohydrate, potentially suitable capture components include, for example, antibodies, lectins and selectins.

For certain embodiments, suitable target molecule (e.g., analyte molecule)/capture component pairs can include, but are not limited to, antibodies/antigens, receptors/ligands, proteins/nucleic acid, nucleic acids/nucleic acids, enzymes/substrates and/or inhibitors, carbohydrates (including glycoproteins and glycolipids)/lectins and/or selectins, proteins/proteins, proteins/small molecules; small molecules/small molecules, etc. According to one embodiment, the capture components are portions (particularly the extracellular portions) of cell surface receptors that are known to multimerize, such as the growth hormone receptor, glucose transporters (particularly GLUT 4 receptor), and T-cell receptors and the target analytes are one or more receptor target ligands.

In a particular embodiment, the capture component may be attached to a binding surface (for example, the surface of a microwell or of a microbead) via a linkage, which may comprise any moiety, functionalization, or modification of the binding surface and/or capture component that facilitates the attachment of the capture component to the surface. The linkage between the capture component and the surface may comprise one or more chemical or physical (e.g., non-specific attachment via van der Waals forces, hydrogen bonding, electrostatic interactions, hydrophobic/hydrophilic interactions; etc.) bonds and/or chemical linkers providing such bond(s). In certain embodiments, the capture component comprises a capture extender component. In such embodiments, the capture component comprises a first portion that binds the analyte molecule and a second portion that can be used for attachment to the binding surface.

In certain embodiments, the substrate surface may also comprise a protective or passivating layer that can reduce or minimize non-specific attachment of non-capture components (e.g., analyte molecules, binding ligands) to the binding surface during the assay which may lead to false positive signals during detection or to loss of signal. Examples of materials that might comprise passivating layers include: polymers, such as poly(ethylene glycol), that repel the non-specific binding of proteins; naturally occurring proteins with this property, such as serum albumin and casein; zwitterionic surfactants, such as sulfobetaines; naturally occurring long-chain lipids; nucleic acids, such as salmon sperm DNA.

The method of attachment of the capture component to the substrate surface depends of the type of linkage employed and may potentially be accomplished by a wide variety of suitable coupling chemistries/techniques known to those of ordinary skill in

the art. The particular means of attachment selected will depend on the material characteristics of the substrate surface and the nature of the capture component. In certain embodiments, the capture components may be attached to the substrate surface through the use of reactive functional groups on each. According to one embodiment, the functional groups are chemical functionalities. That is, the binding surface may be derivatized such that a chemical functionality is presented at the binding surface which can react with a chemical functionality on the capture component resulting in attachment. Examples of functional groups for attachment that may be useful include, but are not limited to, amino groups, carboxy groups, epoxide groups, maleimide groups, oxo groups and thiol groups. Functional groups can be attached, either directly or through the use of a linker, the combination of which is sometimes referred to herein as a "crosslinker." Crosslinkers are known in the art; for example, homo-or hetero-bifunctional crosslinkers as are well known (e.g., see 1994 Pierce Chemical. Company catalog, technical section on crosslinkers, pages 155-200, or "Bioconjugate Techniques" by Greg T. Hermanson, Academic Press, 1996). Non-limiting example of crosslinkers include alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), esters, amide, amine, epoxy groups and ethylene glycol and derivatives. A linker may also be a sulfone group, forming a sulfonamide.

According to one embodiment, the functional group is a light-activated functional group. That is, the functional group can be activated by light to attach the capture component to the substrate surface. One example is PhotoLink™ technology available from SurModics, Inc. in Eden Prairie, MN.

In some cases, the substrate may comprise streptavidin-coated surfaces and the capture component may be biotinylated. Exposure of the capture component to the streptavidin-coated surfaces may cause associated of the capture component wit the surface by interaction between the biotin component and streptavidin.

In certain embodiments, attachment of the capture component to the binding surface may be effected without covalently modifying the binding surface of a substrate. For example, the attachment functionality can be added to the binding surface by using a linker that has both a functional group reactive with the capture component and a group that has binding affinity for the binding surface. In certain embodiments, a linker comprises a protein capable of binding or sticking to the binding surface; for example, in one such embodiment, the linker is serum albumin with free amine groups on its surface.

A second linker (crosslinker) can then be added to attach the amine groups of the albumin to the capture component (e.g., to carboxy groups).

According to one embodiment in which a chemical crosslinker is used to attach the capture components to the substrate, the analyte molecule may be captured on the binding surface of a substrate using a capture component attached via chemical crosslinking in the following manner. First, the binding surface is derivatized with a functional group, such as, an amine group. Next, a crosslinker and the capture component are placed in contact with the binding surface such that one end of the crosslinker attaches to the amine group and the capture component attaches to the other end of the crosslinker. In this way, capture components comprising proteins, lectins, nucleic acids, small organic molecules, carbohydrates can be attached.

One embodiment utilizes proteinaceous capture components. As is known in the art, any number of techniques may be used to attach a proteinaceous capture component to a wide variety of solid surfaces. "Protein" or "proteinaceous" in this context includes proteins, polypeptides, peptides, including, for example, enzymes and antibodies. A wide variety of techniques are known to add reactive moieties to proteins, for example, the method outlined in U.S. Patent No. 5,620,850. The attachment of proteins to surfaces is known, for example, see Heller, Acc. Chem. Res. 23:128 (1990), and many other similar references.

In some embodiments, the capture component (or binding ligand) may comprise Fab' fragments. The use of Fab' fragments as opposed to whole antibodies may help reduce non-specific binding between the capture component and the binding ligand. In some cases, the Fc region of a capture component (or binding ligand) may be removed (e.g., proteolytically). In some cases, an enzyme may be used to remove the Fc region (e.g., pepsin, which may produce $F(ab')_2$ fragments and papain, which may produce Fab fragments). In some instances, the capture component may be attached to a binding surface using amines or may be modified with biotin (e.g., using NHS-biotin) to facilitate binding to an avidin or streptavidin coated substrate surface. $F(ab')_2$ fragments may be subjected to a chemical reduction treatment (e.g., by exposure to 2-mercaptoethylamine) to, in some cases, form two thiol-bearing Fab' fragments. These thiol-bearing fragments can them be attached via reaction with a Michael acceptor such as maleimide. Alternatively, the Fab' fragments may then be treated with a reagent (e.g.,

maleimide-biotin) to attach at least one biotin moiety (i.e., biotinylated) to facilitate attachment to streptavidin-coated surfaces as described above.

Certain embodiments utilize nucleic acids as the capture component, for example for when the analyte molecule is a nucleic acid or a nucleic acid binding protein, or when it is desired that the capture component serve as an aptamer for binding a protein, as is well known in the art.

According to one embodiment, each binding surface of a substrate comprises a plurality of capture components. The plurality of capture components, in some cases, may be distributed randomly on the binding surface like a "lawn." Alternatively, the capture components may be spatially segregated into distinct region(s) and distributed in any desired fashion.

Binding between the capture component and the analyte molecule, in certain embodiments, is specific, e.g., as when the capture component and the analyte molecule are complementary parts of a binding pair. In certain such embodiments, the capture component binds both specifically and directly to the analyte molecule. By "specifically bind" or "binding specificity," it is meant that the capture component binds the analyte molecule with specificity sufficient to differentiate between the analyte molecule and other components or contaminants of the test sample. For example, the capture component, according to one embodiment, may be an antibody that binds specifically to some portion of an analyte molecule (e.g., an antigen). The antibody, according to one embodiment, can be any antibody capable of binding specifically to an analyte molecule of interest. For example, appropriate antibodies include, but are not limited to, monoclonal antibodies, bispecific antibodies, minibodies, domain antibodies, synthetic antibodies (sometimes referred to as antibody mimetics), chimeric antibodies, humanized antibodies, antibody fusions (sometimes referred to as "antibody conjugates"), and fragments of each, respectively. As another example, the analyte molecule may be an antibody and the capture component may be an antigen.

According to one embodiment in which an analyte particle is a biological cell (e.g., mammalian, avian, reptilian, other vertebrate, insect, yeast, bacterial, etc., cell), the capture component may be a ligand having specific affinity for a cell surface antigen (e.g., a cell surface receptor). In one embodiment, the capture component is an adhesion molecule receptor or portion thereof, which has binding specificity for a cell adhesion molecule expressed on the surface of a target cell type. In use, the adhesion molecule

receptor binds with an adhesion molecule on the extracellular surface of the target cell, thereby immobilizing or capturing the cell. In one embodiment in which the analyte particle is a cell, the capture component is fibronectin, which has specificity for, for example, analyte particles comprising neural cells.

In some embodiments, as will be appreciated by those of ordinary skill in the art, it is possible to detect analyte molecules using capture components for which binding to analyte molecules that is not highly specific. For example, such systems/methods may use different capture components such as, for example, a panel of different binding ligands, and detection of any particular analyte molecule is determined via a "signature" of binding to this panel of binding ligands, similar to the manner in which "electronic noses" work. This may find particular utility in the detection of certain small molecule analytes. In some embodiments, the binding affinity between analyte molecules and capture components should be sufficient to remain bound under the conditions of the assay, including wash steps to remove molecules or particles that are non-specifically bound. In some cases, for example in the detection of certain biomolecules, the binding constant of the analyte molecule to its complementary capture component may be between at least about 10^4 and about 10^6 M^{-1}, between at least about 10^5 and about 10^9 M^{-1}, between at least about 10^7 and about 10^9 M^{-1}, greater than about 10^9 M^{-1}, at least about 10^7 M^{-1}, at least about 10^8 M^{-1}, at least about 10^9 M^{-1}.

In certain embodiments, the capture component is chosen to be able to bind to a corresponding binding partner associated with or attached to the analyte molecule For example, the capture component according to one embodiment is a chemical crosslinker as described above able to bind to proteins generally. According to one embodiment, every protein molecule in a fluid sample comprises an analyte molecule that attaches to such a chemical crosslinker. In another example, the capture component comprises streptavidin, which binds with high affinity to biotin, and thus captures any analyte molecules to which biotin has been attached. Alternatively, the capture component may be biotin, and streptavidin may be attached to or associated with the analyte molecules such that the analyte molecules can be captured by the biotin.

According to one embodiment, the binding surfaces of a substrate may be functionalized with capture components in the following manner. First, the surface of a substrate (e.g., the end of a fiber optic bundle) is prepared for attachment of the capture component(s) by being modified to form or directly bind to the capture components, or a

linker may be added to the binding surface of the substrate such that the capture component(s) attaches to the binding surface of the substrate via the linker. In one embodiment, the binding surfaces of the substrate are derivatized with a chemical functionality as described above. Next, the capture component may be added, which binds to and is immobilized by the chemical functionality.

A specific embodiment is depicted in FIG. 7, in which the binding surface comprises an array of microwells functionalized with biotin. As shown in panel (A), an array of microwells 130 in this non-limiting example is formed at one end of a fiber optic bundle 126. To attach the capture component, the binding surface of the microwells 130 are first modified (e.g., with aminopropyl silane), as indicated by arrow 127, which may be bound to both the core 131 and cladding 132 surfaces of the distal end of the fiber bundle 126, as shown in FIG. 7, panel (B). However, in certain embodiments, the capture component should be present only within the microwells, the external surfaces of the fiber optic bundle, such as the external surfaces 133 of the cladding 132, should not be modified. In certain cases, after treatment, chemical functionalities may be removed from the external cladding surface 133 to avoid attachment of a capture component in this region. In this example, as shown in FIG. 7, panel (C), and as indicated by arrow 129, treated binding surface 128 may be removed from the external cladding portion 133, e.g., by polishing the tip of the fiber optical bundle (e.g., for 10 seconds with 0.3 μm lapping film), thereby removing the topmost layer of the cladding in this region, thereby removing the added binding moieties. After functionalization of the binding surface of the microwells, the capture component can be attached, as indicated by arrow 131 and shown in FIG. 7, panel (D). In one embodiment, the surface is treated with aminopropyl silane and the capture component comprises biotin or is labeled with biotin. For example, referring to FIG. 7, panel (D), a capture component comprising biotin succinimidyl ester 136 is attached to the amino groups of treated surface 128 of the microwells 130. The modification with aminopropyl silane is effective in this example because NHS-biotin attaches to an amino-silanized binding surface 128.

Examples of capture components 146 and exemplary association of capture components with an analyte molecule 142 within a reaction vessel 130 are depicted in FIG. 7, panels (E-G). A capture component 146 may be localized directly on the surface of the microwell 130, which may contain an optional seal 138, on a microparticle 134 contained within the microwell 130 (FIG. 7, panel (F)), and/or on the seal 138 of the

microwell 130 (FIG. 7, panel (G)). Additional locations where a capture component may be immobilized and additional substrates that may be used for one or both of capturing an analyte molecule according to certain methods of the invention are discussed more below.

In some embodiments only a single analyte molecule associates with each capture component. However, in some instances, more than one analyte molecule may be immobilized with respect to each capture component. In yet other cases, a single analyte molecule may become immobilized with respect to two or more capture components (either of the same or differing types). In some cases, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, at least about 99%, or more, of analyte molecules in the fluid sample exposed to the binding substrate become immobilized with respect to at least one capture component.

Binding Ligands and Precursor Labeling/Labeling Agent Molecules

Binding ligands may be selected from any suitable molecule, particle, or the like, as discussed more below, able to associate with an analyte molecule and/or to associate with another binding ligand. For example, when only a first binding ligand is employed, a first binding ligand may associate with an analyte molecule. In another example, when a first binding ligand and a second binding ligand are employed, a first binding ligand may associate with an analyte molecule and a second binding ligand may become immobilized with respect to the analyte molecule by becoming associated with the first binding ligand. Some non-limiting examples of potentially suitable binding ligands are discussed more below.

Certain binding ligands can comprise a component that is able to facilitate detection, either directly or indirectly. A component may facilitate indirect detection, for example, by converting a precursor labeling agent molecule into a labeling agent molecule (e.g., an agent that is directly or indirectly detected in an assay). In some embodiments, the binding ligand may comprise an enzymatic component. In some cases, a plurality of types of binding ligands may be employed, for example, a first binding ligand and a second binding ligand. In some cases, at least one, at least two, at least three, at least four, at least five, or the like, types of binding ligands may be employed. In some embodiments, at least one binding ligand employed in the assay may

comprise a converting agent. A precursor labeling agent molecule upon exposure to a converting agent is converted to a labeling agent molecule (e.g., a detectable product), examples of which are discussed herein.

As mentioned above, a precursor labeling agent molecule is any molecule, particle, or the like, that can be converted to a labeling agent molecule upon exposure to a suitable binding ligand (e.g. a binding ligand comprising a converting agent able to convert the precursor labeling agent). In some embodiments, a labeling agent molecule is insoluble (such that it can form a detectable precipitate) and/or immobilized with respect to the reaction vessel. In some case, a labeling agent molecule may be detected directly (e.g., the labeling agent molecule produces a detectable signal, constitutively and/or in response to an external simulation), for example, the labeling agent molecule form a precipitate (e.g., be insoluble), fluorogenic, chromogenic, chemiluminescent, or the like. In other cases, the labeling agent molecule may be detected indirectly (e.g., the labeling agent molecule converts another particle, molecule, or the like into a detectable entity, or immobilizes or captures another agent which is directly or indirectly detectable), as discussed more herein.

In some cases, the precursor labeling agent molecule may be solubilized or suspended in a fluid, such as a liquid. For embodiments where the fluid is a liquid, the liquid may comprise any suitable liquid, and/or more than one type of liquid, and may be selected according to its properties. For example, the liquid may be substantially hydrophobic or substantially hydrophilic, substantially viscous or substantially non-viscous, substantially polar or non-polar, or the like. Non-limiting examples of liquids include water, alcohols (e.g., methanol, ethanol, propanol, isopropanol, butanol, etc.), hydrocarbons (e.g., pentane, hexane, heptane, etc.), organic solvents (e.g., toluene, benzene, etc.), ethers (e.g., diethyl ether, tetrahydrofuran), dimethylsulfoxide, acetone, acetic acid, or the like. The precursor labeling agent molecule may have a solubility of greater than about 1×10^{-6} M, greater than about 1×10^{-5} M, greater than about 1×10^{-4} M, greater than about 1×10^{-3} M, greater than about 1×10^{-2} M, greater than about 1×10^{-1} M, greater than about 1 M, greater than about 10 M, or greater.

In some embodiments, the labeling agent molecule is insoluble. That is, the precursor labeling agent molecule is converted to a product which is insoluble in the liquid in which the labeling agent molecule is contained. In some cases, the labeling agent molecule has a solubility of less than 1×10^{-1} M, less than 1×10^{-2} M, less than $1 \times$

10^{-3} M, less than 1 x 10^{-4} M, less than 1 x 10^{-5} M, less than 1 x 10^{-6} M, less than 1 x 10^{-7} M, less than 1 x 10^{-8} M, less than 1 x 10^{-9} M, less than 1 x 10^{-10} M, less than 1 x 10^{-12} M, less than 1 x 10^{-15} M, less than 1 x 10^{-20} M, less than 1 x 10^{-30} M, or less.

In some embodiments, binding ligands and precursor labeling agents may be selected such that are able to function in the following manner. In some cases, if only a first binding ligand is employed, the first binding ligand may associate with an analyte molecule and may comprise a component which converts a precursor labeling agent molecule into a labeling agent. In other cases, if both a first and a second binding ligand are employed, the first binding ligand may associate with an analyte molecule and comprise a component which is able to interact with the second binding ligand. The second binding ligand may associate with the first binding ligand. At least one of the first and second binding ligand may comprise a converting agent which is able to convert a precursor labeling agent molecule into a labeling agent molecule. The assay may also comprise additional components (e.g., a third binding ligand, labeling agent capture components, etc.), and these additional components may be selected for particular desired functionality (e.g., a third binding agent selected such that it associates with an second binding ligand which is associated with a first binding ligand which is associated with an analyte molecule or particle, etc.). Any assay component may be selected such that it is able to associate with other assay component(s) as outlined above. In some cases, an assay component may be able to associate with more than one other assay component, for example, a third binding ligand may associate with both a first and a second binding ligand. The binding constant of the analyte molecule or particle with a binding ligand and/or the binding constant between two types of binding ligands may be at least about 10^4 M^{-1}, at least about 10^5 M^{-1}, at least about 10^6 M^{-1}, at least about 10^7 M^{-1}, at least about 10^8 M^{-1}, at least about 10^9 M^{-1}, between about 10^4 M^{-1} and about 10^9 M^{-1}, greater than about 10^9 M^{-1}, or greater.

In some cases, at least one binding ligand may comprise biotin and/or streptavidin. As a non-limiting example, the first binding ligand may be a biotinylated detection antibody, wherein the antibody may associate with an analyte molecule. The second binding ligand may be a streptavidin conjugated with a converting agent (e.g., an enzyme such as horseradish peroxidase), wherein the streptavidin of the second binding ligand will associate with the biotin of the first binding ligand. The converting agent of

the second binding ligand may convert a precursor labeling agent molecule into a labeling agent molecule.

A non-limiting embodiment is illustrated in FIG. 8. Reaction vessel 2 comprises a capture component 4 to which analyte molecule 6 associates. Reaction vessel 2 is exposed to first binding ligand 11 which comprises biotin 10 and component 8 which associates with analyte molecule 6. Reaction vessel 2 is also exposed to second binding ligand 13 which comprises streptavidin 12, which associates with biotin from first binding ligand 11, and converting agent 14, which converts precursor labeling agent molecule 16 into labeling agent molecule 18, as indicated by arrow 20.

In some embodiments, at least one binding ligand may comprise a converting agent which facilitates the conversion of a precursor labeling agent molecule into a labeling agent molecule. Non-limiting examples of converting agents include an enzymatic component, a nanoparticle, etc., as discussed more herein.

In some embodiments, at least one binding ligand employed in certain embodiments of the present invention may comprise an enzymatic component as a converting agent. In this instance, the precursor labeling agent molecule may be an enzymatic substrate, for example, a chromogenic, fluorogenic, or chemiluminescent enzymatic substrate, that upon contact with the enzymatic component of the binding ligand, converts to a detectable product. In some cases, the detectable product is insoluble and/or immobilized in the reaction vessel. In some cases, the enzymatic substrate is provided in an amount sufficient to contact every binding ligand associated with an analyte molecule which was partitioned across a plurality of reaction vessels. In some embodiments, the presence of a detectable produce (e.g., a chromogenic, fluorogenic, or chemiluminescent product) in a reaction vessel may provide information about the identity and/or concentration of an analyte molecule in the fluid sample based on the interaction of the analyte molecule with the capture component and the binding ligand, as described herein. Non-limiting examples of enzymatic components that may be employed include beta-galactosidase and horseradish peroxidase.

As will be understood by those of ordinary skill in the art, enzymatic precursor labeling agent molecules may be selected for conversion by many different enzymes. Thus, any known chromogenic, fluorogenic, or chemiluminescent enzyme precursor labeling agent molecule capable of producing a detectable product in a reaction with a particular enzyme can potentially be used in the present invention as the precursor

labeling agent molecule in embodiments where at least one binding ligand comprises an enzymatic component. For example, many chromogenic, fluorogenic, or chemiluminescent precursor labeling agent molecules suitable for use an enzymatic precursor labeling agent molecule are disclosed in *The Handbook -A Guide to Fluorescent Probes and Labeling Technologies,* Tenth Ed., Chapter 10.

A specific example of an enzymatic component for a binding ligand is horseradish peroxidase (HRP). HRP is a common enzymatic component for various assays and is known to those of ordinary skill in the art. HRP may be the enzymatic component of a binding ligand, wherein the binding ligand may also be capable of associating with an analyte molecule, another binding ligand, etc. As a non-limiting example, a binding ligand may be an HRP-labeled antibody or streptavidin conjugate, wherein the analyte molecule is an antigen. In some cases, HRP may convert a precursor labeling agent molecule into a labeling agent molecule that is insoluble and precipitates in the reaction vessel. Many examples include those typically used in Western blotting applications such as chloronapthol and/of diaminobenzidine, as known to those commonly skilled in the art. In some instances, the precipitate will be a darkly colored molecule and the precipitate may be detected optically. For example, the darkly colored molecules may be detected using light as the precipitate may block transmission of light through the well.

A binding ligand that comprises an enzymatic component (e.g., HRP) may be used jointly with a precursor labeling agent molecule (e.g., enzymatic substrate) that may be immobilized and/or insoluble when converted to a labeling agent molecule (e.g., detectable product). For example, HRP in the presence of hydrogen peroxide catalyzes the conversion of tyramide into an activated tryamide that can become immobilized with respect to materials (e.g. glass) of certain reaction vessels. In some embodiments, a tyramide molecule may be attached to any variety of molecules or particles that facilitate detection. For example, a tryamide molecule may be attached to a dye (e.g., a fluorescent dye). Therefore, the presence of the dye in a reaction vessel can be used to detect the presence of an analyte molecule in a reaction vessel. In some cases, the conversion of tyramide to activated tyramide may cause a component associated with the tyramide to become detectable (e.g., may cause a non-fluorescent component to fluoresce upon activation).

An exemplary embodiment is illustrated in FIG. 8. Reaction vessel 2 comprises a capture component 4 with which analyte molecule 6 associates. Reaction vessel 2 is exposed to first binding ligand 11 which comprises component 10 and component 8 which associates with analyte molecule 6. Reaction vessel 2 is also exposed to second binding ligand 13 which comprises component 12, which associates with component 10 from first binding ligand 11, and HRP 14, which converts precursor tyramide 16 (precursor labeling agent) into an activated tyramide 18 (labeling agent), as indicated by arrow 20. The activated tyramide may precipitate in the reaction vessel and/or may associate with a labeling agent capture component in the reaction vessels. The activated tyramide may be detected directed (e.g., if a precipitate or if the activated tyramide comprises a detectable component) or indirectly (e.g., if the activated tyramide comprises a component (e.g., an enzymatic component) which is able to convert a labeling agent reactant into a detectable product).

In other instances, the precursor labeling agent molecule may be used in conjunction with a labeling agent reactant (e.g., FIGS. 3B and 3D, as discussed herein). For example, the precursor labeling agent molecule may comprise tyramide and a component which is able to convert a labeling agent reactant into a detectable product or is able to associate with a labeling agent reactant which is detectable. For example, a tyramide molecule may be associated with biotin. A reaction vessel may be exposed to fluorescently-labeled streptavidin which may associate with the biotin and is able to be detected directly. In other cases, a tyramide molecule may comprise an enzymatic component. The enzymatic component (e.g., streptavidin-beta-galactosidase) may be exposed to a labeling agent reactant (e.g., an enzymatic substrate) which may be converted to a detectable product.

In yet other instances, the precursor labeling agent molecule may be used in conjunction with a first and a second labeling agent reactant (e.g., FIG. 3C, as discussed herein). For example, the precursor labeling agent molecule may comprise tyramide and a component (e.g., biotin) which is able to associate with a first labeling agent reactant (e.g., an enzymatic component). The second labeling agent reactant (e.g., an enzymatic substrate) may be converted to a detectable product upon exposure to the first labeling agent reactant which is associated with the labeling agent molecule.

Other examples materials that may be utilized as enzymatic components, precursor labeling agent molecules and labeling agent reactants will become apparent to

those commonly skilled in the art with the guidance of the present disclosure. As specific examples, HRP may activate a labeled tyramide derivative such as a fluorescent or biotinylated tyramide, hapten-conjugated tyramides, or tyramide labeled with polymeric reagents (e.g., tyramide-conjugated gold particles). Many kits comprising the reagents mentioned above may be purchased from commercial sources.

It is to be understood that a wide variety of labeling agents/detectable products can be used in the practice of the methods described herein For example, it is understood that a variety of colored labels (for example, metallic nanoparticles (for example, gold nanoparticles), semiconductor nanoparticles, semiconductor nanocrystals (for example, quantum dots), spectroscopic labels (for example, fluorescent labels), radiolabels, and enzymatic labels may be used, in the practice of the invention.

In some embodiments, a binding ligand may comprise a nanoparticle, for example, a gold nanoparticle. In this embodiment, the precursor labeling agent molecule may be a material that can associate with the binding ligand (e.g., the nanoparticle) upon conversion to a labeling agent. For example, metal particles may nucleate the highly specific deposition of metal (e.g., silver) from an appropriate metal salt solution in the presence of a suitable reducing agent. In this way, the nanoparticle may be coated in another metal such that the size of the nanoparticle increases and may be detected to determine the presence or absence of an analyte molecule or particle in a reaction vessel. Various techniques, such as electroless deposition, may be employed to make visible particles. In other instances, the nanoparticle may convert the precursor labeling agent molecule into a labeling agent molecule that is immobilized with respect to the reaction vessel or is insoluble. In a specific example, the first binding ligand comprises a gold nanoparticle. In this example, the precursor labeling agent molecule may be a silver salt that in the presence of a reducing agent forms a silver coating on the gold nanoparticle. Functionalization of a component that acts as a binding ligand in the inventive assays will be known to those skilled in the art and many kits and nanoparticle labeled antibodies and the like are commercially available to facilitate functionalization. Molecules, solutions, compounds and the like that may function as precursor labeling agents for deposition on a gold particle to form a labeling agent are also be known to those skilled in the art and are commercially available.

In some embodiments, the plurality of reaction vessels may additionally comprise at least one labeling agent molecule capture component. As describe above, a labeling agent molecule capture component is a capture component that specifically binds to or otherwise captures a labeling agent molecule, such that the labeling agent molecule is immobilized during the assay. Generally, the labeling agent molecule capture component allows the attachment of a labeling agent molecule to a solid support (e.g., the surface of a microwell, a sealing component or a nanoparticle in a reaction vessel) for the purposes of detection, quantification, or other analysis. The labeling agent molecule capture component may or may not be the same as a capture component present in a reaction vessel which is used to capture the analyte molecules. In such instances, of the plurality of capture components in a reaction vessel, at least one capture component may capture an analyte molecule, and the remainder of the capture components would be available to capture labeling agent molecules. In some cases, a method of the present invention will comprise the step of immobilizing at least a portion of the labeling agent molecules with respect to the at least one labeling agent molecule capture component.

In some embodiments, a functional group or other entity facilitating attachment of an analyte capture component to a surface may function as the labeling agent molecule capture component. For example, the plurality of reaction vessels may be functionalized with functional group or other entity facilitating attachment of an analyte capture component to a surface, and at least some of the functional groups/entities may be attached to a capture component and at least some of the groups/entities may remain unattached to analyte capture components. The groups/entities that are not coupled to analyte capture components may act as a labeling agent molecule capture components. For example, in some cases, the entity may comprise biotin and the biotin may also act as a labeling agent molecule capture component, for example, when a precursor labeling agent molecule is converted to a labeling agent molecule that comprises a component that associates with biotin (e.g., activated tyramide or streptavidin).

Detection Methods

In some embodiments, in the systems/methods in which the analyte molecules to be detected are partitioned across a plurality of reaction vessels, the reaction vessels may be interrogated using a variety of techniques, including techniques known to those of ordinary skill in the art.

In a specific embodiment of the present invention, reaction vessels are optically interrogated. The reaction vessels exhibiting changes in their optical signature may be identified by a conventional optical train and optical detection system. Depending on the labeling agent molecules detected and the operative wavelengths, optical filters designed for a particular wavelength may be employed for optical interrogation of the reaction vessels. In one embodiment, the plurality of reaction vessels of the present invention is formed directly as part of a fiber optic bundle.

According to one embodiment, the array of reaction vessels of the present invention can be used in conjunction with an optical detection system such as the system described in U.S. Publication No. 20030027126. For example, according to one embodiment, the array of reaction vessels of the present invention is formed in one end of a fiber optic assembly comprising a fiber optic bundle constructed of clad fibers so that light does not mix between fibers.

FIG. 9A shows a non-limiting example of a system of the present invention according to some embodiments. The system comprises a light source 252, excitation filter 454, dichromatic mirror 458, emission filter 460, objective 470, and substrate 472. Light 453 may be given from light source 452 and passed through excitation filter 454. The light may reflect off dichromatic mirror 458, pass through objective 470 and shine on substrate 472. In some cases, stray light 464 may be reduced by stray light reducing function 468. Light 471 emitted from substrate passes through objective 472 and emission filter 460 and is observed. The system may comprise additional component (e.g., additional filters, mirrors, magnification devices, etc.) , as will be understood by those of ordinary skill in the art.

The optical detection system of U.S. Publication No. 20030027126 operates as follows. Light returning from the distal end of the fiber optic bundle is passed by the attachment to a magnification changer which enables adjustment of the image size of the fiber's proximal or distal end. Light passing through the magnification changer is then shuttered and filtered by a second wheel. The light then is imaged on a charge coupled device (CCD) camera. A computer executes imaging processing software to process the information from the CCD camera and also optionally controls the first and second shutter and filter wheels. As depicted in U.S. Publication No. 20030027126, the proximal end of the bundle is received by a z-translation stage and x-y micropositioner.

For example, FIG. 10 shows a schematic block diagram of a system employing a

fiber optic assembly 400 with an optical detection system. The fiber optic assembly 400 comprises a fiber optic bundle or array 402 that is constructed from clad fibers so that light does not mix between fibers. An array or system, 400 is attached to the bundle's distal end 412, with the proximal end 414 being received by a z-translation stage 416 and x-y micropositioner 418. These two components act in concert to properly position the proximal end 414 of the bundle 402 for a microscope objective lens 420. Light collected by the objective lens 420 is passed to a reflected light fluorescence attachment with three pointer cube slider 422. The attachment 422 allows insertion of light from a 75 Waft Xe lamp 424 through the objective lens 420 to be coupled into the fiber bundle 402. The light from the source 424 is condensed by condensing lens 426, then filtered and/or shuttered by filter and shutter wheel 428, and subsequently passes through a ND filter slide 430. Light returning from the distal end 412 of the bundle 402 is passed by the attachment 422 to a magnification changer 432 which enables adjustment of the image size of the fiber's proximal or distal end. Light passing through the magnification changer 432 is then shuttered and filtered by a second wheel 434. The light is then imaged on a charge coupled device (CCD) camera 436. A computer 438 executes imaging processing software to process the information from the CCD camera 436 and also possibly control the first and second shutter and filter wheels 428, 434.

The array of reaction vessels in certain embodiments of the present invention may be integral with or attached to the end of the fiber optic bundle using a variety of compatible processes. In some cases, microwells are formed at the center of each individual fiber of the fiber optic bundle and the microwells may or may not be sealed. Each optical fiber of the fiber optic bundle may convey light from the single microwell formed at the center of the fiber's end. This feature enables the interrogation of the optical signature of individual reaction vessels to identify reactions/contents in each microwell. Consequently, by imaging the end of the bundle onto the CCD array, the optical signatures of the reaction vessels are individually interrogatable and may be detected substantially simultaneously.

In some embodiments, the methods of the present invention may be performed with or without sealing of the array of reaction vessels. In some cases, there may be substantially no difference between as assay performed with sealing the array and an assay performed using essentially identical conditions without sealing of the array. Without wishing to be bound by theory, this may be attributed to the insolubility and/or

immobilization of labeling agent molecules in the reaction vessels (e.g., such that the labeling agent molecule is unable to diffuse into adjacent reaction vessels and thus additional reaction vessels would comprise a detectable product) provided by certain embodiments of the invention. In other cases, it may be attributed to the direct detection of a detectable molecule (e.g.,, an insoluble labeling agent molecule). For example, in instances where amplification is not employed (e.g., direct detection of a labeling agent molecule, as opposed to further exposure and detection of a labeling agent reactants, a second labeling agent molecule, etc.), there may be no species (e.g., labeling agent reactants, etc.) which diffuse to neighboring reaction vessels and cause additional reaction vessels to comprise a detectable product.

In embodiments where the plurality of reaction vessels are sealed, the plurality of reaction vessels may be sealed, for example, through the mating of the substrate and a sealing component. In some cases, the sealing of the reaction vessels may be such that the contents of each reaction vessel cannot escape the reaction vessel. In some cases, the reaction vessels may be sealed after the addition of a binding ligand and, optionally, a precursor labeling agent molecule to facilitate detection of the analyte molecules. For embodiments employing precursor labeling agent molecules, by sealing the contents in some of each reaction vessel, a reaction to produce the detectable labeling agent molecule can proceed within the sealed reaction vessels, thereby producing a detectable amount of a labeling agent molecule that is retained in the reaction vessel for detection purposes.

In some embodiments, at least a fraction of the number of detectable species (e.g., labeling agent molecules) may be detected substantially simultaneously. "Substantially simultaneously" when used in conjunction with detection, as used herein, refers to detection of the species/molecules/particles of interest at approximately the same time, as opposed to sequentially detected. A plurality of molecules/particles (e.g., labeling agent molecules, labeling agent reactants, etc.) may be detected substantially simultaneously using various techniques, including optical techniques (e.g., CCD detector).

Quantification

According to certain embodiments of the present invention, certain methods, systems, and devices disclosed can be used to detect the presence of an analyte molecule

and/or determine the concentration of analyte molecules in a fluid sample. In some cases, there is a correlation between the percentage of reaction vessels containing one or more analyte molecules or particles and the concentration of the analyte molecules in the fluid sample. Thus, the quantification method of certain embodiments of the present invention allows for calculation of the number of analyte molecules in a fluid sample based on the percentage of reaction vessels that contain an analyte molecule. In some embodiments, the measure of the concentration of analyte molecules in a fluid sample will be determined using a calibration curve. Methods to determine a measure of the concentration of analyte molecules in a fluid sample are discussed more below.

Certain embodiments of present invention are distinguished by the ability to detect and/or quantify low numbers/concentrations of analyte molecules or particles in a fluid sample. It is currently believed that this ability may be achieved by spatially isolating individual or small numbers of analyte molecules or particles, for example, as when they are partitioned across an array of reaction vessels, and then detecting their presence in the reaction vessels. The presence of an analyte molecules or particles in a reaction vessel can be counted in a binary fashion (e.g., zero when an analyte molecule is absent; one when an analyte molecule is present), for example by determining the presence of a detectable molecule or particle (e.g. a labeling agent) in a reaction vessel that contains at least one analyte molecule or particles.

In some embodiments, the plurality of analyte molecules (or particles) may be partitioned such that at least some of the reaction vessels contain no analyte molecules and at least some of reaction vessels contain at least one or, in certain cases, only one analyte molecule. For example, in some cases, the plurality of analyte molecules (or particles) may be partitioned such that a statistically significant fraction of the reaction vessels contain no analyte molecules and a statistically significant fraction of reaction vessels contain at least one analyte molecule. In other cases, the plurality of analyte molecules may be partitioned such that a statistically significant fraction of the reaction vessels contain no analyte molecules and a statistically significant fraction of reaction vessels contain only one analyte molecule. In either case, the number of the plurality of reaction vessels and/or fraction of the plurality of reaction vessels that contain or do not contain an analyte molecule may be determined. The number and/or fraction of the plurality of reaction vessels that contain an analyte molecule can be related to the concentration of analyte molecules or particles in the sample. In some embodiments, a

measure of the concentration of analyte molecules or particles in the fluid sample is determined based on the determination of the number and/or fraction of the plurality of reaction vessels that contain an analyte molecule. In certain such embodiments, the measure of the concentration of the analyte molecules or particles in the fluid sample is determined at least in part comparison of a measured parameter to a calibration standard and/or by a Poisson and/or Gaussian distribution analysis of the number or fraction of the plurality of reaction vessels that contain an analyte molecule, as discussed more below. A "statistically significant fraction" of the reaction vessels that contain a specified quantity of dissociated species is defined as the minimum number of reaction vessels that can be reproducibly determined to contain an analyte molecule or particle with a particular system of detection (i.e., substantially similar results are obtained for multiple essentially identical fluid samples comprising the target analyte molecule) and that is above the background noise (e.g., non-specific binding) that is determined when carrying out the assay with a sample that does not contain any analyte molecules or particles, divided by the total number of reaction vessels. The statistically significant fraction may be experimentally determined for a certain assay type and equipment set up (e.g., for each analyte molecule determined, each binding ligand, etc). In certain embodiments, the percentage of reaction vessels (e.g., the statistically significant fraction) which comprises only one or at least one analyte molecule or particle is less than about 10%,less than about 5%, less than about 1%, less than about 0.5%, or less than about 0.1% of the total reaction vessels. In some cases, the percentage of reaction vessels which do not contain an analyte molecule or particle is at least about 20%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, or at least about 95%, at least about 99%, at least about 99.5%, at least about 99.9%, or greater, of the total number of reaction vessels.

In some embodiments, a measure of the concentration of analyte molecules or particles in the fluid sample may be determined at least in part by comparison of a measured parameter to a calibration standard. For example, the fraction of reaction vessels that comprise an analyte molecule may be compared against a calibration curve to determine a measure of the concentration of the analyte molecule in the fluid sample. The calibration curve may be produced by completing the assay with a plurality of standardized samples of known concentration under the conditions used to analyze the test samples. A reading may be taken for the signal related to the

detection/quantification of the analyte molecules for each standardized sample, therefore allowing for the formation of a calibration curve relating the detection of the analyte molecules with a known concentration of the analyte molecule. The assay may then be completed on a sample comprising the analyte molecule in an unknown concentration, and the detection of the analyte molecules from this assay may be plotted on the calibration curve, therefore determining a measure of the concentration of the analyte molecule in the fluid sample.

In one exemplary calibration (see FIG. 11), four standardized fluid samples comprising an analyte molecule in varying concentration (w, x, y, and z) are provided. An assay (e.g., immobilizing the analyte molecules, detecting the presence of at least a portion of the immobilized analyte molecules) is carried out for each sample, and a value corresponding to the detection of the analyte molecules (b, c, d, and e) may be determined. A plot is produced of the values related to detection of the analyte molecules (b, c, d, and e) versus the concentration of the standardized samples (w, x, y, and z), as depicted in FIG. 11. The assay may be then be carried out under substantially identical conditions on a fluid sample comprising an analyte molecule of unknown concentration t, wherein the resulting value related to detection of the analyte molecules is f. This value is plotted on the graph and a measure of the unknown concentration of the target analyte in the fluid sample may be interpolated from the values of the standardized samples. In some cases, the calibration curve may have a limit of detection, wherein the limit of detection is the lowest concentration of analyte molecules in a fluid sample that may be accurately determined. In some cases, the r^2 value of the calibration curve may be greater than about 0.5, greater than about 0.75, greater than about 0.8, greater than about 0.9, greater than about 0.95, greater than about 0.97, greater than about 0.98, greater than about 0.99, greater than about 0.9999, or about 1.

In some embodiments, the calibration curve and/or the calculation of concentration of the analyte molecules in the fluid sample based on a calibration curve may be stored and/or determined with a system comprising a computer. The computer may comprise software that may use the data collected to produce the calibration curve and/or a determination of the measure of the concentration of the analyte molecules in the fluid sample. For example, a fluorescence image of an array comprising the analyte molecules partitioned across the array (e.g., reaction vessels formed in an optical fiber bundle) may be collected and analyzed using image analysis software (e.g., IP Lab, BD

Biosciences). The analysis software may automatically segment all reaction vessels that have fluorescence intensity over the background intensity and give a number indicative of the total number of reaction vessel which comprise fluorescent intensity above background (e.g., a number that correlates to the number of reaction vessels which comprise an analyte molecule). The number of reaction vessels which comprise fluorescence intensity above background may be divided by the total number of reaction vessels addressed to give a number correlating to the fraction of reaction vessels which comprise an analyte molecule. The active well fraction may be compared to a calibration curve to determine a measure of the concentration of analyte molecules in the fluid sample.

In some cases, the number of analyte molecules that are detected may or may not be approximately equal to the number of analyte molecules in the fluid sample. For example, the ratio of analyte molecules detected in the fluid sample to analyte molecules which are detected may be about 1:1, about 2:1, about 5:1, about 10:1, about 100:1, about 1000:1, about 10000:1, or the like. In some cases, the ratio is greater than about 1:1, greater than about 10:1, great than about 100:1, greater than about 1000:1, greater than about 10000:1, or greater.

In some embodiments, the concentration of analyte molecules or particles in the fluid sample that may be substantially accurately determined is less than about 5000 fM, less than about 3000 fM, less than about 2000 fM, less than about 1000 fM, less than about 500 fM, less than about 300 fM, less than about 200 fM, less than about 100 fM, less than about 50 fM, less than about 25 fM, less than about 10 fM, less than about 5 fM, less than about 2 fM, less than about 1 fM, less than about 0.5 fM, less than about 0.1 fM, or less. In some embodiments, the concentration of analyte molecules or particles in the fluid sample that may be substantially accurately determined is between about 5000 fM and about 0.1 fM, between about 3000 fM and about 0.1 fM, between about 1000 fM and about 0.1 fM, between about 1000 fM and about 1 fM, between about 100 fM and about 1 fM, between about 100 fM and about 0.1 fM. The concentration of analyte molecules or particles in a fluid sample may be considered to be substantially accurately determined if the measured concentration of the analyte molecules or particles in the fluid sample is within about 10% of the actual concentration of the analyte molecules or particles in the fluid sample. In certain embodiments, the measured concentration of the analyte molecules or particles in the fluid sample may be within

about 10%, within about 7%, within about 5%, within about 4%, within about 3%, within about 2%, within about 1%, within about 0.5%, within about 0.4%, within about 0.3%, within about 0.2% or within about 0.1%, of the actual concentration of the analyte molecules or particles in the fluid sample. The accuracy of the assay method may be determined, in some embodiments, by determining the concentration of analyte molecules in a fluid sample of a known concentration using the selected assay method.

Without being limited by theory, the quantification method is believed to be driven in part by the fact that the number and volume of reaction vessels employed govern the dynamic range of concentrations that can be determined. That is, based on the number and volume of the reaction vessels in an array of the present invention, an estimate can be made of the range of concentrations of detected molecules in the solution partitioned across the vessels that allows for a measure of the concentration to be determined using certain methods of the present invention.

For example, for an array comprising approximately 2.4×10^5 reaction vessels, each having a volume of approximately 50 fL, a solution having a concentration of approximately 4×10^{-11} M analyte molecules in a fluid sample will yield, on average, one analyte molecule per reaction vessel. However, it is important to note that distributing a fluid sample having an analyte molecule concentration within the appropriate range into an array of reaction vessels will not result in the distribution of exactly one analyte molecule per each reaction vessel; statistically, some vessels will have multiple analyte molecules while others will have no analyte molecules. In the case where the ratio of vessels containing one or more analyte molecules to the number of vessels containing no analyte molecules is high, the data may be fit to a Gaussian distribution. As the ratio of reaction vessels containing an analyte molecule to the number of vessels containing no analyte molecules approaches zero, the Poisson distribution may be applied to the data. This limiting distribution may be used to calculate the probability of rare events occurring in a large number of trials. For example, based on Poisson statistics, for a concentration of approximately 4×10^{-11} M, a distribution between zero and five analyte molecules per reaction vessel is predicted, with the most probable values being zero and one.

Equation 1 can be used to determine the probability of observing events based on the expected average number of events per trial, μ:

Equation 1: $P\mu(v) = e^{-\mu}(\mu^v/v!)$

where v is the number of events observed (e.g., the number of reaction vessels) and μ is the expected average number of events per trial (e.g., the average number of analyte molecules per reaction vessel.

If the concentrations used are much less than approximately 4×10^{-11} M, the expected average number of analyte molecules per well becomes exceptionally low, the distribution is narrowed, and the probability of observing anything other than zero or one analyte molecules per well is improbable in all experimental cases. At these low concentrations, the relationship between the percentage of active reaction vessels and the bulk analyte molecule concentration is approximately linear. Thus, based on this knowledge, the array of the present invention can be used to determine the concentration of analyte molecules by a simple digital readout system (e.g., "counting" of active wells) as described herein in combination with a suitable calibration.

According to one embodiment, the quantification method of the present invention can be performed as follows. The method employs a digital readout system (also referred to as a "binary readout system") that involves first detecting the analyte molecules in the plurality of reaction vessels by any detection method as described herein. The number of reaction vessels which comprise an analyte molecule is then counted and a percentage of the total number of reaction vessels which comprise an analyte molecule is calculated. That is, utilization of a yes or no response, in conjunction with the high-density array of reaction vessels, permits the digital readout of bulk concentrations of analyte molecules. In some embodiments, this readout is accomplished by counting the number of reaction vessels containing at least one labeling agent molecule, with the resulting number of reaction vessels comprising a labeling agent molecule corresponding to the number of reaction vessels comprising an analyte molecule. Given the large number of reaction vessels simultaneously interrogated in the array of the present invention, the ratio of analyte molecules to reaction vessels may be at least about 1:100, at least about 1:1000, at least about 1:10,000, as the large number of reaction vessels provides a statistically significant signal even at this low ratio.

In some embodiments, without being limited by theory, it is believed that the quantification method of the present invention may only be limited by the number of individual reaction vessels that can be fabricated and interrogated. Thus, expanding the number of reaction vessels may increase both the dynamic range and the sensitivity of the assay. For example, increasing the number of reaction vessels by a factor of ten may

decrease the ratio of analyte molecules to reaction vessels by a factor of ten, thereby increasing the dynamic range and/or sensitivity of the assay. As mentioned above, in some embodiments, an array will comprise between about 1,000 and about 50,000, between about 1,000 and about 1,000,000, between about 1,000 and about 10,000, between about 10,000 and about 100,000, between about 100,000 and about 1,000,000, between about 1,000 and about 100,000, between about 50,000 and about 100,000, between about 20,000 and about 80,000, between about 30,000 and about 70,000, between about 40,000 and about 60,000, or about 50,000 reaction vessels.

In some embodiments, accuracy of this technique may be compromised above and below the thresholds of the dynamic range. For example, as the concentration of the analyte molecules goes below the lower limit of the dynamic range, the number of analyte molecules may be too low to observe a sufficient number of occupied wells. In such a situation, the number of reaction vessels could be decreased in order to make sure that a statistically significant number of them are occupied by an analyte molecule, the volume of the reaction vessels could be increased, and/or the sample tested could be concentrated. Results for extremely dilute concentrations may have large relative errors associated with them, due to the very small number of reaction vessels that are expected to show activity. In other cases, the ultimate upper limit to this technique may occur when 100% of the reaction vessels contain at least one analyte molecule. At this limit, discrimination between two solutions of high analyte molecule concentrations may not be feasible. In such a situation, to provide a more accurate test, a greater number of reaction vessels could be used, and/or the volume of each reaction vessel could be reduced, and/or the concentration of the sample could be reduced, e.g., through dilution.

In the range where the fraction of reaction vessels containing at least one analyte molecule is less than about 20%, the probability that any well contains two or more analyte molecules is very small and the number of analyte molecules closely matches the number of occupied reaction vessels. Between 20% occupied and 100% occupied, an increasing number of wells may contain more than one analyte molecule, however Gaussian statistics can still be used to correlate occupancy fraction with concentration with reasonable accuracy until the occupancy fraction approaches 100%.

As alluded to above, the practical dynamic range of the method may be increased in several ways. In one approach, the sample and/or solution carrying the analyte molecules may be diluted, e.g. by a factor of 10 or more. Both the solution comprising

the analyte molecules and the diluted solution comprising the analyte molecules may be assayed concurrently using the method of this invention. The dynamic ranges of the two assays may overlap, but be offset by the dilution factor, hence extending the dynamic range.

In some embodiments, multiple arrays of reaction vessels can be used, each array having reaction vessels with differing volumes, differing binding surface areas, or differing density and/or type of capture components on the binding surface. These configurations can be constructed as either distinct arrays or as one large array with distinct sub-arrays with varying characteristics. Since the probability of an analyte molecule being detected in a given reaction vessel can be related to volume, binding surface area, and capture component density, the sub-arrays may be designed to provide different sensitivity ranges. Thus, with such configurations the effective range of the combined array may be extended.

In certain embodiments, after partitioning of the solution containing analyte molecules across an array of reaction vessels, less than about 20% of the total number of the plurality of reaction vessels will contain at least one analyte molecule (i.e., at least 80% of reaction vessels will be free of analyte molecules). Under such circumstances, the number of reaction vessels containing at least one analyte molecule will typically fall within the linear range of a Poisson distribution. In another embodiment, more than about 20% but less than about 60% of the total number of reaction vessels contain at least one analyte molecule. Under such circumstances, the number of reaction vessels containing at least one analyte molecule will typically fall within the non-linear range of a Poisson distribution. In another embodiment, more than about 60% but less than about 95% of the total number of reaction vessels contains at least one analyte molecule. Under such circumstances, the number of reaction vessels containing at least one analyte molecule will typically fall within the highly non-linear range of a Poisson distribution. In embodiments where greater than about 60% of the total number of reaction vessel contains at least one analyte molecule, it may be desirable to decrease the percentage of reaction vessels which comprise at least one analyte molecule (e.g., to less than about 20%). This may be accomplished using any suitable technique discussed herein, for example, diluting the fluid comprising the analyte molecules and/or increasing the number of reaction vessels. In some cases, less than about 1%, less than about 5%, less than about 10%, less than about 20%, less than about 40%, less than about 60%, less

than about 80%, less than about 90%, less than about 95%, or less than about 99% of the total number of the plurality of reaction vessels will contain at least one analyte molecule. In certain embodiments, more than about 1%, more than about 5%, more than about 10%, more than about 20%, more than about 40%, more than about 60%, more than about 80%, more than about 90%, more than about 95%, or more than about 99% of the total number of the plurality of reaction vessels will contain no analyte molecules. In some cases, at least about 95%, at least about 90%, at least about 80%, at least about 40%, at least about 5% of the reaction vessels contain no analyte molecule.

In some embodiments, the invention provides a method of determining the concentration of the analyte molecules in a fluid, the method comprising dividing the fluid containing the analyte molecules into a plurality of second, smaller fluid samples of essentially equal volume so that a statistically significant fraction of the second, smaller fluid samples contain either no analyte molecules or a single analyte molecule; determining the presence or absence of an analyte molecule in each of the second, smaller fluid samples so as to identify the number of second, smaller fluid samples that contain an analyte molecule; and determining the concentration of analyte molecules in the fluid sample to be tested from the number of second, smaller samples that contain the analyte molecules.

In certain embodiments, the present invention provides a method for determining the concentration of analyte molecules in a fluid, the method comprising the partitioning at least a portion of the analyte molecules in the fluid across a plurality of reaction vessels so that at least some of the reaction vessels contain an analyte molecule and at least some of the reaction vessels contain no analyte molecules; determining the presence or absence of an analyte molecule in each reaction vessel to identify the number of reaction vessels that contain an analyte molecule and/or to identify the number of reaction vessels that contain no analyte molecules; and determining the concentration of analyte molecules in the fluid at least in part from the number of reaction vessels that do or do not contain an analyte molecule. In some embodiments, at least about 99%, at least about 95%, at least about 90%, at least about 80%, at least about 40%, at least about 20%, at least about 10% at least about 5%, at least about 1%, and the like of the reaction vessels do not contain an analyte molecule. In some embodiments, the concentration of analyte molecules in the fluid sample is determined at least in part using a calibration curve, a Poisson distribution analysis and/or a Gaussian distribution of the number of

reaction vessels that contain at least one or one analyte molecule. In other embodiments, the concentration of analyte molecules in the fluid sample is determined at least in part by a Gaussian distribution analysis of the number of reaction vessels that contain an analyte molecule.

In certain embodiments, the present invention provides a method of determining the concentration of analyte molecules in a fluid, the method comprising exposing the fluid to a plurality of reaction vessels under conditions so that at least one analyte molecule is captured in at least some of the reaction vessels, wherein each reaction vessel comprises a microwell and an optional sealing component and each reaction vessel defines a binding surface that has a capture component immobilized thereon; determining the presence or absence of an analyte molecule in each reaction vessel so as to identify the number of reaction vessels that contain an analyte molecule and/or the number of reaction vessels that do not contain an analyte molecule; and determining the concentration of analyte molecules in the fluid sample to be tested from the number of reaction vessels that contain and/or do not contain an analyte molecule. In some embodiments, at least about 99%, at least about 95%, at least about 90%, at least about 80%, at least about 50%, at least about 20%, at least about 10% at least about 5%, at least about 1%, and the like of the reaction vessels contain either zero or one analyte molecule. In some embodiments, the concentration of analyte molecules in the fluid sample is determined at least in part using a calibration curve, a Poisson distribution analysis and/or a Gaussian distribution analysis of the number of reaction vessels that contain at least one or one analyte molecule.

In certain embodiments, the present invention provides a method of determining the concentration of analyte molecules in a fluid, the method comprising exposing the fluid to a plurality of reaction vessels under conditions so that at least one analyte molecule is captured in at least some of the reaction vessels, wherein each reaction vessel comprises a microwell and an optional sealing component and each reaction vessel defines a binding surface that has a capture component immobilized thereon; determining the presence or absence of an analyte molecule in each reaction vessel so as to identify the number of reaction vessels that contain an analyte molecule and/or the number of reaction vessels that do not contain an analyte molecule; and determining the concentration of analyte molecules in the fluid sample to be tested from the number of reaction vessels that contain and/or do not contain an analyte molecule. In some

embodiments, at least about 99%, at least about 95%, at least about 90%, at least about 80%, at least about 50%, at least about 20%, at least about 10% at least about 5%, at least about 1%, and the like of the reaction vessels contain either zero or one analyte molecule. In some embodiments, the concentration of analyte molecules in the fluid sample is determined at least in part using a calibration curve, a Poisson distribution analysis and/or a Gaussian distribution analysis of the number of reaction vessels that contain at least one or one analyte molecule.

In certain embodiments, the present invention provides a method of determining the concentration of analyte molecules in a fluid sample to be tested, the method comprising partitioning at least a portion of the analyte molecules in the fluid into a plurality of reaction vessels, so that, for substantially all of the reaction vessels, each reaction vessel contains either no analyte molecules or a single analyte molecule; determining the presence or absence of an analyte molecule in a plurality of reaction vessels to provide a fraction of the interrogated reaction vessels that contain an analyte molecule; and determining the concentration of analyte molecules in the fluid from the fraction of interrogated reaction vessels that contain an analyte molecule.

In certain embodiments, the present invention provides a method of determining the concentration of analyte molecules in a fluid, the method comprising partitioning the fluid into a plurality of second, smaller fluid samples of equal volume so that at least some of the second, smaller fluid samples contain either a single analyte molecule or no analyte molecules, (b) determining the presence or absence of an analyte molecule in at least a subset of the second samples so as to identify the fraction of second samples in the subset that contain an analyte molecule; and determining the concentration of analyte molecules in the sample to be tested from the fraction of second samples of the subset that contain the analyte molecules.

In some embodiments, a method of the present invention may be used for the detection of analyte molecules in a fluid. For example, a method of detecting analyte molecules in a fluid may comprise providing a fluid containing the analyte molecules and an array, the array comprising a plurality of reaction vessels; contacting the array with the fluid such that the ratio of the number of analyte molecules in the fluid contacted with the array to the number of reaction vessels in the array is less than 1:1; and determining the number of reaction vessels which contain an analyte molecule. In some cases, the ratio of the number of analyte molecules in the fluid contacted with the

array to the number of reaction vessels in the array is less than about 1:5, less than about 1:10, less than about 1:100, or less than about 1:500.

In certain embodiments, a method of detecting analyte molecules in a fluid according to the invention comprises providing a fluid and an array, the fluid comprising at least one analyte molecule at a first concentration, the array comprising a plurality of reaction vessels; diluting the fluid to create a diluted fluid, wherein the diluted fluid comprises the analyte molecules at a second concentration,; contacting the array with the diluted fluid such that the ratio of analyte molecules to the total number of reaction vessels in the array is between 1:1 and 1:500; and determining the number of vessels of said array which contain an analyte molecule. In some cases, the ratio is less than about 1:1, less than about 1:5, less than about 1:10, less than about 1:100, or less than about 1:500.

Multiplexing and Reuse of an Array

In accordance with one detection embodiment, sensor redundancy is used. In this embodiment, a plurality of reaction vessels comprising identical capture components referred to as "subpopulations" are used. That is, each subpopulation comprises a plurality of identical capture components present in reaction vessels of an array. Further, according to one embodiment, each subpopulation comprises a plurality of reaction vessels comprising identical capture components. By using a number of identical capture components for a given array, the optical signal from each microwell can be combined for the subpopulation and any number of statistical analyses run, as outlined below. This can be done for a variety of reasons. For example, in time varying measurements, redundancy can significantly reduce the noise in the system. For non-time based measurements, redundancy can significantly increase the confidence of the data.

The number of subpopulations, according to one embodiment, can range from 2 to any number of subpopulations possible given the limitations of the overall size of the array(s) and the number of different capture components. Alternatively, the number can range from about 2 to about 10. In a further alternative, the number can range from about 2 to about 5.

In one embodiment, a plurality of identical capture components is used. As will be appreciated by those in the art, the number of identical capture components in a

subpopulation will vary with the application and use of the sensor array (e.g., the number of capture component per well may vary). In general, anywhere from 2 to thousands, from about 2 to 100, from about 2 to about 50, or from about 5 to about 20 of identical capture components may be used in a given subpopulation. In one case, about 10 identical capture components may be used.

A reaction vessel array according to one embodiment, utilizes a plurality of capture components that are directed to a single target analyte but are not identical. In other words, the capture components bind to different binding sites on an analyte molecule. This embodiment thus provides for more than one different capture component on each binding surface or different capture components on different binding surfaces. In one example, a single analyte is interrogated by a first and a second capture component, each of which is capable of binding to a different site on the analyte molecule. This adds a level of confidence to the assay as non-specific binding interactions can be statistically reduced. In this embodiment, when proteinaceous analytes are evaluated, the assay can utilize capture components that bind to different parts of the target. For example, two or more antibodies (or antibody fragments) to different portions of the same analyte protein may be used as capture components, (e.g., antibodies directed towards different epitopes). Similarly, when nucleic acid analyte molecules are to be evaluated, redundant nucleic acid probes may be used as capture components that are overlapping, adjacent, or spatially separated in the gene. In most cases, the two such capture components would not compete for a single binding site, and adjacent or separated probes are used.

In certain such embodiments, a plurality of different capture components may be used. For example, about 2 to about 20, about 2 to about 10, from about 2 to about 5. In other cases, about 2, about 3, about 4, or about 5 different capture components may be used. However, as above, more may also be used, depending on the application.

In certain embodiments, the plurality of reaction vessels of certain embodiments of the present invention use a plurality of different capture components that are directed to a plurality of target analyte molecules, which can be the same or different. Such embodiments can include more than one different capture components on each binding surface or different capture components on different binding surfaces. In one example, a plurality of a first analyte molecule and a plurality of a second analyte molecule may be provided to which a plurality of a first capture component and a plurality of a second

capture component on the same binding surfaces or on different binding surfaces are capable of binding.

In such embodiments, more than one type of analyte molecule may be identified. For example, a first analyte molecule and a second analyte molecule may be identified so long as, in the case of direct assays, each different analyte molecule interacts differently with one or more first binding ligand types. In one embodiment, the analyte molecules may be identified using one or more first binding ligands having specificity to particular analyte molecules or particles wherein each first binding ligand converts precursor labeling agents into a uniquely identifiable labeling agent. Thus, each specific analyte molecule can be distinguished based upon the detectable signal produced by reaction of the first binding ligand with the precursor labeling agent.

In one particular approach, referred to as a sequential approach, the analyte molecules are identified using the same or different binding ligands. In this approach, the one or more analyte molecules of interest are captured on one or more binding surfaces. A first type of first binding ligand associates with the first analyte molecules. The presence of the first type of first binding ligand can be determined. Thereafter, the first type of first binding ligand is removed, for example, by washing. Then a second, different type of first binding ligand associates with a second, different type of analyte molecule. The presence of the second type of first binding ligand may be determined. Thereafter, the second type of first binding ligand may be removed by washing and the process repeated as desired to detect additional types of analyte molecules. It is understood that, in this approach, the same or different precursor labeling agent molecule type may be converted by the first and the second types of first binding ligand. It is understood that essentially any precursor labeling agent described herein can potentially be used in this approach. It is understood that the sequential reactions can be performed using labeling agent molecules (or labeling agent reactants), each of which produce the same or different colors.

In certain embodiments, a plurality of different capture component types can be used, for example, from about 2 to about 100, from about 2 to about 20, from about 2 to about 10, or about 2, 3, 4 or 5, 6, 7, 8, 9. However, as above, more may also be used, depending on the application.

In another approach, referred to as a spatial approach, it is possible to use either (i) two different capture component types disposed within separate regions of a binding

surface, (ii) two different first binding ligand types that are applied to an separate regions of a binding surface, or (iii) a combination or (i) and (ii).

In the first example of the spatial approach, different capture component types having different specificity are applied to different regions of the binding surface. For example, this can be achieved by placing microdroplets on different regions of a fiber microwell array if it is used as a binding surface or to a sealing component if it is used as the binding surface. Each micro-droplet would contain a different type of capture component that would functionalize the surface it contacts with different analyte capture specificity In this way, depending on the size of the droplets, it is possible to make an array of different capture component types on the binding surface that are spatially discrete. The number of multiplexed analyte molecules that could be detected in this manner would depend on the size of the different capture component regions and the size of the array of reaction vessels.

In the second example of the spatial approach, it is understood that the analyte molecules of interest can be captured uniformly across a binding surface or within discrete regions of the binding surface. In this approach, however a first type of first binding ligand is applied to a first region of the binding surface and the presence of a first type of analyte molecule is detected. Simultaneously or sequentially, a second type of first binding ligand is applied to a second region of the binding surface and the presence of a second type of analyte molecule is detected.

It is understood that that each of the different assay configurations above, including the use of different types of capture components directed to different types of analyte molecules and the plurality of capture components directed to the same analyte molecule, can also be utilized for quantification as described herein.

In addition, under certain circumstances it is understood that the array of reaction vessels may be reused during a subsequent assay. In this approach, a log of the wells that contained analyte in a first assay is recorded and then in a subsequent assay the wells that previously contained analyte molecules are mathematically subtracted from the array of reaction vessels interrogated in a subsequent assay.

Systems and Kits

In some cases, the present invention provides systems and/or kits for detecting analyte molecules in a fluid sample. In some cases, the systems comprise an array

comprising a plurality of reaction vessels, at least one binding ligand type, and at least one precursor labeling agent type.

In some embodiments, the system and/or kit may provide an array comprising a plurality of reaction vessels, each reaction vessel having a volume not exceeding about 10 attoliters, about 100 attoliters, about 1 femtoliter, about 50 femtoliters, about 100 femtoliters, about 1 picoliter, about 50 picoliters, and the like. Additionally, each reaction vessel may contain at least one capture component immobilized within each reaction vessel having binding specificity for an analyte molecule. In some cases, the capture component may not have binding specificity for an analyte molecule, but rather for a molecule or other entity included that has binding specificity for an analyte molecule. In some instances, the binding constant of the analyte molecule or particle to the capture component is at least about $10^4\,M^{-1}$, about $10^5\,M^{-1}$, about $10^6\,M^{-1}$, about $10^7\,M^{-1}$, about $10^8\,M^{-1}$, about $10^9\,M^{-1}$ or greater.

In some cases, the system and/or kit provides at least one binding ligand type having binding specificity for the analyte molecules or particles. Examples of binding ligands are described in more detail herein. In some instances, the binding constant of the analyte molecule or particle to a binding ligand is at least about $10^4\,M^{-1}$, at least about $10^5\,M^{-1}$, at least about $10^6\,M^{-1}$, at least about $10^7\,M^{-1}$, at least about $10^8\,M^{-1}$,at least about $10^9\,M^{-1}$ or more.

In some embodiments, the system and/or kit also provides at least one type of precursor labeling agent. The labeling agent molecules may be solubilized or suspended in a liquid. As discusses herein, the precursor labeling agent molecules are converted upon exposure to the first binding ligand to labeling agent molecules that are insoluble and/or that become immobilized within a reaction vessel.

The system and/or kit may also comprise other components, all of which have been discussed herein. For example, the kit may further comprise a second binding ligand type (and third binding ligand type, etc). The kit may also comprise a sealing component. In some cases, the kit may also comprise at least one labeling agent reactant, wherein the at least one labeling agent reactant is able to convert, upon exposure to labeling agent molecules, into a detectable product.

Incorporated herein by reference is U.S. Patent Application Serial No. 12/236,484, filed September 23, 2008, entitled "High Sensitivity Determination of the Concentration of Analyte Molecules or Particles in a Fluid Sample," by Duffy, *et al.*

The following examples are included to demonstrate various embodiments of the invention. Those of ordinary skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. Accordingly, the following examples are intended to illustrate certain embodiments of the present invention, but do not exemplify the full scope of the invention.

Example 1

This example outlines the materials used in the following examples. Optical fiber bundles were purchased from Schott North America, Inc (Southbridge, MA). In some cases, the core glass of the optical fiber bundles comprised barium, lanthanum, boron, silica, and aluminum. The refractive index of the core glass was 1.694, and the density was 4.23 g/cc. The cladding glass comprised silica, lead, potassium, sodium, and aluminum. The refractive index of the clad glass was 1.559, and the density was 3.04 g/cc. The fiber array was a bundle of 50,000 individual fibers, each with a core diameter of 4.5μm and the center-to-center spacing of the cores was 8 μm.

Non-reinforced gloss silicone sheets were obtained from Specialty Manufacturing Inc. (Saginaw, MI). Hydrochloric acid, 4-(dimethylamino) pyridine, 3-aminopropyl trimethoxysilane, triethylamine, N,N-Disuccinimidyl carbonate, anhydrous ethanol, and N,N-Dimethyl formamide were all from Sigma-Aldrich (Saint Louis, MO). Monoclonal anti-human TNF-α antibody, biotinylated polyclonal anti-human TNF-α antibody, and recombinant human TNF-α were purchased from R&D systems, Inc (Minneapolis, MN). Tyramide signal amplification kit was from Invitrogen (Carlsbad, California). Phosphate buffered saline, Blocker ™BSA (10%), and 10% TWEEN 20 were obtained from Pierce (Rockford, IL). Fiber polisher was from Allied High Tech Products, Inc. (Rancho Dominguez, California).

Example 2

Optical fibers were interrogated using the following experimental set-up. An upright epifluorescence microscope was custom built containing a mercury light source, excitation and emission filter wheels, objectives, and a CCD camera for acquiring fluorescence images. A mechanical platform that housed the silicone sheet was built

underneath the microscope stage. The stage may be raised up to bring the silicone sheet into contact with the end of the optical fiber array that was mounted onto the microscope stage. This mechanism allows for individual reaction vessels to be sealed during the signal development for immunoassays.

The set-up is similar to that as described above in FIG. 18A and is also pictured in FIG. 18B. The imaging system used for collecting the data integrated an Olympus BX-61 microscope system with a Cooke Corporation Sensicam QE CCD camera, 474. The BX-61 microscope is an upright microscope specifically designed for fluorescence detection. The scope can house UIS2 optics, which, in comparison to standard optics, offers increased signal-to-noise ratio, high light transmission, and diverse illumination capabilities. 10x and 20x UMPlanFl objectives (e.g., 470), which have a NA of 0.3 and 0.5, respectively, were installed on the BX-61 microscope. Wavelength specific filter sets are held in a cube, which is stored in a rotating wheel within the microscope. The BX-61 can hold 6 cubes for easy access to wavelengths specific for a number of fluorophores. Filter sets are typically purchased from Chroma Technology Corporation and are compatible with the cubes on the BX-61. The resorufin filter cube (Chroma Technology filter set #41010) was used for all of the enzyme amplification experiments described here. This filter set uses a HQ577/10x excition filter, a 585 long-pass dichroic mirror, and a HQ620/60m emission filter.

A 6-axis mechanical platform was constructed beneath the microscope stage, which was used to house a non-reinforced silicone gasket material (sealing component) to be applied to the fiber optic array. Arrays were mounted on the microscope stage using a fiber array holder fabricated to secure and position the fiber array on the stage. A droplet of beta-galactosidase substrate (RDG) (precursor labeling agent molecule) was placed on the silicone gasket material, and put into contact with one end of the fiber array. The mechanical platform was used to move the silicone sheet into contact with the distal end of the etched optical fiber array, creating an array of isolated femtoliter reaction vessels.

Example 3

Optical fiber bundles arrays were prepared using the following techniques. Optical fiber bundles approximately 5 cm long were first polished on the polishing machine using 30, 9, and 1 micron-sized diamond lapping films. The polished fiber bundles were chemically etched in a 0.025 M HCl solution for approximately 115

seconds to generate high-density (50,000) microwell arrays and then immediately submerged into water to quench the reaction. To remove impurities from etching, the etched fibers were sonicated for 5 seconds and washed in water for 5 min. The fibers were then dried under argon and silanized in a 2% silanization solution that was prepared by mixing 950 ul (microliter) of anhydrous ethanol, 50 ul of water, and 20 ul of 3-aminopropyl trimethoxysilane for 30min, and then washed with anhydrous ethanol for 10 min and dried under nitrogen. The silanized fibers were cured at 80 °C for 30 min and cooled to room temperature. The optical fiber arrays were then further activated with N,N-Disuccinimidyl carbonate, a homo-bifunctional cross-linker, to introduce NHS ester groups on the arrays for the next immobilization of capture antibodies. The reaction was completed in a mixture with 80mg N,N-Disuccinimidyl carbonate, 4 mg 4-(dimethylamino)pyridine, and 125 ul of triethylamine in 1.6 ml of DMF after 4 hrs incubation and followed by a thorough washing of fibers using anhydrous ethanol for 10min. Finally, the fiber arrays were incubated with capture antibodies (monoclonal anti-human TNF-α antibody, 250 ug/ml in PBS) overnight at room temperature, and washed with PBS.

Example 4

The following is an example a non-limiting example of one assay of the present invention. A fiber optic array was prepared in a similar manner according to Example 3. A stock solution of recombinant human TNF-α protein (571 nM) was made by reconstituting 50 μg of the protein with 1ml of PBS. Different concentrations of the TNF-α targets (1 pM, 500 fM, 200 fM, 80 fM, and 32 fM) were prepared by diluting the stock solution with PBS+ 1% BSA. The functionalized fiber-optic single molecule arrays were blocked with PBS+1% BSA for 8 hrs at 4 °C. Three blocked single molecule arrays were incubated with each different concentration of TNF-α targets target analyte molecules) as well as the negative control overnight at room temperature, followed by washing with PBS + 1% BSA + 0.1% Tween 20 for 20 min at room temperature. The target-bound single molecule arrays were then incubated with 500 pM of biotinylated anti-human TNF-α antibody (first binding ligand) diluted from the stock (~333 nM) with PBS +1% BSA for 1 hr at room temperature. The arrays were washed with PBS + 1% BSA + 0.1% Tween 20 for 20 min. Streptavidin-HRP conjugate (100 μg) was reconstituted in 200 ul of PBS to make a stock solution of 500 μg/ml. The arrays

were then reacted with the diluted streptavidin-HRP conjugate solution (second binding ligand) at 5 μg /ml for 1 hr, and washed again with PBS+ 1% BSA+0.1% Tween 20 for 20 min.

Example 5

The following is an example of signal development using catalyzed reporter deposition. A tyramide-Alexa 488 stock solution was prepared by dissolving the solid material provided with a tyramide signal amplification kit (e.g., from Molecular Probes, Oregon) in 150 ul of DMSO. A working solution containing 0.015% H_2O_2 was made by adding 1 μl of 30% H_2O_2 to 200 μl of amplification buffer provided with the kit and then addition of 10 μl of this intermediate dilution (0.15%) to 100 μl of amplification buffer. This working solution was then used to make 10- or 100-fold dilution of the tyramide-Alexa 488 stock for the signal development for ELISAs on the fiber-optic single molecule array.

The catalyzed reporter deposition on the single molecule array using the HRP system was performed in two different ways. In one instance, the single molecule arrays prepared as described in Example 4 were incubated with the above working solution containing tyramide-Alexa 488 (precursor labeling agent) in an open vial for 15 min. In the other instance, the tyramide working solution was sealed into individual reaction vessels on the single molecule array on the microscope for 30 min. After the signal development, the arrays were thoroughly washed with PBS for 20 min and imaged on the microscope (excitation: 496 nm/emission: 520 nm).

Example 6

The arrays of the present invention may or may not be sealed when using the catalyzed reporter deposition method. The signal development for single molecule detection using the catalyzed HRP reporter system described in Example 5 was compared between sealing an array and without sealing an array for tyramide-Alexa 488 deposition. To seal the array, each fiber bundle was mounted on the scope individually and the tyramide working solution (1:10 dilution from the stock) was sealed with the silicone sheet and isolated into individual reaction vessels with an incubation time of 30 min for tyramide deposition. Without sealing the single molecule array on the microscope, signal development were performed with multiple arrays in a open vial with

a more diluted tyramide working solution (1:100 dilution) and a shorter reaction time (15 min). In this example, a relatively high background signal was recorded from the image without sealing as compare to the array that was sealed. In addition, a greater viability in number of positive wells caused higher detection limits. Without being bound by theory, both of the above observations may be due to the diffusion and deposition of tyramide molecules in the bulk solution into neighboring wells even with the absence of HRP, resulting in high backgrounds and false positive signals. It should be noted, that in some instance, improved results may be observed when not sealing the array, as opposed to sealing the array. Well-sealed arrays ensure the deposition of reporter molecules only within the reaction vessels with the presence of the bound targets and HRP. FIG. 12A and 12B show images of the fiber optic array analyzed in this example, with and without sealing of the array, respectively. FIG. 10 shows a calibration curve determined using arrays that were not sealed while FIG. 11 shows calibration curve determined using arrays that were sealed. In this embodiment, a greater variability in the number of wells detected was observed when the array was not sealed, which results in a higher limit of detection. In some instances, however, the results may be reversed and not sealing the array may result in a higher limit of detection.

Example 7

The following is an example of the variation in the limit of detection observed for fiber optic array using a sealed array. The detection limit of TNF-α was determined on the fiber-optic single molecule arrays using catalyzed reporter deposition based on the HRP (binding ligand) and tyramide precursor labeling agent) Alexa 488 system with sealing. Five different concentrations of TNF-α (target analyte molecule) were included to generate the calibration curve as well as the negative control with the absence of TNF-α target. The percentage of detectable wells was obtained for each array. For each concentration level, three fiber arrays were used, and the averaged data from the three replicates was used to plot the calibration curve. Overnight target incubation was performed for all target concentrations to obtain a lower detection limit. Buffers with high stringency were used for washing between incubation steps to reduce any nonspecific interactions. As shown in FIG. 15, a relatively low NSB (1.43+/-0.056%) was obtained from the negative control. A detection limit of approximately 32 fM was

achieved for TNF-α with the detectable wells (1.93+/-0.47%) greater than the signal from NSB plus three times the standard deviations of NSB (1.59%).

Fluorescent images of the fiber optic arrays were analyzed using IPlab software (Scanalytics, Fairfax, VA). The number of microwells in the fiber-optic single molecule array with fluorescence intensity above a background threshold (e.g., background signal as determined by completing the assay with a sample that does not comprise analyte molecules or particles) was counted for each image. The percentage of these positive wells was used to quantify the concentration of targets using Poisson statistics, as discussed herein.

While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively.

What is claimed:

Claims

1. A method of detecting analyte molecules or particles in a fluid sample containing or suspected of containing analyte molecules or particles, comprising:

partitioning the fluid sample across an array comprising a plurality of reaction vessels, so that at least some of the reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle;

immobilizing at least one binding ligand with respect to an analyte molecule or particle within each reaction vessel containing at least one analyte molecule or particle;

exposing the at least one binding ligand to a liquid in which is solubilized or suspended precursor labeling agent molecules, wherein exposure of the precursor labeling agent molecules to the at least one binding ligand converts at least some of the precursor labeling agent molecules into a labeling agent molecules which are insoluble in the liquid and/or which become immobilized within the reaction vessel; and

determining from detecting the presence of the labeling agent molecules the number of reaction vessels which contain an analyte molecule or particle.

2. The method of claim 1, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 10% of the total number of reaction vessels.

3. The method of claim 1, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 5% of the total number of reaction vessels.

4. The method of claim 1, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 1% of the total number of reaction vessels.

5. The method of claim 1, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 0.1% of the total number of reaction vessels.

6. The method of claim 1, wherein a first and a second binding ligand is provided.

7. The method of claim 6, wherein the first binding ligand is associated with an analyte molecule or particle and the second binding ligand is associated with the first binding ligand.

8. The method of claim 1, wherein the array of reactions vessels comprises a plurality of fiber optic microwells.

9. The method of claim 1, wherein the volume of a reaction vessel is between about 10 attoliters and about 50 picoliters.

10. The method of claim 1, wherein each reaction vessel comprises at least one analyte capture component.

11. The method of claim 10, where the analyte molecules or particles are immobilized in the reaction vessel by associated with at least one analyte capture component.

12. The method of claim 1, wherein each reaction vessel comprises at least one labeling agent capture component.

13. The method of claim 12, wherein the labeling agent molecules are immobilized in the reaction vessel by associating with the at least one labeling agent capture component.

14. The method of claim 1, wherein the at least one binding ligand comprises an enzymatic component.

15. The method of claim 14, wherein the enzymatic component is horseradish peroxidase.

16. The method of claim 1, wherein the at least one binding ligand comprises a nanoparticle.

17. The method of claim 16, wherein the nanoparticle is a gold nanoparticle.

18. The method of claim 1, wherein the precursor labeling agent molecules are converted to labeling agent molecules by electroless deposition.

19. The method of claim 1, wherein the precursor labeling agent molecules comprise tyramide.

20. The method of claim 6, wherein a third binding ligand is provided, and wherein the first binding ligand is associated with analyte molecules or particles contained in the reaction vessels, the second binding ligand is immobilized with respect to the first binding ligand, and the third binding ligand is immobilized with respect to the second binding ligand.

21. The method of claim 1, wherein the labeling agent molecules are detected directly.

22. The method of claim 1, wherein the labeling agent molecules are detected indirectly.

23. The method of claim 1, wherein at least about 95% of the reaction vessels contain no analyte molecule or particle.

24. The method of claim 1, wherein at least about 90% of the reaction vessels contain no analyte molecule or particle.

25. The method of claim 1, wherein at least about 80% of the reaction vessels contain no analyte molecule or particle.

26. The method of claim 1, wherein at least about 40% of the reaction vessels contain no analyte molecule or particle.

27. The method of claim 1, wherein at least about 5% of the reaction vessels contain no analyte molecule or particle.

28. The method of claim 1, wherein the labeling agent molecules associate with at least one binding ligand.

29. The method of claim 1, wherein the labeling agent molecules form a precipitate.

30. The method of claim 1, wherein the labeling agent molecules are fluorogenic.

31. The method of claim 1, wherein the labeling agent molecules are chromogenic.

32. The method of claim 1, wherein the labeling agent molecules are chemiluminescent.

33. The method of claim 1, wherein the array comprising between about 100 and about 1,000,000 reaction vessels.

34. The method of claim 1, wherein the array of reaction vessels is sealed during at least a portion of the method.

35. The method of claim 1, wherein the array of reactions vessels is not sealed during at least a portion of the method.

36. The method of claim 6, wherein the second binding ligand comprises streptavidin.

37. The method of claim 6, wherein the second binding ligand comprises biotin.

38. The method of claim 1, wherein the at least one binding ligand comprises an antibody that associates with an analyte molecule or particle.

39. The method of claim 1, wherein the labeling agent molecules comprise an enzymatic component.

40. The method of claim 1, wherein a first labeling agent reactant is provided, and wherein the reactant is converted into a detectable product upon exposure to a labeling agent molecule.

41. The method of claim 1, wherein a first labeling agent reactant and a second labeling agent reactant are provided, wherein the first labeling agent reactant associates with the labeling agent molecules and the second labeling agent reactant is converted to a detectable product upon exposure to the first labeling agent reactant.

42. A system for detecting analyte molecules or particles, comprising;

an array comprising a plurality of reaction vessels, wherein at least some of the reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle;

at least one binding ligand immobilized with respect an analyte molecule or particle within each reaction vessel containing an analyte molecule or particle; and

precursor labeling agent molecules solubilized or suspended in a liquid contained within the reaction vessels, wherein the precursor labeling agent molecules are able to convert upon exposure to a binding ligand to labeling agent molecules that are insoluble within the liquid and/or that become immobilized within reaction vessels containing a binding ligand.

43. The system of claim 42, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 10% of the total number of reaction vessels.

44. The system of claim 42, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 5% of the total number of reaction vessels.

45. The system of claim 42, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 1% of the total number of reaction vessels.

46. The system of claim 42, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 0.1% of the total number of reaction vessels.

47. The system of claim 42, wherein a first and a second binding ligand are immobilized with respect an analyte molecule or particle within each reaction vessel containing an analyte molecule or particle.

48. The system of claim 47, wherein the first binding ligand is associated with the analyte molecule or particle and the second binding ligand is associated with the first binding ligand.

49. The system of claim 42, wherein the array of reaction vessels comprises a plurality of fiber optic microwells.

50. The system of claim 42, wherein the volume of a reaction vessel is between about 10 attoliters and about 50 picoliters.

51. The system of claim 42, wherein the array comprising the plurality of reaction vessels comprises at least a portion of a substantially planar surface mated with at least a portion of a sealing component comprising a plurality of microwells.

52. The system of claim 42, wherein the array comprising a plurality of reaction vessels comprises at least a portion of a sealing component mated with at least a portion of a substantially planar substrate.

53. The system of claim 42, wherein each reaction vessel comprises at least one analyte capture component.

54. The system of claim 53, where the analyte molecules or particles are immobilized in the reaction vessel by association with at least one analyte capture component.

55. The system of claim 42, wherein each reaction vessel comprises at least one labeling agent capture component.

56. The system of claim 55, wherein the labeling agent molecules are immobilized in the reaction vessel by association with the at least one labeling agent capture component.

57. The system of claim 42, wherein the at least one binding ligand comprises an enzymatic component.

58. The system of claim 57, wherein the enzymatic component is horseradish peroxidase.

59. The system of claim 42, wherein the at least one binding ligand comprises a nanoparticle.

60. The system of claim 59, wherein the nanoparticle is a gold nanoparticle.

61. The system of claim 42, wherein the precursor labeling agent molecules are converted to labeling agent molecules by electroless deposition.

62. The system of claim 42, wherein the precursor labeling agent comprises tyramide.

63. The system of claim 42, wherein the labeling agent molecules form a precipitate.

64. The system of claim 42, wherein the labeling agent molecules are fluorogenic.

65. The system of claim 42, wherein the labeling agent molecules are chromogenic.

66. The system of claim 42, wherein the labeling agent molecules are chemiluminescent.

67. The system of claim 42, wherein the array comprising between about 100 and about 1,000,000 reaction vessels.

68. The system of claim 42, wherein at least a portion of the array of reaction vessels is sealed.

69. The system of claim 42, wherein at least a portion of the array of reactions vessels is not sealed.

70. The system of claim 42, wherein the at least one binding ligand comprises streptavidin.

71. The system of claim 42, wherein the at least one binding ligand comprises biotin.

72. The system of claim 42, wherein the labeling agent molecules comprise an enzyme.

73. The system of claim 42, wherein a first labeling agent reactant is provided, and wherein the first labeling agent reactant is converted into a detectable product upon exposure to the labeling agent molecules.

74. The system of claim 42, wherein a first labeling agent reactant and a second labeling agent reactant are provided.

75. The system of claim 74, wherein the first labeling agent reactant associates with the labeling agent molecules, and the second labeling agent reactant is converted to a detectable product when exposed to the first labeling agent reactant.

76. A method of detecting analyte molecules or particles in a fluid sample containing or suspected of containing analyte molecules or particles, comprising:

providing a fluid sample containing or suspected of containing analyte molecules or particles;

immobilizing at least one binding ligand with respect to at least some of the analyte molecules or particles;

exposing the at least one binding ligand to a liquid in which is solubilized or suspended precursor labeling agent molecules, wherein exposing the precursor labeling agent molecules to the at least one binding ligand converts at least some of the precursor labeling agent molecules into labeling agent molecules which are insoluble in the liquid and/or which become immobilized within the reaction vessel; and

determining a measure of the concentration of the analyte molecules or particles in the fluid sample based on the detection of labeling agent molecules wherein the true concentration of the analyte molecules or particles in the fluid sample is less than about 100×10^{-15} molar, and wherein the measure of the concentration determined in the determining act differs from the true concentration by no greater than 10%..

77. The method of claim 76, wherein the concentration of analyte molecules or particles in the fluid sample is less than about 50×10^{-15}.

78. The method of claim 76, wherein the concentration of analyte molecules or particles in the fluid sample is less than about 10×10^{-15}.

79. The method of claim 76, wherein the labeling agent molecules are detected directly.

80. The method of claim 76, wherein the labeling agent molecules are detected indirectly.

81. A kit for detecting analyte molecules or particles comprising:

an array comprising a plurality of reaction vessels, each reaction vessel having a volume not exceeding about 100 femtoliters and each reaction vessel containing at least one analyte capture component immobilized or able to become immobilized within the reaction vessels having binding specificity for the analyte molecules or particles;

at least one binding ligand having binding specificity for the analyte molecules or particles; and

precursor labeling agent molecules able to be solubilized or suspended in a liquid, wherein the precursor labeling agent molecules are able to convert upon exposure to the at least one binding ligand to labeling agent molecules that are insoluble within the liquid and/or that become immobilized within the reaction vessels.

82. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the capture component is at least about $10^4\ M^{-1}$.

83. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the capture component is at least about $10^5\ M^{-1}$.

84. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the capture component is at least about $10^6\ M^{-1}$.

85. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the capture component is at least about $10^7\ M^{-1}$.

86. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the capture component is at least about 10^8 M^{-1}.

87. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the capture component is at least about 10^9 M^{-1}.

88. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the first binding ligand is at least about 10^4 M^{-1}.

89. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the first binding ligand is at least about 10^5 M^{-1}.

90. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the first binding ligand is at least about 10^6 M^{-1}.

91. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the first binding ligand is at least about 10^7 M^{-1}.

92. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the first binding ligand is at least about 10^8 M^{-1}.

93. The kit of claim 81, wherein the binding constant of the analyte molecule or particle to the first binding ligand is at least about 10^9 M^{-1}.

94. The kit of claim 81, wherein the kit further comprises a first and a second binding ligand.

95. The kit of claim 94, wherein the first binding ligand has binding specificity for the analyte molecules or particles and the second binding ligand has binding specificity for the first binding ligand.

96. The kit of claim 81, wherein the array of reactions vessels comprises a plurality of fiber optic microwells.

97. The kit of claim 81, wherein the volume of a reaction vessel is between about 10 attoliters and about 50 picoliters.

98. The kit of claim 81, wherein the array comprising between about 100 and about 1,000,000 reaction vessels.

99. The kit of claim 81, wherein the at least one binding ligand comprises an enzymatic component.

100. The kit of claim 99, wherein the enzymatic component is horseradish peroxidase.

101. The kit of claim 81, wherein the at least one binding ligand comprises a nanoparticle.

102. The kit of claim 101, wherein the nanoparticle is a gold nanoparticle.

103. The kit of claim 81, wherein the precursor labeling agent molecules comprise tyramide.

104. The kit of claim 81, wherein the labeling agent molecules form a precipitate.

105. The kit of claim 81, wherein the labeling agent molecules are fluorogenic.

106. The kit of claim 81, wherein the labeling agent molecules are chromogenic.

107. The kit of claim 81, wherein the labeling agent molecules are chemiluminescent.

108. The kit of claim 81, further comprising a sealing component.

109. The kit of claim 94, wherein the second binding ligand comprises streptavidin.

110. The kit of claim 94, wherein the first binding ligand comprises biotin.

111. The kit of claim 81, wherein the labeling agent molecules comprise an enzymatic component.

112. The kit of claim 81, further comprising a first labeling agent reactant and wherein the first labeling agent reactant is able to convert to a detectable product upon exposure to the labeling agent molecules.

113. A method of detecting analyte molecules or particles in a fluid sample containing or suspected of containing analyte molecules or particles, comprising:

partitioning the fluid sample across an array comprising a plurality of reaction vessels, so that at least some of the reaction vessels contain no analyte molecules or particles and at least some of the reaction vessels contain at least one analyte molecule or particle;

immobilizing at least one binding ligand comprising a binding site with respect to an analyte molecule or particle within each reaction vessel containing at least one analyte molecule or particle;

applying an enzymatic component to the array and capturing the enzymatic component with the binding site;

contacting the enzymatic component with precursor labeling agent molecules, wherein the precursor labeling agent molecules are converted to labeling agent molecules upon contact with enzymatic components;

detecting the labeling agent molecules; and

determining from the detection of the labeling agent molecules the number of reaction vessels which contain an analyte molecule or particle.

114. The method of claim 113, wherein a first binding ligand and a second binding ligand are provided.

115. The method of claim 114, wherein the first binding ligand is associated with an analyte molecule or particle and the second binding ligand is associated with the first binding ligand.

116. The method of claim 114, wherein the second binding ligand comprises the binding site.

117. The method of claim 113, wherein the array of reactions vessels comprises a plurality of fiber optic microwells.

118. The method of claim 113, wherein the volume of a reaction vessel is between about 10 attoliters and about 50 picoliters.

119. The method of claim 113, wherein each reaction vessel comprises at least one analyte capture component.

120. The method of claim 119, wherein the analyte molecules or particles are immobilized in the reaction vessel by association with at least one analyte capture component.

121. The method of claim 113, wherein each reaction vessel comprises at least one labeling agent capture component.

122. The method of claim 121, wherein the labeling agent molecules are immobilized in the reaction vessel by associating with the at least one labeling agent capture component.

123. The method of claim 113, wherein the enzymatic component is horseradish peroxidase.

124. The method of claim 113, wherein the precursor labeling agent molecules comprise tyramide.

125. The method of claim 113, wherein at least one binding ligand is associated with each analyte molecule or particle contained in the reaction vessels.

126. The method of claim 113, wherein the labeling agent molecules are detected directly.

127. The method of claim 113, wherein the labeling agent molecules are detected indirectly.

128. The method of claim 113, wherein at least about 95% of the reaction vessels contain no analyte molecule or particle.

129. The method of claim 113, wherein at least about 90% of the reaction vessels contain no analyte molecule or particle.

130. The method of claim 113, wherein at least about 80% of the reaction vessels contain no analyte molecule or particle.

131. The method of claim 113, wherein at least about 40% of the reaction vessels contain no analyte molecule or particle.

132. The method of claim 113, wherein at least about 5% of the reaction vessels contain no analyte molecule or particle.

133. The method of claim 113, wherein the labeling agent molecules form a precipitate.

134. The method of claim 113, wherein the labeling agent molecules are fluorogenic.

135. The method of claim 113, wherein the labeling agent molecules are chromogenic.

136. The method of claim 113, wherein the labeling agent molecules are chemiluminescent.

137. The method of claim 113, wherein the array comprises between about 100 and about 1,000,000 reaction vessels.

138. The method of claim 113, wherein the array comprises at least about 1,000 reaction vessels.

139. The method of claim 113, wherein the array of reaction vessels is sealed during at least a portion of the method.

140. The method of claim 113, wherein the array of reactions vessels is not sealed during at least a portion of the method.

141. The method of claim 113, wherein the at least one binding ligand comprises streptavidin.

142. The method of claim 113, wherein the at least one binding ligand comprises an antibody that associates with the analyte molecule or particle.

143. The method of claim 113, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 10% of the total number of reaction vessels.

144. The method of claim 113, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 5% of the total number of reaction vessels.

145. The method of claim 113, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 1% of the total number of reaction vessels.

146. The method of claim 113, wherein the percentage of reaction vessels which comprise at least one analyte molecule or particle is less than about 0.1% of the total number of reaction vessels.

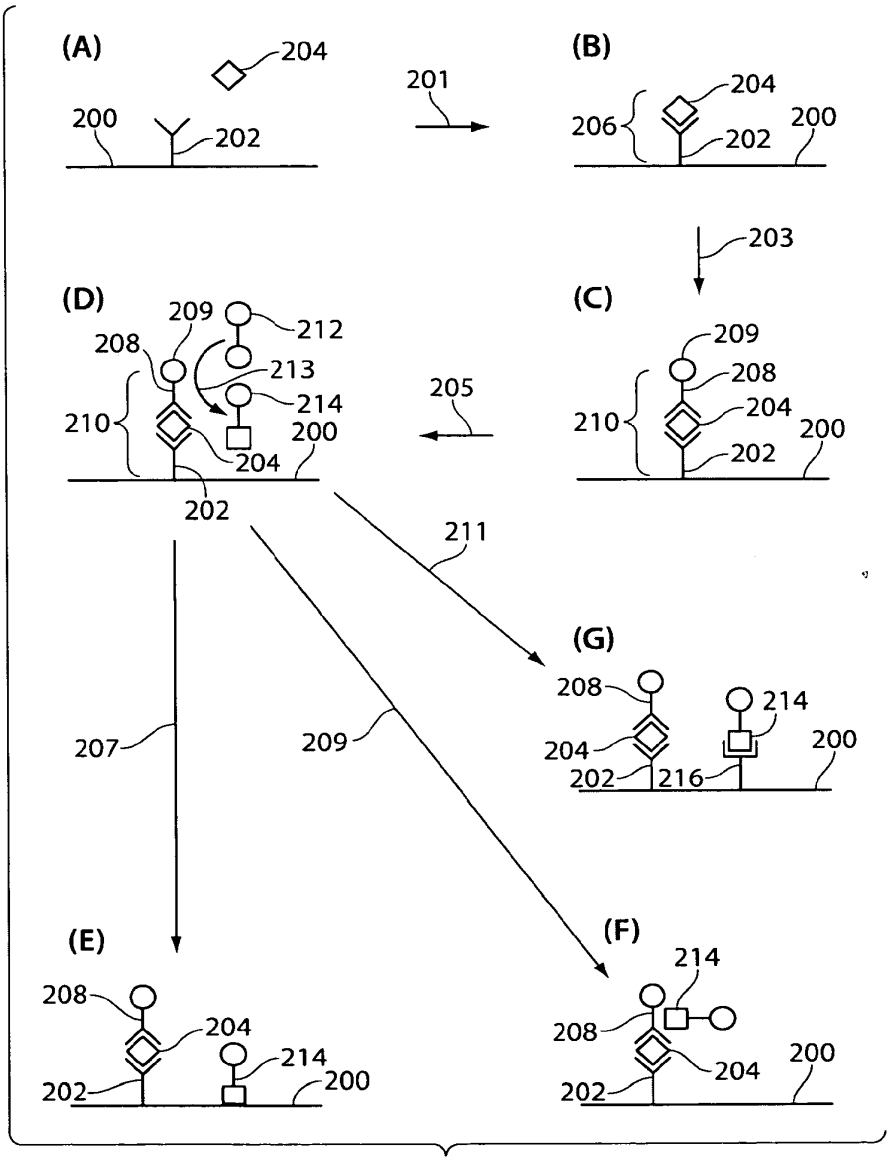


Fig. 1

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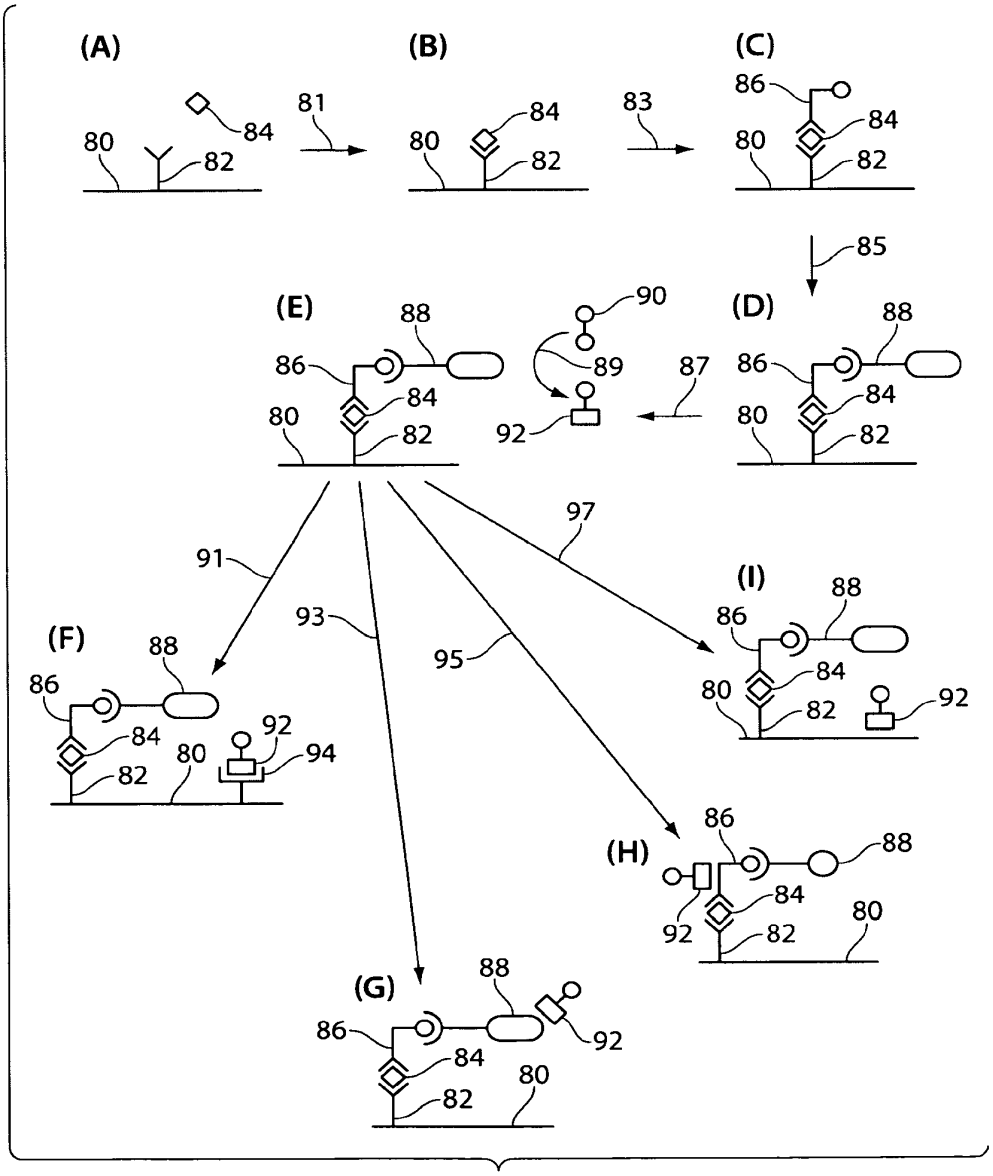


Fig. 2

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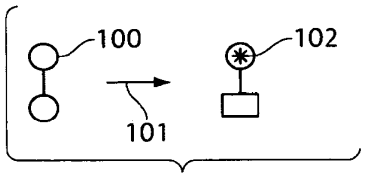


Fig. 3A

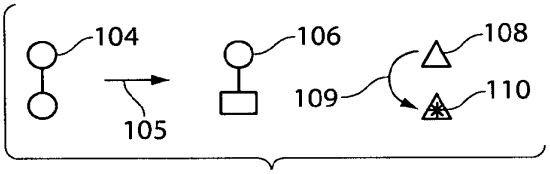


Fig. 3B

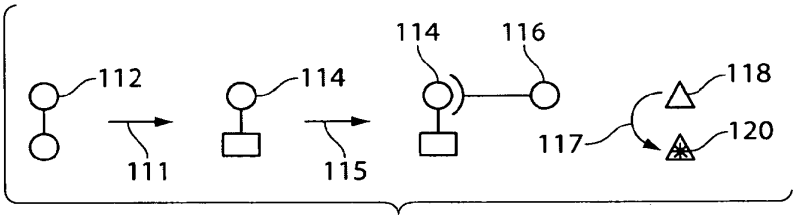


Fig. 3C

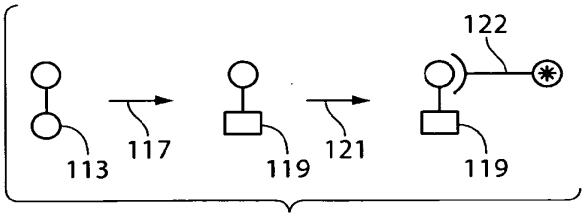


Fig. 3D

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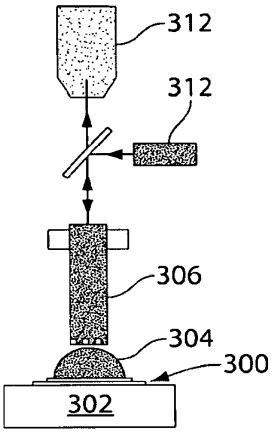


Fig. 4A

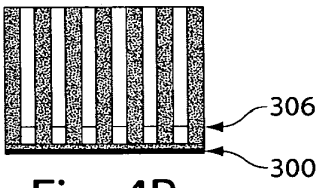


Fig. 4B

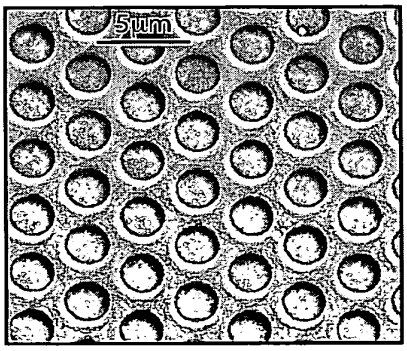


Fig. 4C

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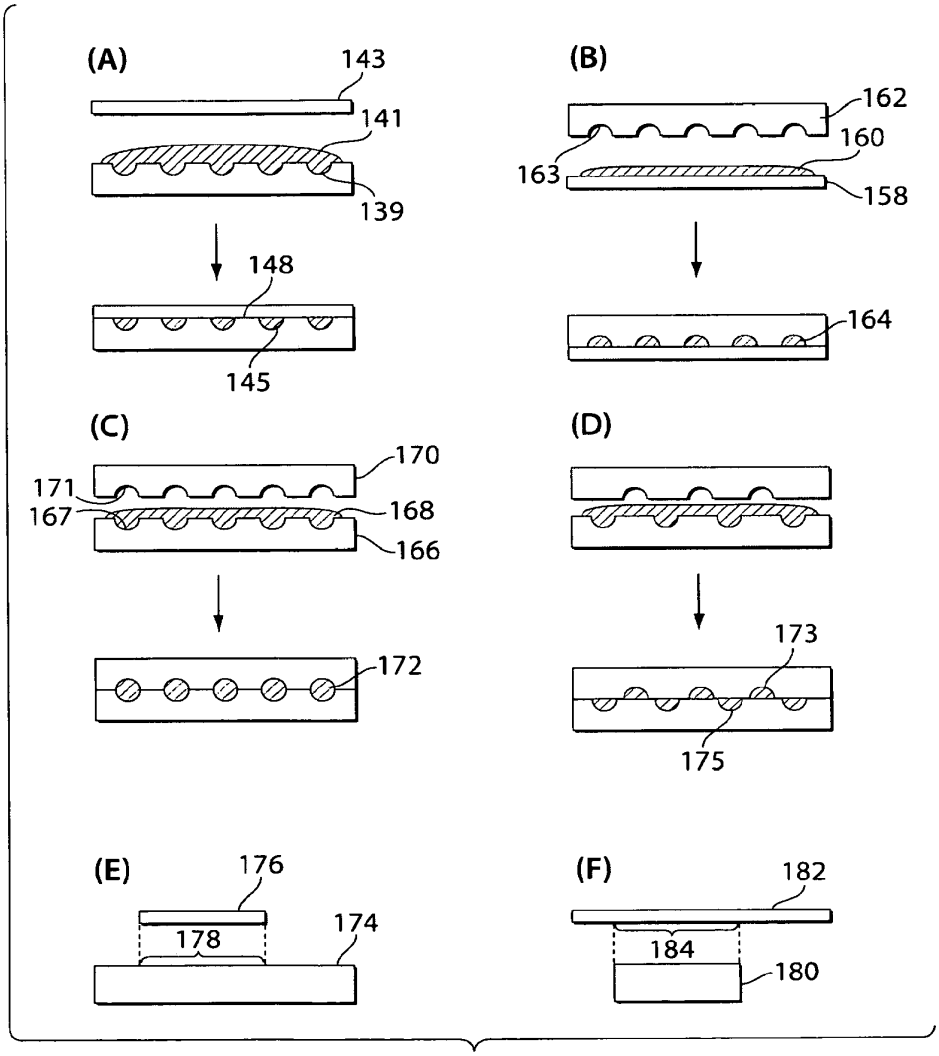


Fig. 5

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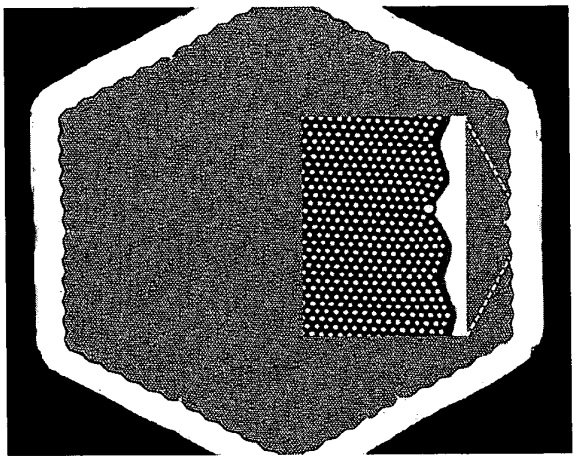


Fig. 6A

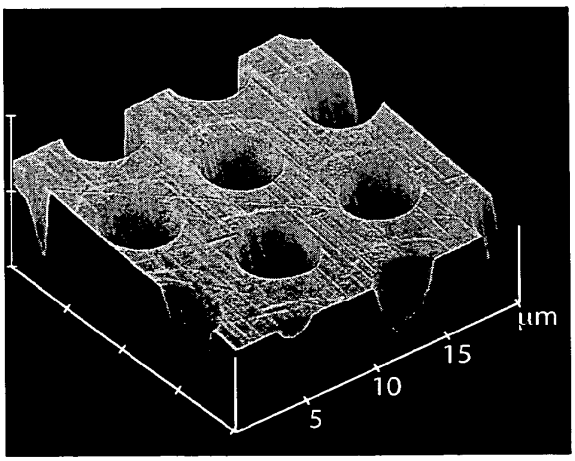


Fig. 6B

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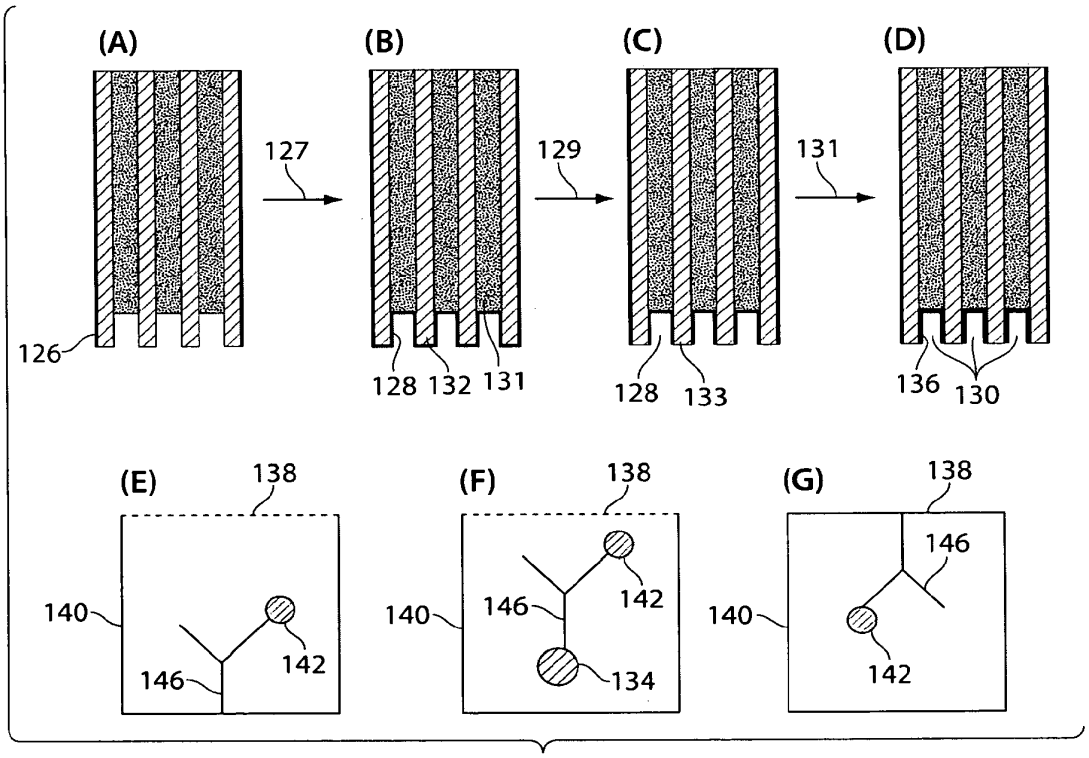


Fig. 7

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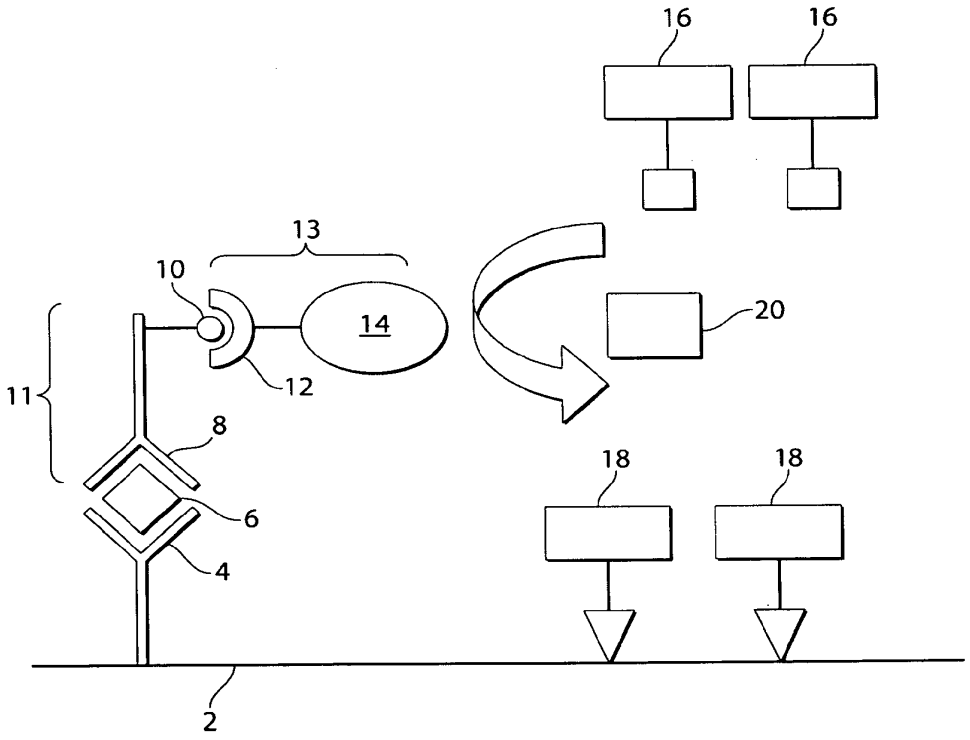


Fig. 8

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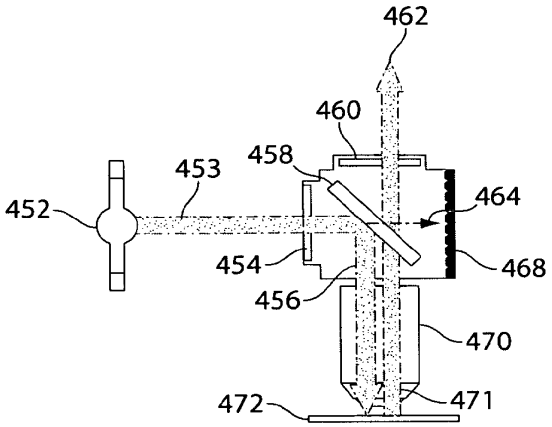


Fig. 9A

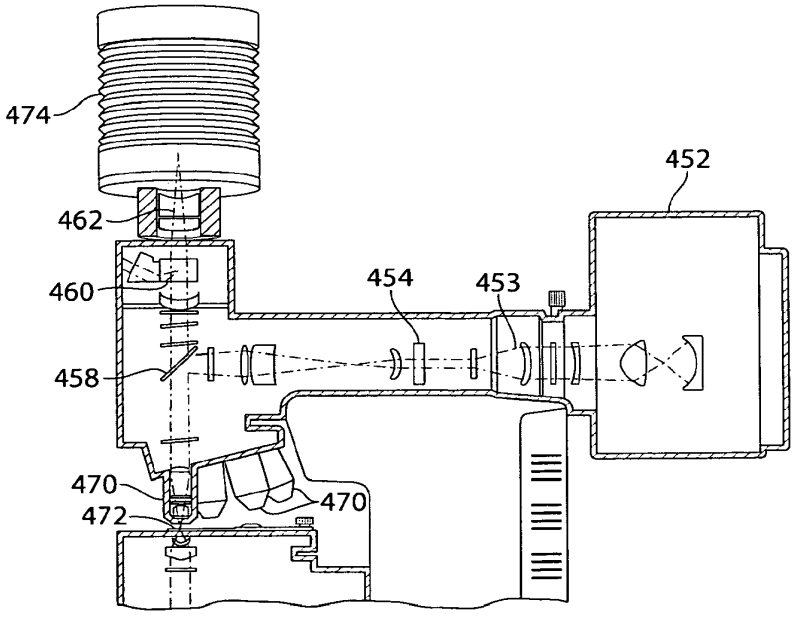


Fig. 9B

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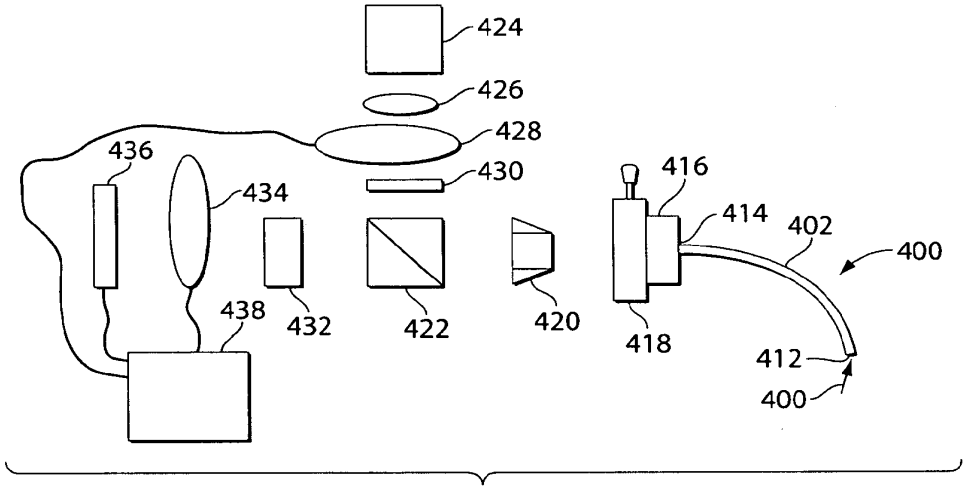


Fig. 10

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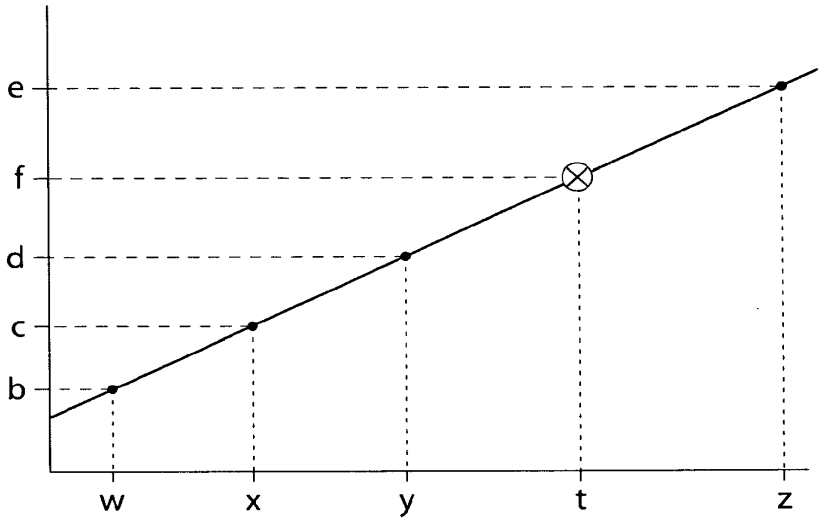


Fig. 11

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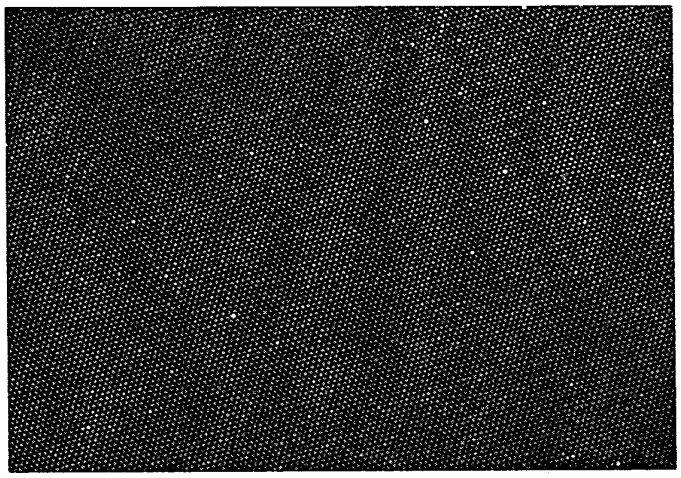


Fig. 12A

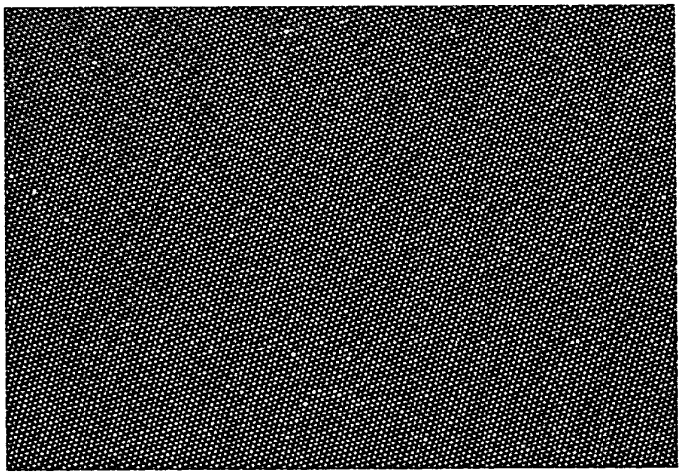


Fig. 12B

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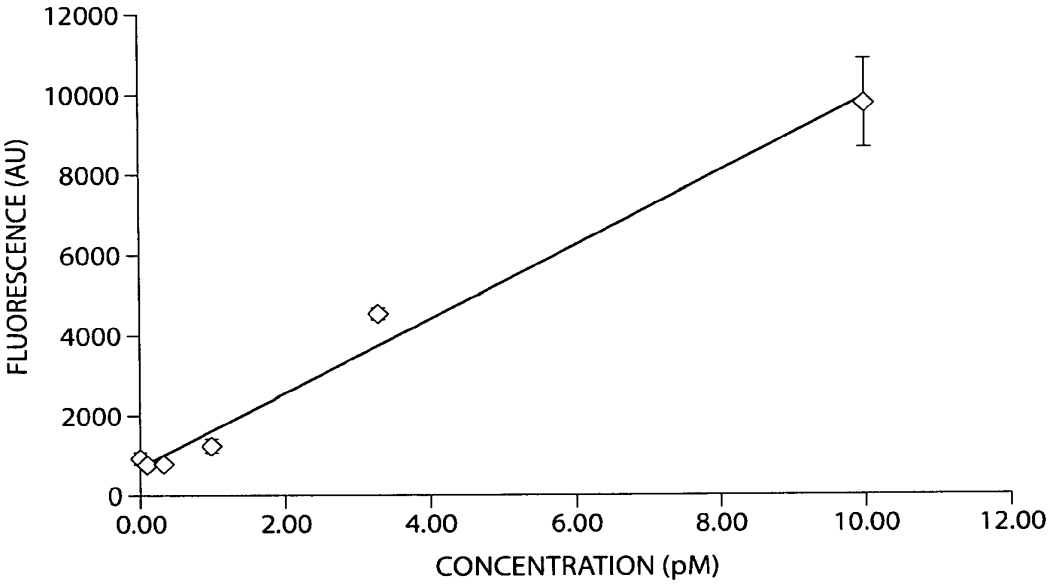


Fig. 13

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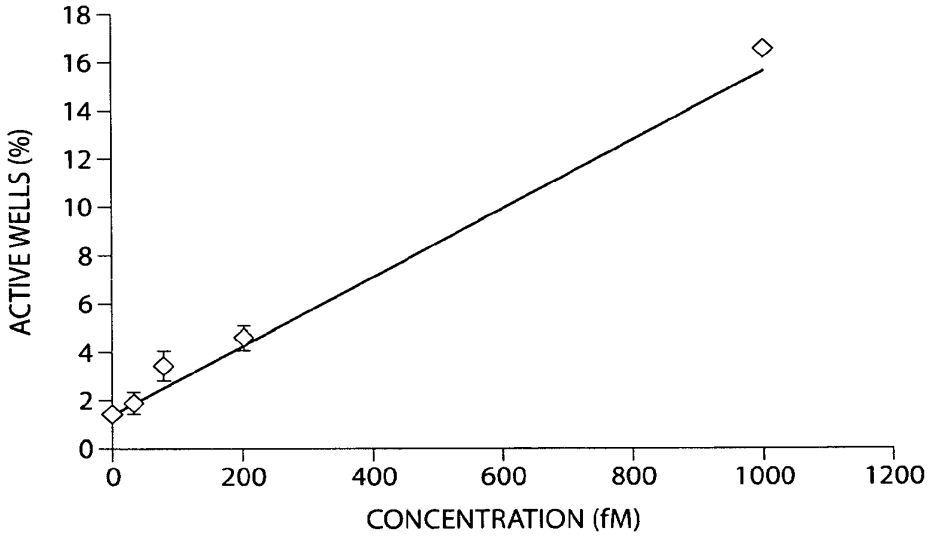


Fig. 14

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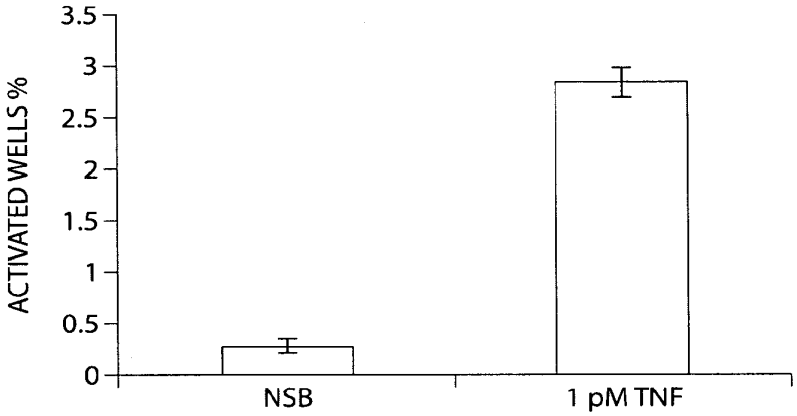


Fig. 15

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Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

[Continued on next page]

(54) Title: MICRO-RNA, AUTOANTIBODY AND PROTEIN MARKERS FOR DIAGNOSIS OF NEURONAL INJURY

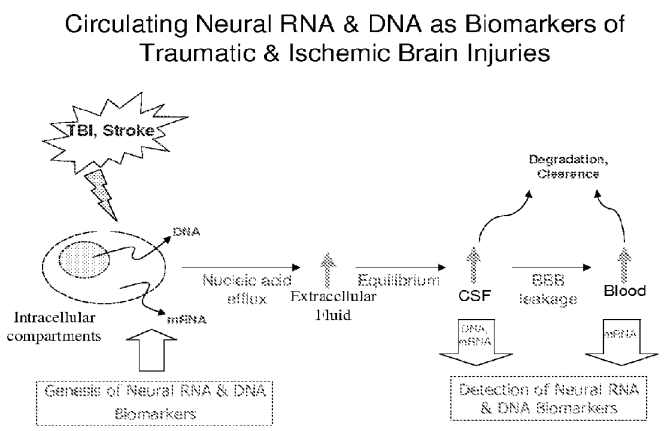


FIG. 34

(57) Abstract: Processes and materials are provided for the detection, diagnosis, or determination of the severity of a neurological injury or condition, including traumatic brain injury, multiple-organ injury, stroke, Alzeimer's disease, Pakinson disease and Chronic Traumatic Encephalopathy (CTE),. The processes and materials include biomarkers detected or measured in a biological sample such as whole blood, serum, plasma, or CSF. Such biomarkers include Tau and GFAP proteins, their proteolytic breakdown products, brain specific or enriched micro-RNA, and brain specific or enriched protein directed autoantibodies. The processes and materials are operable to detect the presence of absence of acute, subacute or chronic brain injuries and predict outcome for the brain injury.

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MICRO-RNA, AUTOANTIBODY AND PROTEIN MARKERS FOR DIAGNOSIS OF NEURONAL INJURY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 61/242,123 filed September 14, 2009, 61/354,504 filed June 14, 2010, 61/355,779 filed June 17, 2010, and 61/380,158 filed September 3, 2010, the contents of each of which are incorporated herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the identification of markers of traumatic brain injury (TBI) or other neurological conditions including stroke, Alzeimer's disease, Pakinson disease and Chronic Traumatic Encephalopathy (CTE).. Inventive markers include auto-antibodies, DNA, RNA, or miRNA that may play a role in central nervous system function and therapy. The invention also relates to diagnostic and therapeutic materials and methods, including methods for detecting biomarkers in brain injury, and the diagnostic method for aiding and monitoring the propensity or progression of brain injury.

BACKGROUND OF THE INVENTION

[0003] The field of clinical neurology remains frustrated by the recognition that secondary injury to a central nervous system tissue associated with physiologic response to the initial insult could be lessened if only the initial insult could be rapidly diagnosed or in the case of a progressive disorder before stress on central nervous system tissues reached a preselected threshold. Traumatic, ischemic, and neurotoxic chemical insult, along with generic disorders, all present the prospect of brain damage. While the diagnosis of severe forms of each of these causes of brain damage is straightforward through clinical response testing, computed tomography (CT), and magnetic resonance imaging (MRI), the imaging diagnostics are limited by both the high cost of spectroscopic imaging and long diagnostic time. The clinical response testing of incapacitated individuals is of limited value and often precludes a nuanced diagnosis. Additionally, owing to the limitations of existing diagnostics, situations arise wherein a subject experiences a stress to their neurological condition but are often unaware that damage has occurred or fail seek treatment as the subtle symptoms often quickly resolve. The lack of treatment of these mild to moderate challenges to neurologic condition of a subject can have a cumulative effect or otherwise result in a severe brain damage event, either of which have a poor clinical prognosis.

[0004] In order to overcome the limitations associated with spectroscopic and clinical response diagnosis of neurological condition, there is increasing attention on the use of biomarkers as internal indicators of change to molecular or cellular level health condition of a subject. As biomarker detection uses a sample obtained from a subject, typically cerebrospinal fluid, blood, or plasma, and detects the biomarkers in that sample, biomarker detection holds the prospect of inexpensive, rapid, and objective measurement of neurological condition. The attainment of rapid and objective indicators of neurological condition allows one to determine severity of a non-normal brain condition with a previously unrealized degree of objectivity, predict outcome, guide therapy of the condition, as well as monitor subject responsiveness and recovery. Additionally, such information as obtained from numerous subjects allows one to gain a degree of insight into the mechanism of brain injury.

[0005] Biomarkers of central nervous system (CNS) injury could provide physicians and laboratory studies with quantifiable neurochemical markers to help determine not only the severity and cellular pathology of injury, but also provide a surrogate marker of therapeutic interventions. While a number of potential biochemical markers for TBI have been proposed, no definitive marker or process has been shown capable of diagnosing TBI, distinguishing between MTBI and TBI, or of demonstrating successful or therapeutic advantage of therapeutic administration. This shortcoming is further apparent should an individual also suffer from multiple organ injury. Brain injuries are commonly difficult to treat effectively, and successful outcome commonly depends on how rapidly an individual is diagnosed with a particular injury subtype. Thus, a need exists for a sensitive and specific biochemical marker(s) of TBI with the diagnostic ability to evaluate post-concussion intracranial pathology to improve patient management and facilitate therapeutic evaluation.

SUMMARY OF THE INVENTION

[0006] Materials and processes for detecting a neurological condition in a subject are provided that include measuring a quantity of one or more neuron specific biomarkers in a biological sample, wherein the synthesis or production of the biomarker is altered following an injury and detecting a neurological condition based on a ratio of the quantity one or more of the biomarkers in said biological sample. A neuron specific biomarker is optionally a protein, a protein breakdown product, nucleic acid molecule (e.g. oligonucleotide such as DNA, RNA, or miRNA), or an autoantibody to any thereof. Optionally combinations of multiple biomarkers are measured in one or more biological samples. Illustrative examples of biomarkers include an

autoantibody directed toward a protein, wherein said protein is: GFAP; Tau; S100β; βIII-tubulin; Neuroflilament light, medium or heavy polypeptide (NF-L, -M and –H); V-type proton ATPase; Gamma-enolase (NSE); vimentin; endophilin-A1; Microtubule-associated protein 2 (MAP-2); alpha-internexin; neuroserpin; neuromodulin; synaptotagmin-1; Voltage-gated potassium channel; collapsin response mediator proteins (CRMP-1 to 5); αII-spectrin; neurofascin; MBP; ubiquitin carboxyl-terminal esterase; Poly (ADP-ribose) polymerase (PARP); breakdown products thereof; derivatives thereof; or combinations thereof. In some embodiments a biomarker is a nucleic acid encoding at least a portion of a protein such as a DNA or RNA oligonucleotide, wherein the protein is GFAP; αII-spectrin; an αII-spectrin breakdown product; neurofascin; MBP; MAP2; ubiquitin carboxyl-terminal esterase; a ubiquitin carboxyl-terminal hydrolase; a neuronally-localized intracellular protein; MAP-tau; C-tau; Poly (ADP-ribose) polymerase (PARP); a collapsin response mediator protein; breakdown products thereof, derivatives thereof, or combinations thereof. A biomarker is optionally a miRNA. A miRNA is optionally brain enriched or brain specific. A miRNA optionally is a at least a portion of a miRNA that regulates the expression of a protein, wherein the protein is GFAP; αII-spectrin; an αII-spectrin breakdown product; neurofascin; MBP; MAP2; ubiquitin carboxyl-terminal esterase; a ubiquitin carboxyl-terminal hydrolase; a neuronally-localized intracellular protein; MAP-tau; p53; SYTL1; calpastatin; Poly (ADP-ribose) polymerase (PARP); CAPN1, 2, or 6; IRS-1; SMAD5; a collapsin response mediator protein; synaptotagmin-1 or -9; Rho kinase; synapsin 1; syntaphilin; ATXN1; derivatives thereof; or combinations thereof. A miRNA optionally is one of SEQ ID NOs: 51-113, optionally one of SEQ ID NOs: 51-60 or 84-93, or optionally miR-9, miR-34, miR-92b, miR-124a, miR-124b, miR-135, miR-153, miR-183, miR-219, miR-222, miR-125a, miR-125b, miR-128, miR-132, miR-135, miR-137, miR-139, miR-218a, or combinations thereof.

[0007] In some embodiments, a biomarker is a breakdown product of a neuronally localized or protein. Optionally a breakdown product is a breakdown product of GFAP such as GBDPs or a breakdown product of Tau such as TBDPs. A GFAP breakdown product is optionally produced by cleavage at Asn59, Thr383, or both, in SEQ ID NO: 114. A TBDP is optionally produced by cleavage C-terminal to amino acid 25, 44, 129, 157, 229, 421, or combinations thereof, each in SEQ ID NO: 11. Alternatively, or additionally, a TBDP is a breakdown product of rat Tau (SEQ ID NO: 5) from cleavage C-terminal to amino acid 43, 120, 220, 370, 412, or combinations thereof.

[0008] The measured concentration of one or more biomarkers is optionally compared to the level of the biomarker in a similar biological sample from one or more control subjects to provide a ratio of concentrations. The ratio is optionally positive where the level of biomarker in a biological sample from a subject is higher than that of a control subject, or negative where the level of biomarker in a biological sample from a subject is lower than that of a control subject.

[0009] It is appreciated that any suitable biological sample is operable. Illustrative examples include blood, plasma, serum, CSF, urine, saliva, or tissue. A biological sample is optionally cell free plasma or cell free serum.

[0010] An injury is optionally induced by a compound. Illustratively, a compound is administered to a subject as a therapeutic, or as a candidate therapeutic such as in drug discovery or development.

[0011] The materials and processes detect, diagnose, or measure the level of one or more conditions such as brain injury (e.g. traumatic brain injury), multiple-organ injury, stroke, neurodegenerative disease, or combinations thereof.

[0012] Also provided is a detection agent for detecting and optionally quantifying a biomarker in a biological sample. A detection agent optionally binds to a portion of GFAP within 10 amino acids of the cleavage site at amino acid position 59 or 383 in SEQ ID NO: 114 or variants thereof. A detection agent optionally binds human Tau (SEQ ID NO: 11) amino sequence within ten amino acids of the cleavage site at amino acid position 25, 44, 129, 157, 229, or 421. Optionally, a detection agent binds an amino acid sequence SEQ ID NO: 11 or variants thereof within 10 amino acids C-terminal to Ser129, within 10 amino acids N-terminal to Val229, within 10 amino acids N-terminal to Asp421, within 10 amino acids C-terminal to Lys44, within 10 amino acids N-terminal to Ser129. It is appreciated that a detection agent optionally binds an amino acid sequence that is at least a portion of SEQ ID NOs: 20, 21, 24, 25, 26, 27, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, or 44.'

[0013] In some embodiments, a detection agent binds an amino acid sequence that is SEQ ID NO: 5 or a variant thereof within: ten amino acids of the cleavage site at amino acid position 43, 121, 229, 38, or 412; optionally within 10 amino acids N-terminal to Asp421, within 10 amino acids N-terminal to Val220, within 10 amino acids C-terminal to Ser120, within 10 amino acids C-terminal to Lys43, or within 10 amino acids C-terminal to Arg370. A detection agent optionally binds at least a portion of SEQ ID NOs: 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 38, 39, 40, or 41.

DETAILED DESCRIPTION OF THE DRAWINGS

[0014] FIG. 1 are western blots for GFAP and GBDP in rat tissue following traumatic brain injury and quantification;

[0015] FIG. 2 are western blots of GFAP and GBDP in rat CSF following traumatic brain injury;

[0016] FIG. 3 are western blots of *in vitro* digested brain lysates compared to rat CCI samples and a digested human sample illustrating levels of GBDP (A) and SBDP (B);

[0017] FIG. 4 illustrates differential proteolysis of GFAP to GBDP upon varying neurotoxic insult;

[0018] FIG. 5 illustrates calpain induced cleavages of recombinant human GFAP;

[0019] FIG. 6 is a schematic of Tau cleavage in neurons to produce TBDPs;

[0020] FIG. 7 illustrates TBDP specific antibodies by western blot using anti-total Tau (A), anti-TauBDP-45K (B), and anti-TauBDP-35K (C);

[0021] FIG. 8 illustrates breakdown products of human and rat Tau as probed by total Tau antibody (A); rat TBDP-35K antibody (B) or rat TBDP-13K specific antibody (C);

[0022] FIG. 9 illustrates rat cerebrocortical cultures challenged by various agents by (A) phase contrast microscopy or (b) assessed by using propidium iodine labeling of nuclei at 24 h for necrosis, and Hoechst 33342 stained condensed nuclear DNA as evidence for % apoptosis;

[0023] FIG. 10 illustrates rat cerebrocortical cultures challenged by various agents and probed for Tau and TBDPs;

[0024] FIG. 11 illustrates Tau and TBDPs following CCI challenge in rats in either cortex (A, B) or hippocampus (C, D);

[0025] FIG. 12 illustrates calpain-1 specific cleavage in rat cortex following experimental CCI;

[0026] FIG. 13 illustrates activation of calpain-1 and calpain-2 in rat cortex following CCI;

[0027] FIG. 14 illustrates the calpain specificity of Tau-BDP35K by administration of SNJ-1945 *in vivo* immediately after CCI as observed by western blot (A) and densitometric analyses thereof (B);

[0028] FIG. 15 are western blots of human CSF for GFAP and GBDPs in two patients (A and B) at various time points following injury;

[0029] FIG. 16 illustrates the presence of human serum autoantibodies directed to brain specific proteins found in post-mortem human brain protein lysate where (A) is total protein load

measured by Coomassie brilliant blue stain, (B) western blot probing with control serum, or (C) western blot probing with pooled post-TBI patient serum;

[0030] FIG. 17 illustrates the presence of autoantibodies in serum from human subjects at 72 hours and 30 days post-TBI;

[0031] FIG. 18 illustrates the presence of autoantibody in the serum from a human subject detectable within 5 days following TBI (A) and their IgG specificity is confirmed (B);

[0032] FIG. 19 illustrates that the TBI induced autoantigens are brain specific;

[0033] FIG. 20 illustrates that autoantibodies in human serum recognize calpain specific cleavage products of rat brain;

[0034] FIG. 21 illustrates the presence of autoantibodies to GFAP, neurofascin, and MBP in serum from a human TBI subject obtained 10 days following injury;

[0035] FIG. 22 illustrates ion exchange fractions of human brain lysate stained by Coomassie blue (A) and probed by western blot (B) using serum from a human subject obtained 10 days following TBI where sequencing of overlapping bands indicates that autoantibodies are directed to GFAP and other proteins listed in Table 2;

[0036] FIG. 23 illustrates antigen competition experiments indicating the presence of autoantibodies to GFAP in human serum post-TBI;

[0037] FIG. 24 illustrates autoantibody preferential recognition of calpain mediated GFAP breakdown products instead of intact GFAP;

[0038] FIG. 25 illustrates the presence of autoantibodies in serum from human TBI patients across several study groups;

[0039] FIG. 26 illustrates gender and age differences with respect to the levels of autoantibodies in serum following TBI in a human study;

[0040] FIG. 27 illustrates increased levels of autoantibodies in TBI diagnosed subjects broken down by GCS score;

[0041] FIG. 28 illustrates the correlation of autoantibodies with survival at 6 months;

[0042] FIG. 29 illustrates the correlation between serum GFAP and UCHL1 levels and autoantibody intensity (A) illustrates scatter plots and (B) illustrates correlations;

[0043] FIG. 30 illustrates the correlation between CSF GFAP, UCHL1, and SBDP145 and autoantibody intensity;

[0044] FIG. 31 illustrates autoantibody intensity as a function of gender (A); autoantibody intensity as a function of outcome (B); and autoantibody intensity and mortality as a function of gender (C);

[0045] FIG. 32 illustrates the intensity of autoantibodies as a function of survival (A) and the intensity and propensity of autoantibodies as a function of survival for females (B) and males (C);

[0046] FIG. 33 illustrates the intensity of autoantibodies as a function of GCS score;

[0047] FIG. 34 is a schematic of nucleic release by neuronal cells during neuronal injury;

[0048] FIG. 35 illustrates the level of GFAP, UCHL1 and S100β in serum from TBI human subjects with mild and moderate injury magnitude;

[0049] FIG. 36 depicts the results of miRNA cluster analyses;

[0050] FIG. 37 is a schematic of the number of identified miRNAs in biological samples.

DESCRIPTION OF THE INVENTION

[0051] Injury to the brain tissue or the BBB leads to the release of intracellular molecules such as proteins, degraded protein fragments, DNA and RNA (including miRNA) into the cerebrospinal fluid (CSF) or the blood stream. The leakage of these antigens may lead to formation of the autoantibodies against them. The present invention capitalizes on measurement the increased or decreased quantity of these inventive neuron specific biomarkers in a biological sample, and as such, has utility in the diagnosis and management of abnormal neurological condition. Specifically, the invention has utility as a diagnostic to identify or classify neuronal injury including injury related to disease, illustratively a traumatic brain injury (TBI) and subtypes thereof, as well as for identifying potential therapeutics effective for the particular brain injury type the subject has endured.

[0052] The processes have utility to detect a neurological trauma or condition that is predictive or indicative of future disease or injury. Illustratively, the processes have utility as a safety or efficacy screening protocol for *in vivo* or *in vitro* for drug development. Drug development is not limited to drugs directed to neurological conditions. The inventive biomarkers also have utility to detect expected or unexpected neurological side effects in *in vivo* animal studies as a means of selecting a lead compound for analyses or as a means of assessing safety of a previously identified drug candidate.

[0053] As used herein an "injury" is an alteration in cellular or molecular integrity, activity, level, robustness, state, or other alteration that is traceable to a singular or continuing event, optionally a plurality of events. An injury illustratively includes an event that is physical, mechanical, chemical, biological, functional, infectious, or other modulator of cellular or molecular characteristics. An injury is illustratively the result of a physical trauma such as an

impact (e.g. percussive) or a biological abnormality such as a stroke resulting from either blockade or leakage of a blood vessel. An exemplary impact injury is traumatic brain injury (TBI). An injury is optionally an infection by an infectious agent. In some embodiments, an injury is exposure to a chemical compound. A person of skill in the art recognizes numerous equivalent injuries that are encompassed by the terms injury or event.

[0054] An injury is optionally a physical event such as a percussive impact. An impact is the like of a percussive injury such as resulting from a blow to the head that either leaves the cranial structure intact or results in breach thereof. Experimentally, several impact methods are used illustratively including controlled cortical impact (CCI) such as at a 1.6 mm depression depth, equivalent to severe TBI in humans. This method is described in detail by Dixon, CE, et al., *J Neurotrauma*, 1999; 16(2):109-22. It is appreciated that other experimental methods producing impact injury are similarly operable.

[0055] TBI may also result from stroke. Ischemic stroke is optionally modeled by middle cerebral artery occlusion (MCAO) in rodents. UCHL1 protein levels, for example, are increased following mild MCAO, which is further increased following severe MCAO challenge. Mild MCAO challenge may result in an increase of biomarker levels within two hours that is transient and returns to control levels within 24 hours. In contrast, severe MCAO challenge results in an increase in biomarker levels within two hours following injury and may be much more persistent demonstrating statistically significant levels out to 72 hours or more.

[0056] Without being restricted to one theory or model, one proposed delineation between mild-traumatic brain injury (mTBI) and TBI are the recognizable increase or decrease in molecular biomarkers in biological fluids following injury. Illustrative examples of molecular markers include those described by Kobeissy FH, et al., *Mol Cell Proteomics*, 2006; 5:1887-1898. An exemplary definition of TBI is the presence of at least one recognizable biomarker with at least two-fold increased or decreased biomarker levels.

[0057] The term "biomarker" as used herein is a protein, nucleic acid, or other differentiator useful for measurement of biological activity or response. A neuron specific biomarker is a biomarker with relevance to neuronal or glial structure, function, or activity. A protein is illustratively an antibody or a breakdown product of a physiological protein. A "nucleic acid" or "oligonucleotide" is defined herein as a macromolecule composed of two or more nucleotides such as deoxyriboncucleotides, or ribonucleotides. Biomarkers used herein are illustratively neuron specific or enriched biomarkers, that is to say that biomarkers are molecules that are not normally found at appreciable levels outside of one or more neuron types. Biomarkers

illustratively include: one or more breakdown products of a protein, illustratively GFAP, αII-spectrin, UCHL1, among others; antibodies; DNA; RNA; miRNA; or one or more fragments of RNA, DNA, peptides, proteins, or other biological material whose presence, absence, level or activity is correlative of or predictive of neurological damage or disease.

[0058] In some embodiments, a biomarker is an antibody, illustratively, an autoantibody. An autoantibody is an antibody that recognizes, binds, or otherwise interacts with an antigen normally found in a subject, or a tissue or cell of a subject. An antigen is illustratively GFAP; αII-spectrin; neurofascin; MBP; MAP2; a ubiquitin carboxyl-terminal esterase; a ubiquitin carboxyl-terminal hydrolase; a neuronally-localized intracellular protein; microtubule associated protein tau (MAP-tau); Poly (ADP-ribose) polymerase (PARP); collapsin response mediator proteins (CRMP-1 to 5); breakdown products thereof, any other biomarker listed in one or more tables or references herein, and combinations thereof.

[0059] Alternatively or in addition, a biomarker is a portion of a protein that is GFAP, neuron specific enolase (NSE), ubiquitin C-terminal hydrolase L1 (UCHL1), Neuronal Nuclei protein (NeuN), 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), Soluble Intercellular Adhesion Molecule-1 (sICAM-1), inducible nitric oxide synthase (iNOS), any other biomarker listed in one or more tables or references herein, and combinations thereof. Illustratively, neuron specific enolase (NSE) is found primarily in neurons; GFAP is found in astrocytes; and CNPase is found in the myelin of the central nervous system. As such, a biomarker is illustratively a breakdown product (BDP) of a protein. Numerous methods of producing breakdown products are known in the art. Illustratively, calpain or caspase proteolytic cleavage of a neuronal specific protein produce one or more BDPs of that protein.

[0060] A biomarker is optionally a nucleic acid. Nucleic acids illustratively are oligoribonucleotides or oligodeoxyribonucleotides. As such a nucleic acid biomarker is optionally DNA or RNA that encodes at least a portion of a protein expressed in a neuron. Circulating DNA has been used for studies in systemic lupus and rheumatoid arthritis because in these diseases the amount of DNA in the blood is rather high (Tan et al, 1966). Free DNA in serum has also been suggested as a biomarker for specific cancers (Leon et al., 1977). Methylated DNA (Muller et al., 2003) and free DNA with specific mutations (Silva et al, 1999) have been found in plasma and linked to cancer. It has also been possible to find fetal DNA in maternal plasma and serum (Lo et al., 1997). Circulating DNA has been found in the plasma of patients with acute stroke (Rainer et al., 2003). Thus, circulating levels of DNA may correlate with disease. The inventors have discovered that neuronal injury leads to increases in the levels

of circulating DNA of neuronal origin. Measuring increased DNA levels in the CSF or serum is an indicator of neuronal injury and injury severity.

[0061] It has been believed that RNA would not function as a marker of disease or injury due to RNA being easily degraded in the blood. While this is the case for exogenously added RNA, the endogenous RNA from tissues that have suffered trauma may show greater stability and thus be useful as a biomarker. Without being limited to one particular theory, endogenous RNA may be complexed with proteins and protected from degradation when in apoptotic bodies (Hasselman et al., 2001). Moreover, RNA may be more specific to site of injury and type of disease due to the ability to readily identify its sequence and correlate this sequence with a cell type specific expression of the RNA. As such, a biomarker is optionally an RNA molecule. RNA molecules include mRNA, rRNA, miRNA, tRNA, and miRNA.

[0062] A biomarker is optionally a miRNA. MicroRNAs (miRNAs) are non-coding RNA molecules that are able to regulate gene expression post-transcriptionally through degradation of the messenger RNA hence reducing protein expression. A miRNA biomarker is a miRNA that regulates the expression of a gene encoding one or more neuron specific proteins such as any of those listed in the tables or otherwise herein.

[0063] A process includes measuring the quantity of one or more biomarkers in a biological sample. A biomarker is measured by any method or process known in the art or described herein for determining an absolute quantity, relative quantity, or value representative of quantity e.g. increased or decreased fluorescence relative to background or other comparator. Optionally, one, two, three, four, or more biomarkers are measured simultaneously or sequentially from one or more biological samples. A second or additional biomarker is optionally different from a first biomarker.

[0064] A biomarker illustratively is a breakdown product of a protein, binds to a protein or portion thereof, encodes a protein or portion thereof, or regulates the expression of a protein, where the protein is: ubiquitin carboxyl-terminal esterase L1 (UCHL1); neuron specific enolase (NSE); a spectrin breakdown product (SBDP), illustratively SBDP150, SBDP150i SBDP145, or SBDP120; S100 calcium binding protein B (S100β); microtubule associated protein (MAP), optionally MAP2, MAP1, MAP3, MAP4, MAP5; myelin basic protein (MBP); Tau, illustrativlye MAP-tau or tau BDPs; Neurofilament protein (NF); Cannabinoid Receptor (CB); CAM proteins; synaptic protein; collapsin response mediator proteins (CRMP-1 to 5); inducible nitric oxide synthase (iNOS); neuronal nuclei protein (NeuN); cysteinyl-specific peptidase (CSPase); neuroserpin; alpha-internexin; light chain 3 protein (LC3); neurofascin; the glutamate

transporters (EAAT); nestin; cortin-1, 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase); βIII-tubulin, or any biomarker listed in Table 1, or other table herein, or combinations thereof.

Table 1:

	Glycogen phosphorylase, (BB-form)GP-	
UCHL1	BB	Precerebellin
MBP isoforms	CRMP-2	Cortexin
SBDP150 (calpain)	NP25, NP22; Transgelin-3	EMAP-II
SBDP120 (caspase)	SBDP150i (caspase)	Calcineurin-BDP
MBP—fragment (10/8K)	CaMPK-IIα	MAP2
SBDP145	MOG	N-Cadherin
Synaptophysin	PLP	N-CAM
βIII-Tubulin	PTPase (CD45)	Synaptobrevin
Tau-BDP-35K (calpain)	Nesprin-BDP	MAP1A (MAP1)
NF-L-BDP1	OX-42	MAP1B (MAP5)
NF-M-BDP1	OX-8	Prion-protein
NF-H-BDP1	OX-6	PEP19; PCP4
Synaptotagmin	CaMPKIV	Synaptotagmin-BDP1
PSD93-BDP1	Dynamin	BDNF
AMPA-R-BDP1	Clathrin HC	Nestin
NMDA-R-BDP	SNAP25	IL-6
SBDP150i (caspase)	Profilin (BDP?)	IL-10
MAP2-BDP1 (calpain)	Cofilin (BDP?)	αII-spectrin SBDP 150+145
MAP2-BDP2 (caspase)	APP –BDP (Calpain)	NG2; Phosphacan, neruocan; versican
		Ach Receptor fragment (Nicotinic,
alpha-synuclein	NSF	Muscarinic)
Synapsin 1	IL-6	I-CAM
Synapsin 2-BDP	MMP-9	V-CAM
NeuN	S100β	AL-CAM
GFAP	Neuroglobin	CNPase
p24; VMP	UCHL1 autoantibody	Neurofascins
PSD95	Tau-BDP-35K (calpain)	Neuroserpin
α1,2-Tubulin	Tau-BDP-45K (caspase)	EAAT(1 and 2)
β1,2-Tubulin	Huntingtin-BDP-1 (calpain)	Nestin
Stathmin-2,3,4 (Dendritic)	Huntingtin-BDP-2 (caspase)	Synaptopodin
Striatin-BDP1	Prion-protein BDP	
Snaptojanin-1,2-BDP1	MBP (N-term half)	
betaIII-Spectrin		β-synuclein

betaII-Spectrin-BDP110 (calpain)	Calbindin-9K	Resistin
betaII-Spectrin-BDP85 (caspase)	Tau-Total	Neuropilins
Cannabinoid-receptor1(CB1)	NSE	Orexin
Cannabinoid-receptor2(CB2)	CRMP-1	Fracktalkine
MBP isoforms 14K+17K	CRMP-3	β-NGF
Neurocalcin-delta (Glia)	CRMP-4	L-selectin
Iba1 (Microglia)	CRMP-5	iNOS
		DAT
Peripherin (PNS)		Vimentin
LC3	Crerbellin 3	Beclin-1

[0065] A biomarker is optionally a breakdown product of GFAP or Tau. The amino acid sequence of human GFAP is found at GenBank Accession Nos. NP_002046 and NP_001124491 for isoforms 1 and 2 respectively. The amino acid sequence human tau is found at GenBank Accession Nos. NP_005901 and NP_776088. The amino acid sequence rat tau is found at GenBank Accession No. NP_058908 for the 374 amino acid version. The sequences found at each of these accession numbers and other accession numbers found herein are incorporated herein by reference as if each sequence were fully and explicitly listed.

[0066] A biomarker is optionally any protein or portion thereof, nucleic acid, or antibody that shows either increased expression or decreased expression during or following an injury or event. Expression is recognized as synthesis whereby increased expression is increased synthesis of the biomarker. Optionally, synthesis is generation of a cleavage product where the protease, e.g. calpain or caspase, cleaves a protein to synthesize or produce a breakdown product. Illustratively, neuronal injury may activate a calpain or caspase that in turn enzymatically cleaves GFAP to produce one or more GBDPs. Thus, the GBDPs are understood to be produced, i.e. synthesized, during or following injury or event. Similarly, autoantibodies are produced over the course of time following or during an injury or event. As such, autoantibodies are understood to be synthesized. Nucleic acids are typically expressed at no or certain levels within a cell. Injury or event may alter the rate at which RNA such as miRNAs are translated from genomic DNA. Thus, it is understood that nucleic acids show either increase or decrease in synthesis during or following an injury or event.

[0067] Any number of biomarkers can be detected or measured such as 2, 3, 4, 5, 6, 7, 8, 9, 10, or more. Detection can be either simultaneous or sequential and may be from the same biological sample or from multiple samples from the same or different subjects. Detection of

multiple biomarkers is optionally in the same assay chamber. The inventive process optionally further includes comparing the quantity of a first biomarker and the quantity of the at least one additional neuroactive biomarker to normal levels of each of the first biomarker and the one additional neuroactive biomarker to determine the neurological condition of the subject.

[0068] The present invention is optionally described with respect to UCHL1 or GFAP. It is appreciated that these biomarkers are presented for illustrative purposes only and are not meant to imply expressly or otherwise that the scope of the present invention is limited to UCHL1 or GFAP.

[0069] In some embodiments a biomarker is a breakdown product of GFAP (GBDP). GFAP is associated with glial cells such as astrocytes. A biomarker is optionally one or more GBDP. GDBPs optionally have a migratory molecular weight between 38 to 52 kDa. A GDBP is optionally a product of proteolytic digestion of GFAP. Illustrative proteases include calpain and caspase.

[0070] In some embodiments a biomarker is a nucleic acid such as an oligonucleotide. An oligonucleotide is a DNA or RNA molecule. Preferred examples of RNA molecules are mRNA and miRNA molecules.

[0071] RNA molecules were historically believed to have short half-lives in plasma. More recently, studies indicated that RNA molecules may be protected in plasma by protein or lipid vesicles. As such, RNA molecules released following or during an injury, for example, can be detected in whole blood, plasma, serum, CSF, or other biological material and be associated with the presence of injury in the inventive processes. Numerous methods are known in the art for isolating RNA from a biological sample. Illustratively, the methods described by El-Hefnaway, T, et al., *Clinical Chem.*, 2004; 50(3);564-573, the contents of which are incorporated herein by reference, are operable in the present invention. A biological sample is optionally filtered prior to detecting or measuring a nucleic acid. Filtering removes additional cellular material producing more accurate measurements of biomarker nucleic acids. Methods of filtering a biological sample are known in the art, illustratively as described by Rainer, TH, *Clin. Chem.*, 2004; 50:206-208, the contents of which are incorporated herein by reference.

[0072] In some embodiments, RNA encoding or regulating the expression of UCHL1 or GFAP is detected or measured. Human UCHL1 RNA or cDNA derived therefrom is of known sequence and can be found in the NCBI database at accession number NM_004181. A person of ordinary skill in the art knows that other TBI relevant RNA sequences can similarly be found in the NCBI database such as those encoding proteins listed in the tables herein. As further

examples, mRNA sequence for GFAP is found at accession number NM_001131019.1 and NM_002055.3 for two isoforms of GFAP.

[0073] Exemplary methods of detecting or measuring nucleic acids include methods based on the polymerase chain reaction (PCR), illustratively, PCR or RT-PCR. Nucleic acids can be measured using PCR independent methodologies illustratively, mass spectrometry, fluorescence, staining, other method known in the art. The use of microarrays are optionally employed to detect or measure the level of one or many nucleic acid biomarkers in a sample. Creating and using microarrays for the detection and measurement of nucleic acids is known in the art. Primer and probe designs are also within the level of skill in the art. Any suitable primer and probe as well as labels thereon are operable for the detection of nucleic acid biomarkers in the subject invention. Illustratively, primer and probe design can be performed using automated programs available from commercial sources. Alternatively, numerous commercial suppliers provide primer and probe design services including Applied Biosystems (Foster City, CA).

[0074] A process of detecting a neurological condition optionally includes obtaining a biological sample from a subject that may be suspected of having a neurological condition; measuring a quantity of a neuron specific biomarker in the sample; comparing the level of the biomarker detected with the level of the same or different biomarker from a subject without a neurological condition to obtain a ratio; and diagnosing or detecting the presence or absence of a neurological condition in the subject based on the ratio.

[0075] Optionally, a process involves analyzing the biological sample for the presence of a plurality of biomarkers. A plurality can be any number greater than one. Optionally, two biomarkers are analyzed. Illustratively, the biomarkers are UCHL1 and GFAP related biomarkers. More biomarkers may be simultaneously or sequentially assayed for in the inventive processes illustratively including three, four, five, six, seven, eight, nine, 10, 20, 50, 100, 1000, or any number between or greater.

[0076] Illustrative methods for the detection and quantification of biomarkers include real-time PCR (RT-PCR). RT-PCR allows for the simultaneous amplification and quantitation of a plurality of biomarkers simultaneously. Alternatively, mass spectrometry such as electrospray ionization mass spectrometry coupled with time of flight detection and high performance liquid chromatography are similarly operable. It is appreciated that other methods are similarly operable for detection as will be appreciated by one of ordinary skill in the art.

[0077] Numerous miRNA molecules are operable as biomarkers in the subject invention. The term "miRNA" is used according to its ordinary and plain meaning and refers to a

microRNA molecule found in eukaryotes that is involved in RNA-based gene regulation. Examples include miRNA molecules that regulate the expression of one or more proteins listed in Table 1. Several miRNA molecules have been identified and are operable as biomarkers in the inventive methods. Illustratively, miRNA molecules described by Redell, JB, et al., *J. Neurosci. Res.*, 2009; 87:1435-48; Lei, P., et al., *Brain Res.*, doi:10.1016/j.brainres.2009.05.074; Lu, N, et al., *Exp. Neurology*, 2009; doi:10.1016/j.expneurol.2009.06.015; and Jeyaseelan, K, et al., *Stroke*, 2008; 39:959-966, the contents of each are incorporated herein by reference for the miRNAs defined therein, but also specifically for methods of isolation and quantitation of miRNA described in each reference. These methods, or modifications thereof, are recognized by one of ordinary skill in the art and may be used in the present inventive method.

[0078] A process of determining a neurological condition optionally includes detection or measurement of one or more antibodies in a biological sample. An antibody is optionally an autoantibody. Autoantibodies are directed to antigens released from a site of neurological trauma such as TBI, disease, injury or other abnormality. Without being limited to a particular theory, a TBI causes cellular damage that releases intracellular or cell membrane contents into the CSF or bloodstream. The levels of many of these proteins such as those listed in Table 1, and other tables herein, or nucleic acids that encode the proteins or regulate their expression, are not normally present in biological fluids other than the cytoplasm or cell membrane of neuronal tissue such as brain tissue. The presence of these antigens leads to the production of autoantibodies to these antigens within a subject. Detection of an autoantibody as a biomarker is optionally used to diagnose the presence of an abnormal neurological condition in a subject.

[0079] US Patent No. 6,010,854 describes methods of producing antigens and methods of screening for autoantibodies to neuronal glutamate receptors. These methods are equally applicable to the subject invention. As such, US Patent No. 6,010,854 is incorporated herein by reference for its teaching of methods of producing screening antigens that are operable for screening for autoantibodies. US Patent No. 6,010,854 is similarly incorporated herein by reference for its teaching of methods of detecting autoantibodies. It is appreciated that other methods of detecting antibodies illustratively including ELISA, western blotting, mass spectrometry, chromatography, staining, and others known in the art are similarly operable.

[0080] Several antigens have been discovered as producing autoantibodies following onset of a neurological condition. Such antigens are those illustratively listed in Table 2.

Table 2: Exemplary protein autoantigens identified by MS/MS in FIG. 22

GFAP and GBDP

Neurofilament light polypeptide (NF-L)

Neurofilament Medium polypeptide (NF-M)

Neurofilament heavy polypeptide (NF-H)

V-type proton ATPase

Endophilin-A1

Vimentin

Gamma-enolase (NSE)

Microtubule-associated protein 2

Dihydropyrimidinase-related protein 2

Alpha-internexin

Neuroserpin

Neuromodulin

Synaptotagmin-1

Voltage-gated potassium channel

[0081] In addition several of these and other antigens are associated with brain injury. Illustrative specific examples of autoantigens related to brain injury are listed in Table 3.

Table 3: Exemplary autoantigens related to brain injury

αII-spectrin

SBDPs

NSE

UCHL1

MAP2

MBP

Tau

NF-L,M,H

S100B

βIII-tubulin

5

[0082] Table 4: Exemplary brain injury-induced autoantigens based on reported antigenicity.

Voltage-gated calcium channel VGCC (P/Q-type) (as in Lambert-Eaton myasthenic syndrome)

Voltage-gated potassium channel (VGKC) (as in Limbic encephalitis, Isaac's Syndrome. Autoimmune Neuromyotonia)

Ri (Anti-neuronal nuclear antibody-2) (as in Opsoclonus)

Hu and Yo (cerebellar Purkinje Cells) (as in Paraneoplastic cerebellar syndrome)

Amphiphysin (as in Paraneoplastic cerebellar syndrome)

Glutamic acid decarboxylase (GAD) (as in Diabetes mellitus type 1, Stiff person syndrome)

Aquaporin-4 (Neuromyelitis optica ; evic's syndrome)

Basal ganglia neurons (as in Sydenham's Chorea, Paediatric Autoimmune Neuropsychiatric Disease Associated with Streptococcus (PANDAS)

Homer 3 (subacute idiopathic cerebellar ataxia)

Zic proteins (zinc finger proteins) (as in Joubert syndrome – cerebellum malformation)

ANNA 3 (brain autoantigen)

Purkinje cell antibody (PCA-2)

PKC γ (paraneoplastic cerebellar degeneration)

SOX1 (Myasthenic Syndrome Lambert-Eaton (LEMS))

Gephyrin (Stiff Man Syndrome)

Ma2

CV2 (= CRMP5)

N-methyl-D-aspartate (NMDA) - develop memory impairment

mGluR1 (Cerebellar ataxia)

Nicotinic acetylcholine receptor (as in Myasthenia gravis)

Recoverin

Enolase

TULIP-1 (tubby-like protein 1)

[0083] In some embodiments, full length protein such as any protein listed in Tables 1-4, or a breakdown product thereof, is operable as a screening antigen for autoantibodies. For example, UCHL1 is antigenic and produces autoantibodies in a subject. The sequence for human UCHL1 protein is found at NCBI accession number NP_004172.2. Similarly, the sequence for human GFAP is found at NCBI accession number NP_002046.1. Other illustrative antigens illustratively include αII-spectrin or breakdown products thereof, MAP, Tau, Neurofascin, CRMP-2, MAP2 crude sample, MBP, and human brain lysate or any subfraction thereof.

[0084] Any suitable method of producing peptides and proteins of Tables 1-4 is operable herein. Illustratively, cloning and protein expression systems used with or without purification tags are optionally used. Illustrative methods for production of immunogenic peptides include synthetic peptide synthesis by methods known in the art. Chemical methods of peptide synthesis are known in the art and include solid phase peptide synthesis and solution phase peptide synthesis or by the method of Hackeng, TM, et al., *Proc Natl Acad Sci U S A*, 1997; 94(15):7845-50, the contents of which are incorporated herein by reference. Either method is operable for the production of antigens operable for screening biological samples for the presence of autoantibodies.

[0085] As used herein, "peptide" means peptides of any length and includes proteins. The terms "polypeptide" and "oligopeptide" are used herein without any particular intended size limitation, unless a particular size is otherwise stated.

[0086] It is appreciated that the patterns of biomarkers such as RNA, miRNA, DNA, and autoantibodies is operable to locate the site and severity of neuronal abnormality. Illustratively, damage to the brain reveals a different pattern of a plurality of biomarkers than does damage to other regions of the central nervous system. Also, damage to the hippocampus will produce a different pattern of biomarkers than damage to the frontal lobe. As such, localization of injury is achieved by comparative detection of a plurality of biomarkers. For example, miRNA levels within cells are altered in specific patterns in response to TBI. (See Redell, J, et al., *J. Neurosci. Res.*, 2009; 87:1435–1448.) The inventors have surprisingly discovered that the levels of miRNA biomarkers that regulate expression of the proteins in Table 1 are similarly altered by either upregulation or downregulation dependent on the severity of injury or the time since onset of injury. The pattern of miRNA and other biomarkers changes as injury or disease progresses. This may be a result of secondary injury events, delayed cell apoptosis, or other mechanism altering the release of RNA, DNA, or protein. Redell, J, incorporated herein by reference above, illustrates alteration of miRNA biomarkers within cells at 3 hours and 24 hours. Some miRNAs are upregulated at 3 hours whereas others are only upregulated at 24 hours. Similar results are observed for downregulation of miRNA. As such, the regulation of miRNA biomarkers, the method of their detection, and the temporal alteration in expression of Redell, J, et al., *J. Neurosci. Res.*, 2009; 87:1435–1448 are each incorporated herein by reference as equally applicable to the subject invention. Similarly, the temporal nature of miRNA expression in response to stroke as observed by Jeyaseelan, K, et al., *Stroke*, 2008; 39:959-966 is also incorporated herein by reference for the particular miRNAs taught therein as well as the methods

of isolation, quantification, and detection taught therein. What was neither appreciated, nor contemplated by prior researchers is that nucleic acid and proteins migrate into the blood or CSF following injury. It was believed that the levels of biomarkers, RNA in particular, would remain localized to the nucleus or possibly the cytoplasm of a cell. The inventor's ability to detect levels of these biomarkers in non-cellular biological samples such as blood (or fraction thereof) or CSF provided a much more robust and simplified detection process for determining the neurological condition or risk of neurological condition in a subject.

[0087] As such, in some embodiments screens a biological sample for a first and a second biomarker. Greater numbers are similarly operable. GFAP biomarkers are preferred first biomarkers. As GFAP is associated with glial cells such as astrocytes, preferably the other biomarker is associated with the health of a different type of cell associated with neural function. More preferably, the other cell type is an axon, neuron, or dendrite. Through the use of an inventive assay inclusive of biomarkers associated with glial cells as well as at least one other type of neural cell, the type of neural cells being stressed or killed as well as quantification of neurological condition results. A synergistic measurement of GFAP biomarker optionally along with at least one additional biomarker and comparing the quantity of GFAP biomarker and the additional biomarker to normal levels of the markers provides a determination of subject neurological condition. Specific biomarker levels that when measured in concert with a GFAP biomarker afford superior evaluation of subject neurological condition illustratively include SBDP145 (calpain mediated acute neural necrosis), SBDP120 (caspase mediated delayed neural apoptosis), UCHL1 (neuronal cell body damage marker), and MAP-2.

[0088] The nature of a particular protein associated with an inventive biomarker allows tight determination of extent, location, and severity of injury. Table 5 represents biological locations of proteins related to inventive biomarkers. It is appreciated that increases in autoantibodies or RNA, for example, to peripherin equates to different abnormalities than increases in autoantibodies or RNA to UCHL1.

[0089] **Table 5:**

Class	Gen#1	Gen#2	Gen#3
Axonal	SBDP145 SBDP120 αII-Spectrin	βII-SBDP110, βII-SBDP-108,-85 SBDP150	Tau-BDP-14K(calp) Tau-BDP-40K (casp)
Dendritic	MAP2 βIII-tubulin	P24	MAP2-BDP
Cell body	UCH-L1	GP-BB Stathmin-2,3 PrionProtein	NP25 α-synuclein β-synuclein
Neuroregen.	CRMP-2	Nestin	
Nucleus	NeuN		Ox-GAPDH
Presynaptic	Synaptotagmin	Synaptophysin	Synapsin1,2
Postsynaptic	CaMPKIIa	PSD-93 PSD-95	
Myelin	MBP-fragment MBP	MOG	MBP-frag (New-C)
Glia	GFAP		
Microglia	IL-6	Iba1	OX-8, OX-6 OX-42
Neurovascular.	N-CAM	N-Cadherin	I-CAM, L-CAM
PNS		Peripherin	

[0090] Detection of inventive biomarkers is also operable to screen potential drug candidates or analyze safety of previously identified drug candidates. These assays may be either *in vitro* or *in vivo*. *In vivo* screening or assay protocols illustratively include measurement of a biomarker in an animal illustratively including a mouse, rat, or human. Studies to determine or monitor levels such as UCHL1 or GFAP biomarkers are optionally combined with behavioral analyses or motor deficit analyses such as: motor coordination tests illustratively including Rotarod, beam walk test, gait analysis, grid test, hanging test and string test; sedation tests illustratively including those detecting spontaneous locomotor activity in the open-field test; sensitivity tests for allodynia - cold bath tests, hot plate tests at 38°C and Von Frey tests; sensitivity tests for hyperalgesia - hot plate tests at 52°C and Randall-Sellito tests; and EMG evaluations such as sensory and motor nerve conduction, Compound Muscle Action Potential (CMAP) and h-wave reflex.

[0091] The inventive biomarker analyses are illustratively operable to detect, diagnose, or treat a disease state or screen for chemical or other therapeutics to treat disease. Diseases or conditions illustratively include but are not limited to: neurodegenerative diseases; myelin involving diseases such as multiple sclerosis; stroke; amyotrophic lateral sclerosis (ALS);

chemotherapy; cancer; Parkinson's disease; nerve conduction abnormalities stemming from chemical or physiological abnormalities such as ulnar neuritis and carpel tunnel syndrome; other peripheral neuropathies illustratively including sciatic nerve crush (traumatic neuropathy), streptozotozin (STZ) (diabetic neuropathy), and antimitotic-induced neuropathies (chemotherapy-induced neuropathy); experimental autoimmune encephalomyelitis (EAE); delayed-type hypersensitivity (DTH); rheumatoid arthritis; epilepsy; pain; neuropathic pain; and intra-uterine trauma.

[0092] Analyses of blast injury to a subject reveal several inventive correlations between biomarkers and neuronal injury. Neuronal injury is optionally the result of whole body blast, blast force to a particular portion of the body, or the result of other neuronal trauma or disease that produces detectable or differentiatable levels of biomarkers. Thus, identifying pathogenic pathways of primary blast brain injury (BBI) in reproducible experimental models is helpful to the development of diagnostic algorithms for differentiating severe, moderate and mild (mTBI) from posttraumatic stress disorder (PTSD). Accordingly, a number of experimental animal models have been implemented to study mechanisms of blast wave impact and include rodents and larger animals such as sheep. However, because of the rather generic nature of blast generators used in the different studies, the data on brain injury mechanisms and putative biomarkers have been difficult to analyze and compare.

[0093] To provide correlations between neurological condition and measured quantities of biomarkers, samples of CSF or serum, illustratively, are collected from subjects with the samples being subjected to measurement of biomarkers. A sample is optionally a biological sample. Detected levels of GFAP biomarkers are then optionally correlated with CT scan results as well as GCS scoring. Based on these results, an inventive assay is developed and validated (Lee et al., Pharmacological Research 23:312-328, 2006). It is appreciated that GFAP biomarkers, in addition to being obtained from CSF and serum, are also readily obtained from blood, plasma, saliva, urine, as well as solid tissue biopsy. While CSF is a commonly used sampling fluid owing to direct contact with the nervous system, it is appreciated that other biological fluids have advantages in being sampled for other purposes, and therefore, allow for inventive determination of neurological condition as part of a battery of tests performed on a single sample such as blood, plasma, serum, saliva or urine.

[0094] A biological sample is obtained from a subject by conventional techniques. For example, CSF is optionally obtained by lumbar puncture. Blood is optionally obtained by venipuncture, while plasma and serum are obtained by fractionating whole blood according to

known methods. It is appreciated that CSF is optionally obtained by cannulation or other technique. Blood is optionally obtained by other technique, illustratively cardiac puncture. Surgical techniques for obtaining solid tissue samples are well known in the art. For example, methods for obtaining a nervous system tissue sample are described in standard neurosurgery texts such as Atlas of Neurosurgery: Basic Approaches to Cranial and Vascular Procedures, by F. Meyer, Churchill Livingstone, 1999; Stereotactic and Image Directed Surgery of Brain Tumors, 1st ed., by David G. T. Thomas, WB Saunders Co., 1993; and Cranial Microsurgery: Approaches and Techniques, by L. N. Sekhar and E. De Oliveira, 1st ed., Thieme Medical Publishing, 1999. Methods for obtaining and analyzing brain tissue are also described in Belay et al., Arch. Neurol. 58: 1673-1678 (2001); and Seijo et al., J. Clin. Microbiol. 38: 3892-3895 (2000).

[0095] Optionally, a biomarker is selective for detecting or diagnosing neurological conditions such as brain injury and the like. A biomarker is optionally both specific and effective for the detection and distinguishing levels of TBI. Such biomarkers are optionally termed neurospecific or neuroactive biomarkers.

[0096] It is appreciated that the temporal nature of biomarker presence or activity is operable as an indicator or distinguisher of TBI subtype. In a non-limiting example, the severity of experimental middle cerebral artery occlusion (MCAO) correlates with the temporal maintenance of UCHL1 biomarkers in CSF. MCAO of 30 minutes produces transient UCHL1 biomarker levels peaking at 6 hours and rapidly decreasing, whereas MCAO of 2 hours produces sustained UCHL1 biomarker levels for as many as three days. Similarly, the prevalence of other biomarkers at various timepoints following injury is operable to distinguish TBI subtype. GBDP appear in biological samples including human CSF following percussive blast injury within 24 hours after injury and increase in intensity out to as much as 7 days following injury. Similar results are observed for SBDPs such as SBDP 150/145. Autoantibodies are generally observed within five days following injury with increasing amounts to 30 days following injury.

[0097] Biomarker analyses are optionally performed using biological samples or fluids. Illustrative biological samples operable herein illustratively include, cells, tissues, cerebral spinal fluid (CSF), artificial CSF, whole blood, serum, plasma, cytosolic fluid, urine, feces, stomach fluids, digestive fluids, saliva, nasal or other airway fluid, vaginal fluids, semen, buffered saline, saline, water, or other biological fluid recognized in the art. In some embodiments, a biological sample is CSF or serum. It is appreciated that two or more separate biological samples are

optionally assayed to elucidate the neurological condition of the subject. Optionally, a biological sample is not a cell or cell cytoplasmic or nucleoplasmic material.

[0098] In addition to increased cell expression, biomarkers also appear in biological fluids in communication with injured cells. Obtaining biological fluids such as cerebrospinal fluid (CSF), blood, plasma, serum, saliva and urine, from a subject is typically much less invasive and traumatizing than obtaining a solid tissue biopsy sample. CSF, in particular, is commonly used for detecting nerve damage in a subject as it is in immediate contact with the nervous system and is readily obtainable. Serum is a commonly used biological sample as it is easily obtainable and presents much less risk of further injury or side-effect to a donating subject.

[0099] To provide correlations between neurological condition and measured quantities of biomarkers, some embodiments of the invention collecting samples of CSF or serum as particular examples from subjects with the samples being subjected to measurement of GFAP related nucleic acids, GBDPs, or GFAP related autoantibodies. The subjects vary in neurological condition. Detected levels of biomarkers are optionally then correlated with CT scan results or GCS scoring. Based on these results, an inventive assay is developed and validated (Lee et al., Pharmacological Research 23:312-328, 2006). It is appreciated that biomarkers, in addition to being obtained from CSF and serum, are also readily obtained from blood, plasma, saliva, urine, as well as solid tissue biopsy. While CSF is a sampling fluid in many embodiments of the invention owing to direct contact with the nervous system, it is appreciated that other biological fluids have advantages in being sampled for other purposes and therefore allow for inventive determination of neurological condition optionally as part of a battery of tests performed on a single sample such as blood, plasma, serum, saliva or urine.

[00100] After insult, nerve cells in a subject express altered levels or activities of one or more proteins or nucleic acid molecules than do such cells not subjected to the insult. Thus, samples that contain nerve cells, e.g., a biopsy of a central nervous system or peripheral nervous system tissue are suitable biological samples for use in the invention in some embodiments. In addition to nerve cells, however, other cells expressing illustratively GFAP include, for example, cardiomyocytes, myocytes in skeletal muscles, hepatocytes, kidney cells and cells in testis. A biological sample including such cells or fluid secreted from these cells might also be used in an adaptation of the inventive methods to determine and/or characterize an injury to such non-nerve cells.

[00101] A subject illustratively includes a dog, a cat, a horse, a cow, a pig, a sheep, a goat, a chicken, non-human primate, a human, a rat, guinea pig, hamster, and a mouse. Because the

present invention optionally relates to human subjects, a subject for the methods of the invention is optionally a human.

[00102] Subjects who most benefit from the present invention are those suspected of having or at risk for developing abnormal neurological conditions, such as victims of brain injury caused by traumatic insults (e.g., gunshot wounds, automobile accidents, sports accidents, shaken baby syndrome, other percussive injuries), ischemic events (e.g., stroke, cerebral hemorrhage, cardiac arrest), neurodegenerative disorders (such as Alzheimer's, Huntington's, and Parkinson's diseases; prion-related disease; other forms of dementia), epilepsy, substance abuse (e.g., from amphetamines, Ecstasy/MDMA, or ethanol), and peripheral nervous system pathologies such as diabetic neuropathy, chemotherapy-induced neuropathy and neuropathic pain.

[00103] Baseline levels of biomarkers are those levels obtained in the target biological sample in the species of desired subject in the absence of a known neurological condition. These levels need not be expressed in hard concentrations, but may instead be known from parallel control experiments and expressed in terms of fluorescent units, density units, and the like. Typically, in the absence of a neurological condition GBDPs are present in biological samples at a negligible amount. Illustratively, autoantibodies to GFAP or GBDPs are absent in a biological sample form a subject not suspected of having a neurological condition. However, GFAP and GBDPs are often highly abundant in neurons. Determining the baseline levels of GFAP, GBDPs, autoantibodies, and RNA in neurons, plasma, or CSF, for example, of particular species is well within the skill of the art. Similarly, determining the concentration of baseline levels of other biomarkers is well within the skill of the art.

[00104] As used herein the term "diagnosing" means recognizing the presence or absence of a neurological or other condition such as an injury or disease. Diagnosing is optionally referred to as the result of an assay wherein a particular ratio or level of a biomarker is detected or is absent.

[00105] As used herein a "ratio" is either a positive ratio wherein the level of the target biomarker is greater than the target biomarker in a second sample or relative to a known or recognized baseline level of the same target biomarker. A negative ratio describes the level of the target biomarker as lower than the target biomarker in a second sample or relative to a known or recognized baseline level of the same target biomarker. A neutral ratio describes no observed change in target biomarker. Thus, a quantity of biomarker measured in a biological sample is optionally compared to the level in a control subject to determine if the level in the subject is altered. As such, an altered quantity of a biomarker is a change in the level of biomarker in the

biological fluid from a measured or expected value in a control subject. A control subject is a subject with no known neurological injury or disease.

[00106] As used herein the term "administering" is delivery of a therapeutic to a subject. The therapeutic is administered by a route determined to be appropriate for a particular subject by one skilled in the art. For example, the therapeutic is administered orally, parenterally (for example, intravenously, by intramuscular injection, by intraperitoneal injection, intratumorally, by inhalation, or transdermally. The exact amount of therapeutic required will vary from subject to subject, depending on the age, weight and general condition of the subject, the severity of the neurological condition that is being treated, the particular therapeutic used, its mode of administration, and the like. An appropriate amount may be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein or by knowledge in the art without undue experimentation.

[00107] An exemplary process for detecting the presence or absence of one or more neuroactive biomarkers in a biological sample involves obtaining a biological sample from a subject, such as a human, contacting the biological sample with a compound or an agent capable of detecting of the biomarker being analyzed (i.e. a detection agent), illustratively including a primer, a probe, antibody, aptamer, or an antigen such as in the case of detection of autoantibody biomarkers, and analyzing binding of the compound or agent to the sample after washing. Those samples having specifically bound compound or agent express the marker being analyzed. It is appreciated that other detection methods are similarly operable illustratively contact with a protein or nucleic acid specific stain.

[00108] An inventive process can be used to detect biomarkers in a biological sample *in vitro*, as well as *in vivo*. The quantity of biomarker in a sample is compared with appropriate controls such as a first sample known to express detectable levels of the marker being analyzed (positive control) and a second sample known to not express detectable levels of the marker being analyzed (a negative control). For example, *in vitro* techniques for detection of a marker include enzyme linked immunosorbent assays (ELISAs), radioimmuno assay, radioassay, western blot, Southern blot, northern blot, immunoprecipitation, immunofluorescence, mass spectrometry, RT-PCR, PCR, liquid chromatography, high performance liquid chromatography, enzyme activity assay, cellular assay, positron emission tomography, mass spectroscopy, combinations thereof, or other technique known in the art. Furthermore, *in vivo* techniques for detection of a marker include introducing a labeled agent that specifically binds the marker into a biological sample or test subject. For example, the agent can be labeled with a radioactive

marker whose presence and location in a biological sample or test subject can be detected by standard imaging techniques.

[00109] Any suitable molecule that can specifically bind or otherwise be used to recognize a biomarker is operative in the invention. An agent for detecting an autoantibody is illustratively an antigen capable of binding to an autoantibody or an antibody capable of binding a biomarker being analyzed. Optionally, an antibody is conjugated with a detectable label. Such antibodies can be polyclonal or monoclonal. An intact antibody, a fragment thereof (e.g., Fab or $F(ab')_2$), or an engineered variant thereof (e.g., sFv) can also be used. Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Antibodies for numerous inventive biomarkers are available from vendors known to one of skill in the art. Illustratively, antibodies directed to inventive biomarkers are available from Santa Cruz Biotechnology (Santa Cruz, CA).

[00110] RNA and DNA binding antibodies are known in the art. Illustratively, an RNA binding antibody is synthesized from a series of antibody fragments from a phage display library. Illustrative examples of the methods used to synthesize RNA binding antibodies are found in Ye, J, et al., *PNAS USA*, 2008; 105:82-87 the contents of which are incorporated herein by reference as methods of generating RNA binding antibodies. As such, it is within the skill of the art to generate antibodies to RNA based biomarkers.

[00111] DNA binding antibodies are similarly well known in the art. Illustrative methods of generating DNA binding antibodies are found in Watts, RA, et al., *Immunology*, 1990; 69(3): 348–354 the contents of which are incorporated herein by reference as an exemplary method of generating anti-DNA antibodies.

[00112] An agent or compound for detecting or measuring a biomarker is optionally labeled. A person of ordinary skill in the art recognizes numerous labels operable herein. Labels and labeling kits are commercially available optionally from Invitrogen Corp, Carlsbad, CA. Labels illustratively include, fluorescent labels, biotin, peroxidase, radionucleotides, or other label known in the art.

[00113] Antibody-based assays are useful for analyzing a biological sample for the presence of one or more biomarkers. Suitable western blotting methods are described below in the examples section or are known in the art. For more rapid analysis (as may be important in emergency medical situations), immunosorbent assays (e.g., ELISA and RIA) and immunoprecipitation assays may be used. As one example, the biological sample or a portion thereof is immobilized on a substrate, such as a membrane made of nitrocellulose or PVDF; or a

rigid substrate made of polystyrene or other plastic polymer such as a microtiter plate, and the substrate is contacted with an antibody that specifically binds a GFAP biomarker, or one of the other biomarkers under conditions that allow binding of antibody to the biomarker being analyzed. After washing, the presence of the antibody on the substrate indicates that the sample contained the marker being assessed. If the antibody is directly conjugated with a detectable label, such as an enzyme, fluorophore, or radioisotope, the presence of the label is optionally detected by examining the substrate for the detectable label. Alternatively, a detectably labeled secondary antibody that binds the marker-specific antibody is added to the substrate. The presence of detectable label on the substrate after washing indicates that the sample contained the marker. Alternatively, a sandwich assay is used where a specific primary antibody directed to a biomarker is bound to a solid substrate. A biological sample is incubated with the plate and non-specifically bound material is washed away. A labeled or otherwise detectable secondary antibody is used to bind the biomarker affixed to the substrate by the primary antibody. Detection of secondary antibody binding indicates the presence of the biomarker in the biological sample.

[00114] Numerous permutations of these basic immunoassays are also operative in the invention. These include the biomarker-specific antibody, as opposed to the sample being immobilized on a substrate, and the substrate is contacted with a biomarker conjugated with a detectable label under conditions that cause binding of antibody to the labeled marker. The substrate is then contacted with a sample under conditions that allow binding of the marker being analyzed to the antibody. A reduction in the amount of detectable label on the substrate after washing indicates that the sample contained the marker.

[00115] Although antibodies are useful in the invention because of their extensive characterization, any other suitable agent (e.g., a peptide, an aptamer, or a small organic molecule) that specifically binds a biomarker is optionally used in place of the antibody in the above described immunoassays. For example, an aptamer might be used. Aptamers are nucleic acid-based molecules that bind specific ligands. Methods for making aptamers with a particular binding specificity are known as detailed in U.S. Patent Nos. 5,475,096; 5,670,637; 5,696,249; 5,270,163; 5,707,796; 5,595,877; 5,660,985; 5,567,588; 5,683,867; 5,637,459; and 6,011,020.

[00116] A myriad of detectable labels that are operative in a diagnostic assay for biomarker expression are known in the art. Agents used in methods for detecting GFAP related or other neuron specific protein related biomarkers are conjugated to a detectable label, e.g., an enzyme such as horseradish peroxidase. Agents labeled with horseradish peroxidase can be detected by

adding an appropriate substrate that produces a color change in the presence of horseradish peroxidase. Several other detectable labels that may be used are known. Common examples of these include alkaline phosphatase, horseradish peroxidase, fluorescent compounds, luminescent compounds, colloidal gold, magnetic particles, biotin, radioisotopes, and other enzymes. It is appreciated that a primary/secondary antibody system is optionally used to detect one or more biomarkers. A primary antibody that specifically recognizes one or more biomarkers is exposed to a biological sample that may contain the biomarker of interest. A secondary antibody with an appropriate label that recognizes the species or isotype of the primary antibody is then contacted with the sample such that specific detection of the one or more biomarkers in the sample is achieved.

[00117] In some embodiments an antigen is used to detect an autoantibody. Illustratively, an antigen such as GFAP or one or more GBDPs are separated or placed on a substrate such as a PVDF membrane, the membrane is probed with a biological sample such as serum derived from a subject suspected of having a neurological condition, and the presence of an autoantibody is detected by contacting an autoantibody with an antibody type specific antibody such as an anti-IgG alone or combined with anti-IgM antibody that may or may not have a detectable label attached thereto.

[00118] A process optionally employs a step of correlating the presence or amount of biomarker such as, GBDPs, or autoantibodies thereto in a biological sample with the severity and/or type of nerve cell injury. The amount of biomarker in the biological sample is associated with a neurological condition such as traumatic brain injury. The results of an assay to measure biomarkers can help a physician or veterinarian determine the type and severity of injury with implications as to the types of cells that have been compromised. These results are in agreement with CT scan and GCS results, yet are quantitative, obtained more rapidly, and at far lower cost.

[00119] The present invention provides a step of comparing the quantity of one or more biomarkers to normal levels to determine the neurological condition of the subject. It is appreciated that selection of additional biomarkers allows one to identify the types of cells implicated in an abnormal neurological condition as well as the nature of cell death such as in the case of an axonal injury marker. The practice of an inventive process provides a test that can help a physician determine suitable therapeutics or treatments to administer for optimal benefit of the subject. While the data provided in the examples herein are provided with respect to a full spectrum of traumatic brain injury, it is appreciated that these results are applicable to ischemic

events, neurodegenerative disorders, prion related disease, epilepsy, chemical etiology and peripheral nervous system pathologies. A gender difference is optionally considered.

[00120] An assay for analyzing cell damage in a subject is also provided. The assay optionally includes: (a) a substrate for holding a biological sample isolated from a subject suspected of having a damaged nerve cell, the sample being a fluid in communication with the nervous system of the subject prior to being isolated from the subject; (b) a biomarker specific binding agent; (c) optionally a binding agent specific for another biomarker; and (d) printed instructions for reacting: the agent with the biological sample or a portion of the biological sample to detect the presence or amount of biomarker, and optionally the agent specific for another biomarker with the biological sample or a portion of the biological sample to detect the presence or amount of the at least one biomarker in the biological sample. The inventive assay can be used to detect a neurological condition for financial renumeration.

[00121] The assay optionally includes a detectable label such as one conjugated to the agent, or one conjugated to a substance that specifically binds to the agent, such as a secondary antibody.

[00122] The present invention optionally includes the presence of one or more therapeutic agents such as compounds that may alter one or more characteristics of a target biomarker. A therapeutic optionally serves as an agonist or antagonist of a target biomarker or upstream effector of a biomarker. A therapeutic optionally affects a downstream function of a biomarker. For example, Acetylcholine (Ach) plays a role in pathological neuronal excitation and TBI-induced muscarinic cholinergic receptor activation may contribute to excitotoxic processes. As such, biomarkers optionally relate to levels or activity of Ach or muscarinic receptors. Optionally, an operable biomarker is a molecule, protein, nucleic acid or other that is effected by the activity of muscarinic receptor(s). As such, therapeutics operable in the subject invention illustratively include those that modulate various aspects of muscarinic cholinergic receptor activation.

[00123] Specific mucarinic receptors operable as therapeutic targets or modulators of therapeutic targets include the M_1, M_2, M_3, M_4, and M_5 muscarinic receptors.

[00124] The suitability of the muscarinic cholinergic receptor pathway in detecting and treating TBI arises from studies that demonstrated elevated ACh in brain cerebrospinal fluid (CSF) following experimental TBI (Gorman et al., 1989; Lyeth et al., 1993a) and ischemia (Kumagae and Matsui, 1991), as well as the injurious nature of high levels of muscarinic cholinergic receptor activation through application of cholinomimetics (Olney et al., 1983;

Turski et al., 1983). Furthermore, acute administration of muscarinic antagonists improves behavioral recovery following experimental TBI (Lyeth et al., 1988a; Lyeth et al., 1988b; Lyeth and Hayes, 1992; Lyeth et al., 1993b; Robinson et al., 1990).

[00125] A therapeutic compound operable in the subject invention is illustratively any molecule, compound, family, extract, solution, drug, pro-drug, or other mechanism that is operable for changing, preferably improving, therapeutic outcome of a subject at risk for or victim of a neuronal injury such as TBI or MTBI. A therapeutic is optionally a muscarinic cholinergic receptor modulator such as an agonist or antagonist. An agonist or antagonist may by direct or indirect. An indirect agonist or antagonist is optionally a molecule that breaks down or synthesizes acetylcholine or other muscarinic receptor related molecule illustratively, molecules currently used for the treatment of Alzheimer's disease. Cholinic mimetics or similar molecules are operable herein. An exemplary list of therapeutics operable herein include: dicyclomine, scoplamine, milameline, N-methyl-4-piperidinylbenzilate NMP, pilocarpine, pirenzepine, acetylcholine, methacholine, carbachol, bethanechol, muscarine, oxotremorine M, oxotremorine, thapsigargin, calcium channel blockers or agonists, nicotine, xanomeline, BuTAC, clozapine, olanzapine, cevimeline, aceclidine, arecoline, tolterodine, rociverine, IQNP, indole alkaloids, himbacine, cyclostellettamines, deriviatives thereof, pro-drugs thereof, and combinations thereof. A therapeutic is optionally a molecule operable to alter the level of oractivity of a calpain or caspase. Such molecules and their administration are known in the art.

[00126] An inventive method illustratively includes a process for diagnosing a neurological condition in a subject, treating a subject with a neurological condition, or both. In some embodiments a process illustratively includes obtaining a biological sample from a subject. The biological sample is assayed by mechanisms known in the art for detecting or measuring the presence of one or more biomarkers present in the biological sample. Based on the amount or presence of a target biomarker in a biological sample, a ratio of one or more biomarkers is optionally calculated. The ratio is optionally the level of one or more biomarkers relative to the level of another biomarker in the same or a parallel sample, or the ratio of the quantity of the biomarker to a measured or previously established baseline level of the same biomarker in a subject known to be free of a pathological neurological condition. The ratio allows for the diagnosis of a neurological condition in the subject. An inventive process also optionally administers a therapeutic to the subject that will either directly or indirectly alter the ratio of one or more biomarkers.

[00127] A therapeutic is optionally designed to modulate the immune response in a subject. Illustratively, the levels, production of, breakdown of, or other related parameters of autoantibodies are altered by immunomodulatory therapy. Illustrative examples of immunomodulatory therapies are known in the art that are applicable to the presence of autoantibodies to illustratively GFAP or one or more GBDPs such as therapies used for multiple sclerosis. Such therapies illustratively include administration of glatiramer acetate (GA), beta-interferons, laquinimod, or other therapeutics known in the art. Optionally, combinations of therapeutics are administered as a form of immunomodulatory therapy. Illustrative combinations include IFNβ-1a and methotrexate, IFNβ-1a and azathioprine, and mitoxantrone plus methylprednisolone. Other suitable combinations are known in the art.

[00128] An inventive process is also provided for diagnosing and optionally treating a multiple-organ injury. Multiple organs illustratively include subsets of neurological tissue such as brain, spinal cord and the like, or specific regions of the brain such as cortex, hippocampus and the like. Multiple organ injuries illustratively include apoptotic cell death which is detectable by the presence of caspase induced GBDPs, and oncotic cell death which is detectable by the presence of calpain induced GBDPs. The inventive process illustratively includes assaying for a plurality of biomarkers in a biological sample obtained from a subject wherein the biological fluid sampled was optionally in fluidic contact with an organ suspected of having undergone injury or control organ when the biological sample was obtained from the subject. The inventive process determines a first subtype of organ injury based on a first ratio of a plurality of biomarkers. The inventive process also determines a second subtype of a second organ injury based on a second ratio of the plurality of biomarkers in the biological sample. The ratios are illustratively determined by processes described herein or known in the art.

[00129] Treatment of a multiple organ injury in the inventive process is illustratively achieved by administering to a subject at least one therapeutic antagonist or agonist effective to modulate the activity of a protein or nucleic acid whose activity or level is altered in response to the first organ injury, and administering at least one therapeutic agonist or antagonist effective to modulate the activity or level of a protein or nucleic acid whose activity is altered in response to a second organ injury.

[00130] The invention illustratively includes a composition for distinguishing the magnitude of a neurological condition in a subject. An inventive composition is either an agent entity or a mixture of multiple agents. In some embodiments a composition is a mixture. The mixture optionally contains a biological sample derived from a subject. The subject is optionally

suspected of having a neurological condition. The biological sample in communication with the nervous system of the subject prior to being isolated from the subject. In inventive composition also optionally contains at least two primary agents, optionally antibodies or nucleic acids, that specifically and independently bind to at least two biomarkers that may be present in the biological sample. In some embodiments the first primary agent is in antibody that specifically binds GFAP or one or more GBDPs. A second primary agent is optionally an antibody that specifically binds a ubiquitin carboxyl-terminal hydrolase, preferably UCHL1, or a spectrin breakdown product.

[00131] The agents of the inventive composition are optionally immobilized or otherwise in contact with a substrate. The inventive agents are also optionally labeled with at least one detectable label. In some embodiments the detectable label on each agent is unique and independently detectable in either the same assay chamber or alternate chambers. Optionally, a secondary agent specific for detecting or binding to the primary agent is labeled with at least one detectable label. In the nonlimiting example the primary agent is a rabbit derived antibody. A secondary agent is optionally an antibody specific for a rabbit derived primary antibody. Mechanisms of detecting antibody binding to an antigen are well known in the art, and a person of ordinary skill in the art readily envisions numerous methods and agents suitable for detecting antigens or biomarkers in a biological sample.

[00132] A kit is also provided that encompasses a substrate suitable for associating with the target biomarker in a biological sample. The biological sample is optionally provided with the kit or is obtained by a practitioner for use with an inventive kit. An inventive kit optionally includes at least two antibodies that specifically and independently bind to at least two biomarkers. The antibodies may distinguish between the two biomarkers. Optionally, a first antibody is specific and independent for binding and detecting a first biomarker. A second antibody is specific and independent for binding and detecting a second biomarker. In this way the presence or absence of multiple biomarkers in a single biological sample can be determined or distinguished. Antibodies in the biological sample illustratively include those for biomarkers of αII-spectrin, an αII-spectrin breakdown product (SBDP), a ubiquitin carboxyl-terminal hydrolase, GFAP, GBDP, and a MAP2 protein. An inventive kit also includes instructions for reacting the antibodies with the biological sample or a portion of the biological sample so as to detect the presence of or amount of the biomarkers in the biological sample.

[00133] In the kit, the biological sample can be CSF, blood, urine or saliva , and the agent can be an antibody, aptamer, primer, probe, or other molecule that specifically binds at least one

biomarker for a neurological condition. Suitable agents are described herein. The kit can also include a detectable label such as one conjugated to the agent, or one conjugated to a substance that specifically binds to the agent (e.g., a secondary antibody).

[00134] The invention optionally employs a step of correlating the presence or amount of a biomarker in a biological sample with the severity and/or type of nerve cell (or other biomarker-expressing cell) injury. The amount of biomarker(s) in the biological sample directly relates to severity of nerve tissue injury as a more severe injury damages a greater number of nerve cells which in turn causes a larger amount of biomarker(s) to accumulate in the biological sample (e.g., CSF; serum). Whether a nerve cell injury triggers an apoptotic and/or necrotic type of cell death can also be determined by examining illustratively the GBDPs present in the biological sample. Necrotic cell death preferentially activates calpain, whereas apoptotic cell death preferentially activates caspase-3. Because calpain and caspase-3 GBDPs can be distinguished, measurement of these markers indicates the type of cell damage in the subject. Also, the level of or kinetic extent of UCHL1, and or GFAP present in a biological sample may optionally distinguish mild injury from a more severe injury. In an illustrative example, severe MCAO (2h) produces increased UCHL1 in both CSF and serum relative to mild challenge (30 min) while both produce UCHL1 levels in excess of uninjured subjects. Moreover, the persistence or kinetic extent of the markers in a biological sample is indicative of the severity of the injury with greater injury indicating increases persistence of illustratively GBDP, UCHL1, or SBDP in the subject that is measured by an inventive process in biological samples taken at several time points following injury.

[00135] The results of such a test can help a physician determine whether the administration a particular therapeutic such as calpain and/or caspase inhibitors or muscarinic cholinergic receptor antagonists or any immunomodulators might be of benefit to a patient. This method may be especially important in detecting age and gender difference in cell death mechanism.

[00136] It is appreciated that other reagents such as assay grade water, buffering agents, membranes, assay plates, secondary antibodies, salts, and other ancillary reagents are available from vendors known to those of skill in the art. Illustratively, assay plates are available from Corning, Inc. (Corning, NY) and reagents are available from Sigma-Aldrich Co. (St. Louis, MO).

[00137] Methods involving conventional biological techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 3rd ed., vol. 1-3, ed. Sambrook et al., Cold

Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates); and Short Protocols in Molecular Biology, ed. Ausubel et al., 52 ed., Wiley-Interscience, New York, 2002. Immunological methods (e.g., preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, e.g., in Current Protocols in Immunology, ed. Coligan et al., John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff et al., John Wiley & Sons, New York, 1992.

[00138] Additional protocols such as PCR Protocols can be found in A Guide to Methods and Applications Academic Press, NY. Methods for protein purification include such methods as ammonium sulfate precipitation, column chromatography, electrophoresis, centrifugation, crystallization, and others. See, e.g., Ausubel, et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification," Methods in Enzymology vol. 182, and other volumes in this series; Current Protocols in Protein Science, John Wiley and Sons, New York, NY; and manufacturer's literature on use of protein purification products known to those of skill in the art.

[00139] Assays for neural cell biological activities are described, e.g., in Wouterlood (ed. 1995) Neuroscience Protocols modules 10, Elsevier; Methods in Neurosciences Academic Press; and Neuromethods Humana Press, Totowa, NJ.

[00140] Various aspects of the present invention are illustrated by the following non-limiting examples. The examples are for illustrative purposes and are not a limitation on any practice of the present invention. It will be understood that variations and modifications can be made without departing from the spirit and scope of the invention. While the examples are generally directed to mammalian tissue, specifically, analyses of rat tissue, a person having ordinary skill in the art recognizes that similar techniques and other techniques known in the art readily translate the examples to other mammals such as humans. Reagents illustrated herein are commonly cross reactive between mammalian species or alternative reagents with similar properties are commercially available, and a person of ordinary skill in the art readily understands where such reagents may be obtained. Variations within the concepts of the invention are apparent to those skilled in the art.

Example 1: Materials for Biomarker Analyses.

[00141] Illustrative reagents used in performing the subject invention include Sodium bicarbonate (Sigma Cat #: C-3041), blocking buffer (Startingblock T20-TBS) (Pierce Cat#: 37543), Tris buffered saline with Tween 20 (TBST; Sigma Cat #: T-9039). Phosphate buffered

saline (PBS; Sigma Cat #: P-3813); Tween 20 (Sigma Cat #: P5927); Ultra TMB ELISA (Pierce Cat #: 34028); and Nunc maxisorp ELISA plates (Fisher). Monoclonal and polyclonal GFAP and UCHL1 antibodies are made in-house or are obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies directed to α-II spectrin, GFAP, and breakdown products as well as to MAP2, MBP, neurofascin, IgG, and IgM are available from Santa Cruz Biotechnology, Santa Cruz, CA.

[00142] The anti-tau antibody directed to full length tau is purchased from Santa Cruz Biotechnology, Santa Cruz, CA. To generate antibodies specific to tau-BDPs, the synthetic peptide (Cys-C_6-SIDMVD$_{-COOH}$) (SEQ ID NO: 1) which is the sequence of the caspase-3 generated C-terminal tau breakdown product (tauBDP-45K) (Chung et al., 2001), and the second peptide ($_{NH2}$-KDRTGN -C_6-Cys) (SEQ ID NO: 2) representing the N-terminal of the calpain mediated TauBDP-35K are custom-made by peptide synthesis (California Peptide, Napa, CA). A C_6 linker and N-terminal cysteine are introduced for the subsequent coupling of the peptide to Keyhole Limpet Hemocyanin (KLH) protein using a sulfo-link crosslinking reagent (Pierce). After coupling efficiency determinations, peptides are dialyzed and concentrated. Rabbits are immunized with 2 mg of conjugated protein by injection. Serum samples are collected after three months and are affinity purified using the synthetic peptide against which they were raised coupled to sulfo-linked resins (Pierce). Affinity-purified antibody is then dialyzed against TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) and concentrated for storage in 50% glycerol at –20° C.

[00143] Labels for antibodies of numerous subtypes are available from Invitrogen, Corp., Carlsbad, CA. Protein concentrations in biological samples are determined using bicinchoninic acid microprotein assays (Pierce Inc., Rockford, IL, USA) with albumin standards. All other necessary reagents and materials are known to those of skill in the art and are readily ascertainable.

Example 2: Biomarker Assay Development

[00144] Anti-biomarker specific rabbit polyclonal antibody and monoclonal antibodies as well as antigens are produced in the laboratory or purchased commercially. To determine reactivity specificity of the antibodies to detect a target biomarker a known quantity of isolated or partially isolated biomarker is analyzed or a tissue panel is probed by western blot. An indirect ELISA is used with the recombinant biomarker protein attached to the ELISA plate to determine optimal concentration of the antibodies used in the assay. Microplate wells are coated

with rabbit polyclonal anti-human biomarker antibody. After determining the concentration of rabbit anti-human biomarker antibody for a maximum signal, the lower detection limit of the indirect ELISA for each antibody is determined. An appropriate diluted sample is incubated with a rabbit polyclonal antihuman biomarker antibody for 2 hours and then washed. Biotin labeled monoclonal anti-human biomarker antibody is then added and incubated with captured biomarker. After thorough wash, streptavidin horseradish peroxidase conjugate is added. After 1 hour incubation and the last washing step, the remaining conjugate is allowed to react with substrate of hydrogen peroxide tetramethyl benzadine. The reaction is stopped by addition of the acidic solution and absorbance of the resulting yellow reaction product is measured at 450 nanometers. The absorbance is proportional to the concentration of the biomarker. A standard curve is constructed by plotting absorbance values as a function of biomarker concentration using calibrator samples and concentrations of unknown samples are determined using the standard curve.

Example 3: *In vivo* model of TBI:

[00145] A controlled cortical impact (CCI) device is used to model TBI on rats as previously described (Pike et al, 1998). Adult male (280-300 g) Sprague-Dawley rats (Harlan: Indianapolis, IN) are anesthetized with 4% isoflurane in a carrier gas of 1:1 O_2/N_2O (4 min.) and maintained in 2.5% isoflurane in the same carrier gas. Core body temperature is monitored continuously by a rectal thermistor probe and maintained at 37±1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals are mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. Following a midline cranial incision and reflection of the soft tissues, a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) is performed adjacent to the central suture, midway between bregma and lambda. The dura mater is kept intact over the cortex. Brain trauma is produced by impacting the right (ipsilateral) cortex with a 5 mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a 1.6 mm compression and 150 ms dwell time. Sham-injured control animals are subjected to identical surgical procedures but do not receive the impact injury. Appropriate pre- and post-injury management is preformed to insure compliance with guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and Use of Laboratory Animals. In addition, research is conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles

stated in the "Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition."

Example 4: Middle cerebral artery occlusion (MCAO) injury model:

[00146] Rats are incubated under isoflurane anesthesia (5% isoflurane *via* induction chamber followed by 2% isoflurane *via* nose cone), the right common carotid artery (CCA) of the rat is exposed at the external and internal carotid artery (ECA and ICA) bifurcation level with a midline neck incision. The ICA is followed rostrally to the pterygopalatine branch and the ECA is ligated and cut at its lingual and maxillary branches. A 3-0 nylon suture is then introduced into the ICA *via* an incision on the ECA stump (the suture's path was visually monitored through the vessel wall) and advanced through the carotid canal approximately 20 mm from the carotid bifurcation until it becomes lodged in the narrowing of the anterior cerebral artery blocking the origin of the middle cerebral artery. The skin incision is then closed and the endovascular suture left in place for 30 minutes or 2 hours. Afterwards the rat is briefly reanesthetized and the suture filament is retracted to allow reperfusion. For sham MCAO surgeries, the same procedure is followed, but the filament is advanced only 10 mm beyond the internal-external carotid bifurcation and is left in place until the rat is sacrificed. During all surgical procedures, animals are maintained at 37 ± 1°C by a homeothermic heating blanket (Harvard Apparatus, Holliston, MA, U.S.A.). It is important to note that at the conclusion of each experiment, if the rat brains show pathologic evidence of subarachnoid hemorrhage upon necropsy they are excluded from the study. Appropriate pre- and post-injury management is preformed to insure compliance with all animal care and use guidelines.

Example 5: Tissue and Sample Preparation:

[00147] At the appropriate time points (2, 6, 24 hours and 2, 3, 5 days) after injury, animals are anesthetized and immediately sacrificed by decapitation. Brains are quickly removed, rinsed with ice cold PBS and halved. The right hemisphere (cerebrocortex around the impact area and hippocampus) is rapidly dissected, rinsed in ice cold PBS, snap-frozen in liquid nitrogen, and stored at -80°C until used. For immunohistochemistry, brains are quick frozen in dry ice slurry, sectioned via cryostat (20 μm) onto SUPERFROST PLUS GOLD® (Fisher Scientific) slides, and then stored at -80°C until used. For the left hemisphere, the same tissue as the right side is collected. For western blot analysis, the brain samples are pulverized with a small mortar and pestle set over dry ice to a fine powder. The pulverized brain tissue powder is then lysed for 90 min at 4°C in a buffer of 50 mM Tris (pH 7.4), 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT, 1x protease inhibitor cocktail (Roche Biochemicals). The brain lysates are then centrifuged

at 15,000xg for 5 min at 4°C to clear and remove insoluble debris, snap-frozen, and stored at -80°C until used.

[00148] For gel electrophoresis and electroblotting, cleared CSF samples (7 µl) are prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 2X loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H_2O. Twenty micrograms (20 µg) of protein per lane are routinely resolved by SDS-PAGE on 10-20% Tris/glycine gels (Invitrogen, Cat #EC61352) at 130 V for 2 hours. Following electrophoresis, separated proteins are laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer containing 39 mM glycine, 48 mM Tris-HCl (pH 8.3), and 5% methanol at a constant voltage of 20 V for 2 hours at ambient temperature in a semi-dry transfer unit (Bio-Rad). After electro-transfer, the membranes are blocked for 1 hour at ambient temperature in 5% non-fat milk in TBS and 0.05% Tween-2 (TBST) then are incubated with the primary monoclonal GFAP antibody in TBST with 5% non-fat milk at 1:2000 dilution as recommended by the manufacturer at 4°C overnight. This is followed by three washes with TBST, a 2 hour incubation at ambient temperature with a biotinylated linked secondary antibody (Amersham, Cat # RPN1177v1), and a 30 min incubation with Streptavidin-conjugated alkaline phosphatase (BCIP/NBT reagent: KPL, Cat # 50-81-08). Molecular weights of intact biomarker proteins are assessed using rainbow colored molecular weight standards (Amersham, Cat # RPN800V). Semi-quantitative evaluation of intact GFAP, UCHL1, or SBDP protein levels is performed via computer-assisted densitometric scanning (Epson XL3500 scanner) and image analysis with ImageJ software (NIH).

Example 6: Breakdown products as biomarkers of neuronal injury.

[00149] CSF is prepared as per Example 5 following CCI in rats as described in Example 3. Western blotting using anti-GFAP antibodies reveal an increase in GFAP with increasing time following CCI injury (FIG. 1A, B). Similarly, the levels of GFAP increase with time following CCI with statistically significant maximal levels at day 14 following injury (FIG. 1C). The levels of GFAP and GBDP in rat ipsilateral cortex are also measured and demonstrate increased levels relative to sham (S) treated animals (FIG. 2). These data demonstrate an increase in GFAP and GBDP in CSF and neuronal tissue following CCI similar to that of severe TBI.

[00150] The breakdown of GFAP in neurons is injury specific. Rat brain tissue lysates from sham treated animals of Example 3 as well as a human brain sample from a non-TBI cadaver are treated with two levels of calpain-2 and caspase-3 followed by western blotting using primary antibodies to either GFAP and GBDPs or SBDPs. FIG. 3 demonstrates that *in vitro* digestion of

rat brain lysates with calpain-2 show overlapping GBDPs with digested human brain lysate. Caspase-3 cleavage of rat brain revealed similar GBDPs to CCI treated rats (FIG. 3 A).

[00151] Similarly, the levels of SBDP 150/145 are increased in rat CSF following CCI at both 24 hours and 7 days following injury (FIG. 3B).

[00152] Lysates of mixed glial/neuron cultures are examined for the presence of GBDPs following various treatments. To obtain the cultures cerebrocortical cells are harvested from 1-day old Sprague-Dawley rat brains and plated on poly-L-lysine coated on 6-well culture plates (Erie Scientific, Portsmouth, NH, USA) according to the method of Nath et al., *J. Neurochem.*, 1998; 71:186-195 at a density of 4.36 × 105 cells/mL. Cultures are maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum in a humidified incubator in an atmosphere of 10% CO_2 at 37°C. After 5 days in culture, the media are changed to DMEM with 5% horse serum. Subsequent media changes are performed three times a week. Experiments are performed on days 10 to 11 *in vitro* when astroglia had formed a confluent monolayer beneath morphologically mature neurons.

[00153] In addition to untreated controls, the following conditions are examined: methotrexate (MTX) (1μM) ; apoptotic inducers staurosporine (STS) (0.5 μM; Sigma, St. Louis, MO) that activates calpain and caspase-3 for 24 hours (Zhang et al., 2009); the Ca^{2+} chelator ethylene diamine tetra-acetic acid (EDTA) (5 mM; Sigma) for up to 24 hours as a caspase-dominated challenge (Waterhouse et al., 1996; Chiesa et al., 1998; Mizuno et al., 1998; McGinnis et al., 1999; Zhang et al., 2009). For pharmacologic intervention, cultures were pretreated one hour before the STS, EDTA or MTX challenge with the calpain inhibitor SNJ1945 (Senju Pharmaceuticals, Kobe, Japan) (Shirasaki et al., 2005; 3Oka et al. 2006; Koumura et al., 2008), or the pan-caspase inhibitor Z-VAD (OMe)-FMK (R&D, Minneapolis, MN).

[00154] MTX treated cultured cells showed calpain specific cleavage of GFAP as illustrated by reduction in GBDPs using SJA treatment and the lack of inhibition by treatment with VAD (FIG. 4). In contrast, EDTA treatment produces caspase mediated GDBP formation as illustrated by no effect by SJA treatment and no GBDP with treatment by VAD. Treatment with the apoptosis inducer STS illustrated a balanced cleavage of GFAP by both calpain and caspase.

[00155] To identify the cleavage sites in GFAP mediated by calpains and caspases in neuronal cells, recombinant GFAP is subjected to cleavage with differing levels of calpain or caspase. FIG. 5 illustrates GFAP digestion as detected by Coomassie blue staining, detection of

a C-terminal tag (DDK) associated with recombinant GFAP or by western blot with anti-GFAP antibody illustrates successful cleavage by both calpain and caspase. Excision of GBDP bands from Coomassie blue stained gels and subjecting the bands to N-terminal sequencing reveals cleavage by calpain 2 at Asn59 (GALN*$_{59}$AGFKETRASERAE) (SEQ ID NO: 3) to produce a GBDP with new C-terminus VDFSLAGALN-COOH (SEQ ID NO: 139) and a GBDP with new N-terminus NH_2-AGFKETRASE (SEQ ID NO: 140), and Thr383 (TIPVQT*$_{383}$FSNLQIRET) (SEQ ID NO: 4) producing a GBDP with new C-terminus ENRITIPVQT-COOH (SEQ ID NO: 141) and a GBDP with new N-terminus NH_2-FSNLQIRETS (SEQ ID NO: 142), overall producing multiple GBDPs between 49 and 38 kDa in recombinant human GFAP.

[00156] Breakdown products of Tau in rat hippocampus 48 hours following CCI are identified in U.S. Patent No. 7,456,027, the contents of which are incorporated herein by reference. It was shown that following experimental TBI by CCI injury that rat Tau is cleaved to produce TBDPs of molecular weight 40-55 kDa, 36 kDa, 26 kDa, 18, kDa, and 13 kDa. The cleavage sites and their relevance to specific types of neuronal injury were unknown. The cleavage sites in Tau are identified by *in vitro* digestion of recombinant rat tau using calpain 2 or calpain 1. Following digestion, cleavage fragments are separated by SDS-PAGE, stained with Coomassie blue and bands excised and subjected to N-terminal sequencing. The sequence results are compared to the known sequence of rat tau. Full length rat Tau with calpain and caspase cleavage sites are illustrated in Table 6

[00157] **Table 6:** (SEQ ID NO: 5) The * represents a cleavage site; the bold lettering represents an epitope used to raise a TBDP specific antibody.

Rat Tau

1 MAEPRQEFDT MEDQAGDYTM LQDQEGDMDHGLK * **ESPPQPPADD** GSEEPGS ETSDAKSTPT
61 AEDVTAPLVE ERAPDKQATA QSHTEIPEGT TAEEAGIGDT PNMEDQAAG HVTQARVAGVS*
121 **KDRTGNDEK** KAKGADGKTGA KIATPRGAAT PGQKGTSNAT RIPAKTTPSP KTPPGSGEPP
181 KSGERSGYSS PGSPGTPGSR SRTPSLPTP **PTREPKKVAVV** * RTPPKSPSAS KSRLQTAPVP
241 MPDLKNVRSK IGSTENLKHQ PGGGKVQIIN KKLDLSNVQS KCGSKDNIKH VPGGGSVQIV
301 YKPVDLSKVT SKCGSLGNIH HKPGGGQVEV KSEKLDFKDR VQSKIGSLDN ITHVPGGGNK
361 KIE**THKLTFR***E**NAKAKTD**HG AEIVYKSPVV SGDTSPRHLSNV **SSTGSIDMVD** * SPQLATLA
421 DEVSASLAKQ GL

[00158] Calpain cleaves rat tau at Lys43 (LK*$_{43}$ESPPQPPADD (SEQ ID NO: 6)) producing a TBDP with new C-terminus QEGDMDHGLK-COOH (SEQ ID NO: 115) and a TBDP with new N-terminus NH_2-QEGDMDHGLK (SEQ ID NO: 116); Ser120 (AGHVTQARMVS*$_{120}$KDRTGNDEK (SEQ ID NO: 7) producing a TBDP with new C-terminus VTQARVAGVS-COOH (SEQ ID NO: 117) and a TBDP with new N-terminus NH_2-KDRTGNDEKK (SEQ ID NO: 118), Val220 (PTREPKKVAVV*$_{220}$RTPPKSPSAS (SEQ ID

NO: 8)) producing a TBDP with new C-terminus TREPKKVAVV-COOH (SEQ ID NO: 119) and a TBDP with new N-terminus NH_2-RTPPKSPSAS (SEQ ID NO: 120); and Arg370 (KIETHKLTFR*$_{370}$ENAKAKTDHGAEI (SEQ ID NO: 9)) (FIG. 5) producing a TBDP with new C-terminus KIETHKLTFR-COOH (SEQ ID NO: 121) and an TBDP with new N-terminus NH_2-ENAKAKTDHG (SEQ ID NO: 122). Caspase-3 cleaves rat Tau at Asp412 (SSTGSIDMVD*$_{412}$SPQLATLA (SEQ ID NO: 10)) to produce a TBDP with new C-terminus SSTGSIDMVD-COOH (SEQ ID NO: 123) and a TBDP with new N-terminus NH_2-SPQLATLADE (SEQ ID NO: 124).

[00159] Human Tau is cleaved by calpain and caspase at similar locations. Table 7 illustrates the cleavage locations in human tau.

[00160] **Table 7:** (SEQ ID NO: 11) The * represents a cleavage site; the bold lettering represents an epitope used to raise a TBDP specific antibody.

Human Tau
1 MAEPRQEFEV MEDHAGTYGLGDRKD*QGGYTMHQD QEGDTDAGLK * **ESPLQTPTED** GSEEPG
61 SETSDAKSTP TAEDVTAPLV DEGAPGKQAA AQPHTEIPEG TTAEEAGIGD TPSLEDEAAG
121 **HVTQARMVS** * **KSKDGTGSDD**K KAKGADGKTK IATPRG *AAPP GQKGQANATR IPAKTPPAPK
181 TPPSSGEPPK SGDRSGYSSP GSPGTPGSRS RTPSLPTP **PTREPKKVAVV** * RTPPKSPSSAK
241 SRLQTAPVPM PDLKNVKSKI GSTENLKHQP GGGKVQIINK KLDLSNVQSK CGSKDNIKHV
301 PGGGSVQIVY KPVDLSKVTS KCGSLGNIHH KPGGGQVEVK SEKLDFKDRV QSKIGSLDNI
361 THVPGGGNKK IETHKLTFR*E NAKAKTDHGA EIVYKSPVVS GDTSPRHLSNV **SSTGSIDMVD***
422 SPQLATLADEVSASLAKQG L

[00161] Human TBDPs are produced from Human Tau by calpain cleavage at Ser129 (AGHVTQARMVS$_{129}$KSKDGTGSDD (SEQ ID NO: 12)) to produce a TBDP with new C-terminus GHVTQARMVS-COOH (SEQ ID NO: 125) and a TBDP with new N-terminus NH_2-KSKDGTGSDD (SEQ ID NO: 126), Val229 (PTREPKKVAVV*$_{229}$RTPPKSPSSA (SEQ ID NO: 13)) to produce a TBDP with new C-terminus TREPKKVAVV-COOH (SEQ ID NO: 127) and a TBDP with new N-terminus NH_2-RTPPKSPSSA (SEQ ID NO: 128), Gly157 (GKTKIATPRG*$_{157}$AAPPGQKGQANATRITA (SEQ ID NO: 14)) to produce a TBDP with new C-terminus GKTKIATPRG-COOH (SEQ ID NO: 129) and a TBDP with new N-terminus NH_2-AAPPGQKGQA (SEQ ID NO: 130), Lys44 (LK*$_{44}$ESPLQTPTED (SEQ ID NO: 15)) to produce a TBDP with new C-terminus QEGDTDAGLK-COOH (SEQ ID NO: 131) and a TBDP with new N-terminus NH_2-ESPLQTPTED (SEQ ID NO: 132), and Arg379 (KIETHKLTFR*$_{379}$ENAKAKTDHGAEI (SEQ ID NO: 16)) to produce a TBDP with new C-terminus KIETHKLTFR-COOH (SEQ ID NO: 133), and a TBDP with new N-terminus NH2-ENAKAKTDHG (SEQ ID NO: 134). Caspase cleavage sites in human Tau are Asp25 (GDRKD*$_{25}$QGGYTMHQD (SEQ ID NO: 17)) to produce a TBDP with new C-terminus

GTYGLGDRKD–COOH (SEQ ID NO: 135) and a TBDP with new N-terminus NH2-QGGYTMHQDQ (SEQ ID NO: 136), and Asp421 (SSTGSIDMVD*$_{421}$SPQLATLA (SEQ ID NO: 18)) to produce a TBDP with new C-terminus SSTGSIDMVD-COOH (SEQ ID NO: 137) and a TBDP with new N-terminus NH_2-SPQLATLADE (SEQ ID NO: 138). The overall neuronal injury cleavage pathways of human Tau are illustrated in FIG. 6.

[00162] Antibodies are raised to TAUBDP-45K (caspase) and TAUBDP-35K (calpain). A synthetic peptide (Cys-C_6-SIDMVD) (SEQ ID NO: 1) based on Tau C-terminal of tauBDP-45K generated by caspase-3 (Chung et al., 2001) and another peptide (KDRTGNDEK -C_6-Cys) (SEQ ID NO: 19) based on the new N-terminal of the calpain mediated TauBDP-35K are custom-made (California Peptide, Napa, CA). Other exemplary epitopes for antibodies specific for TBDPs are listed in Table 8.

Table 8:

Tau Fragment-specific peptide epitopes	
Human, rat: SSTGSIDMVD-$_{COOH}$	(SEQ ID NO: 20)
Human, rat: $_{NH2}$-SPQLATLA	(SEQ ID NO: 21)
Rat: AGHVTQARVAGVS –$_{COOH}$	(SEQ ID NO: 22)
Rat: $_{NH2}$-KDRTGNDEK	(SEQ ID NO: 23)
Human, rat: PTREPKKVAVV-$_{COOH}$	(SEQ ID NO: 24)
Human, rat: $_{NH2}$-RTPPKSPSAS	(SEQ ID NO: 25)
Human, rat, mouse: KIETHKLTFR-$_{COOH}$	(SEQ ID NO: 26)
Human, rat, mouse: $_{NH2}$-ENAKAKTDHGAEI	(SEQ ID NO: 27)
Rat: QEGDMDHGLK-$_{COOH}$	(SEQ ID NO: 28)
Rat: $_{NH2}$-ESPPQPPADD	(SEQ ID NO: 29)
Human: QEGDTDAGLK-$_{COOH}$	(SEQ ID NO: 30)
Human: $_{NH2}$-ESPLQTPTED	(SEQ ID NO: 31)
Human: GTYGLGDRKD-$_{COOH}$	(SEQ ID NO: 32)
Human: $_{NH2}$-QGGYT MHQD	(SEQ ID NO: 33)
Human: GKTKIATPRG-$_{COOH}$	(SEQ ID NO: 34)
Human: $_{NH2}$-AAPPGQKGQA	(SEQ ID NO: 35)
Human: AGHVTQARMVS -$_{COOH}$	(SEQ ID NO: 36)
Human: $_{NH2}$-KSKDGTGSDD	(SEQ ID NO: 37)

Calpain cleavage site peptide epitopes		
Pro-calpain-2 (human, rat):	SHERAIK	(SEQ ID NO: 38)
Activated calpain-1 (human, rat):	$_{NH2}$-LGRHENA peptide	(SEQ ID NO: 39)

[00163] A C_6 linker and N-terminal cysteine are introduced for the subsequent coupling of the peptide to Keyhole Limpet Hemocyanin (KLH) protein using a sulfo-link crosslinking reagent (Pierce). After coupling efficiency determinations, peptides are dialyzed, concentrated,

and 2 mg of conjugated protein is used for multiple antigen injections into two rabbits. After 3 months, collected serum samples from the rabbits are subjected to affinity purification using the same synthetic peptide-coupled to sulfo-linked resins (Pierce). Affinity-purified antibody is dialyzed against TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl), before it is concentrated and stored in 50% glycerol at –20° C.

[00164] Additional antibodies to specific cleavage products are also produced. Antigens used include: Cys-eAhx-SSTGSIDMVD-OH (SEQ ID NO: 40) to produce an antibody specific to TBDP-45K (caspase) in human and rat; Cys-C6-PTREPKKVAVV (SEQ ID NO: 41) to produce an antibody specific to human and rat TBDP-14K-20K (calpain); NH2-KSKDGTGSDD-C6-Cys (SEQ ID NO: 42) to human TBDP-35K (calpain); ESPLQTPTED-C6 (SEQ ID NO: 43) to human TBDP-14k; and Cs-Cy6-HVTQARMVS (SEQ ID NO: 44) to human TBDP-10k C-terminal. Other antibodies presented in U.S. Applications Publication No: 2005/0260697, incorporated herein by reference, are also produced.

[00165] Rat tau protein (100 ng) is digested with either calpain-2 or caspase-3. The resulting fragments are separated by SDS-PAGE and probed by western blot with the raised antibodies. Both calpain-1 and -2 digest tau into several immunoreactive fragments (42 kDa, 35 kDa and 15 kDa), while caspase-3 digestion only produces limited fragment doublet of 48kDa/45/kDa (FIG. 7). Probing with TauBDP-35K (Calpain) and TauBDP-45K (caspase) antibodies confirms the fragment-specificity of the antibodies and no cross-reactivity with intact protein or other fragments. (FIG. 7).

[00166] The TBDP antibodies are analyzed for cross reactivity with human Tau BDPs. Purified human Tau is left either untreated, calpain-2 treated, or caspase-3 treated to produce TBDPs. These are compared to TBDPs produced by MTX, or EDTA treated rat cerebrocortical culture cells (CTX) as described above as well as to naïve or TBI ipsilateral hippocampus (IH) or ipsilateral cortex (IC) models. The resulting fragments are separated by SDS-PAGE and probed with anti-total tau monoclonal antibody as illustrated in FIG. 8. Treatment of human tau with calpain or caspase produce numerous breakdown products. MTX treated CTX cells reveals calpain specific cleavages of Tau and EDTA treated CTX cells shows caspase specific cleavages of Tau. The protease mediated cleavage of Tau following TBI is illustrated in both IH and IC TBI models. (FIG. 8A) The anti-TBDP-35K specific antibody shows no cross reactivity to human Tau (FIG. 8B).

[00167] The TBDP specific antibodies are used to probe rat cerebrocortical cultures that are either naïve or subjected to treatment with: an excitotoxic challenge NMDA – (Nath et al.,

1998); the apoptotic inducer staurosporine (STS) (0.5 μM) that activates calpain and caspase-3 for 24 hours (Zhang et al., 2009); or the Ca^{2+} chelator and apoptotic inducer ethylene diamine tetra-acetic acid (EDTA) as a caspase-dominated challenge (Waterhouse et al., 1996; Chiesa et al., 1998; Mizuno et al., 1998; McGinnis et al., 1999; Zhang et al., 2009). NMDA , EDTA and STS treatments each produce extensive neurodegeneration *in vitro*. NMDA induces a mixed necrosis /apoptosis phenotype, while both STS and EDTA produce a robust apoptosis phenotype (FIG. 9).

[00168] Rat cerebrocortical culture lysates are probed with either total-Tau antibody or fragment specific antibodies. With NMDA treatment, Tau is significantly degraded into multiple fragments (42K, 35K, and 15K) including a dominant signal of calpain-mediated TauBDP-35K with minimal caspase-mediated Tau-BDP45K (FIG. 10). The caspase inhibitor (Z-VAD; 20 μM) produces no significant changes of Tau breakdown pattern. The inhibitor (SNJ-1945; 20 μM) significantly reduces the lower molecular weight fragment, including complete blockade of the calpain-mediated TauBDP-35K, although some high molecular weight fragments (425-48K) persist. Interestingly, when a blot was probed with the anti-caspase-mediated Tau-BDP45K antibody, the TauBDP-45/48K are detected in the calpain inhibitor-NMDA co-treatment lane. NMDA treatment yields prominent calpain-mediated SBDP150/SBDP145, with minor bands of caspase-3-mediated SBDP120. These fragments are strongly inhibited with their respective protease inhibitors (SNJ, Z-VAD). Taken together, these data suggest that in NMDA paradigm, calpain is the dominant pathway in tau fragmentation with a more minor contribution of caspase.

[00169] Using the apoptosis inducer EDTA, Tau is truncated only to Tau-BDP48K/45K, as confirmed by total tau blot and caspase-mediated anti-Tau-BDP45K blot (FIG. 10). Both fragments are caspase inhibitor (Z-VAD)-sensitive but insensitive to calpain inhibitor (SNJ-1945). Thus, EDTA challenge produces a straight caspase-dominant tau fragmentation condition. αII-Spectrin breakdown pattern from EDTA challenge confirms the presence of caspase-mediated SBDP120, but not calpain generated fragment SBDP145.

[00170] Staurosporine treatment illustrates a balance of higher molecular weight (45-48K) and low molecular weight (35K, 15K) tau-BDPs (FIG. 10). The 48/45K fragments are caspase-mediated as confirmed by the tau-45K fragment-specific antibody blot as well as its sensitivity to caspase inhibitor (Z-VAD). Similarly, the involvement of calpain is confirmed by the TauBDP-35K-specific antibody and its sensitivity to calpain inhibitor SNJ-1945. Importantly, the presence of calpain inhibitor strongly elevated the Tau-DBP-48k/45K by both total tau blot and anti-Tau-48/45K blot suggesting the dual involvement of both calpain and caspase. This is also

consistent with αII-spectrin breakdown pattern. STS treatment produces a neurodegenerative paradigm where there is a dual and balanced contribution of both calpain and caspase in tau fragmentation.

[00171] TBDPs are produced by CCI injury in rats. Cortical and hippocampal tissue samples obtained following CCI injury as in Example 3 are lysed and the proteins are separated by SDS-PAGE followed by western blotting to identify the presence of specific TBDPs. In rat cortex TBDP-15K rapidly increases with early presentation at 2 hours and recuing maximal levels by 6 hours post-CCI (FIG. 11A, B). The levels then drop after 48 hours post-injury. TBDP-25K shows more gradual increases reaching a maximal level at 48 hours (FIG. 11A, B). Other Tau-BDPs are also observed. The numerous bands observed in both the sham treated as well as the post-CCI represent the numerous phosphorylation states and isoforms of the Tau protein. In the contralateral cortex, no tau proteolysis is observed in all three groups even when tau immunoblots are intentionally over-developed (data not shown). In rat hippocampus TBDP-15K is present at 2 hours post injury with a maximal level observed at 24 hours (FIG. 11C, D). Similar results are observed for TBDP-35K (FIG. 11C, D). Overall, the calpain mediated tau protein fragmentation pattern *in vitro* (TauBDP-35K, 25K, and 15K) (FIG. 7) matches well with *in vivo* tau proteolysis after TBI (FIG. 11). Since the controlled cortical impact device targets the cortex, greater focal injury occurs in the ipsilateral cortex tissue than in the hippocampal tissue, which is impacted indirectly by contusive force. As a result, there is more extensive tau proteolysis in the cortex than in the hippocampus.

[00172] The same samples are probed using the TBDP specific antibodies. FIG. 12A illustrates specific calpain activity producing TBDP-35K in rat cortex and no caspase mediated cleavages in the rat cortex following CCI. Similar results are observed in the rat hippocampus (FIG. 12B). Thus, CCI induced neurodegeneration is accompanied by the formation and accumulation of calpain-mediated TBDPs with only minor contributions to caspase-generated Tau fragments in the injured cortex.

[00173] The calpain-1 and calpain-2 temporal activation profiles of tauBDP-35K are examined using anti-activated calpain-1 new-N-terminal (anti-LGRHENA) (SEQ ID NO: 39) antibody, or pro-calpain-2 N-terminal (anti-SHERAIK) (SEQ ID NO: 38) antibody. FIG. 13 illustrates activation of both calpain-1 (A) and calpain-2 (B) in injured cortex. Calpain-1 has an early peak on day 2 and 3 and subsided afterward (FIG. 13A) while calpain-2 also peaked on day 2 but was sustained to 3 and 5 days (FIG. 13.B). The calpain-1 activation matches very well with the appearance of calpain specific TBDP-35K.

[00174] The calpain mediated cleavage of Tau is further demonstrated by its inhibition using the calpain inhibitor SNJ-1945 which is administered (100 mg/kg, i.v. bolus) immediately following CCI. Probing of the cortical tissue is subjected to immunoblotting with the TBDP-35K specific antibody. The increase in TBDP-35K is suppressed by SNJ-1945 (FIG. 14). Thus, calpain-1 acts as a major Tau cleaving protease following TBI.

Example 7: Severe Human Traumatic Brain Injury Study

[00175] A study included 46 human subjects suffering severe traumatic brain injury. Each of these subjects is characterized by being over age 18, having a GCS of less than or equal to 8 and required ventriculostomy and neuromonitoring as part of routine care. A control group A, synonymously detailed as CSF controls, included 10 individuals also being over the age of 18 or older and no injuries. Samples are obtained during spinal anesthesia for routine surgical procedures or access to CSF associated with treatment of hydrocephalus or meningitis. A control group B, synonymously described as normal controls, totaled 64 individuals, each age 18 or older and experiencing multiple injuries without brain injury. Further details with respect to the demographics of the study are provided in Table 9.

Table 9: Subject Demographics for Severe Traumatic Brain Injury Study

		TBI	CSF Controls	Normal Controls
Number		46	10	64
	Males	34 (73.9%)	29 (65.9%)	26 (40.6%)
	Females	12 (26.1%)	15 (34.1%)	38 (59.4%
Age:	Average	50.2	58.2 1, 2	30.09 2, 3
	Std Dev	19.54	20.52	15.42
	Minimum	19	23	18
	Maximum	88	82	74
Race:	Caucasian			
	Black	45	38 (86.4%)	52 (81.2%)
	Asian	1	6 (13.6)	4 (6.3%)
	Other			7 (10.9%)
				1 (1.6%)
GCS in Emergency Department				
	Average	5.3		

Std Dev	1.9

[00176] The level of biomarkers found in the first available CSF samples for a first and second patient (FIG. 15A and B) illustrate elevated GFAP and GBDP at emergency room admission for a first patient (FIG. 15A). Similarly, a second patient who had CSF drawn within 6 hours following TBI revealed the presence of elevated levels of GFAP and GBDP at this time point (FIG. 15B). The second patient also demonstrated detectable but lower levels of GFAP and GBDP at 18 hours following injury. The levels of SBDP 150/145 and SBDP 120 are also elevated in CSF of the first patient at emergency room admission with relatively lower levels at later time points (FIG. 15A).

Example 8:

[00177] Identification of autoantibodies as biomarkers of TBI. Following severe TBI autoantibodies to neuronal proteins are produced and detectable in the blood of subjects. Brain lysate from a human TBI subject is obtained post-mortem and solubilized with lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM NaF, 1 mM Na3VO4, and a protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Lysates are separated by SDS-PAGE and subsequently stained for total protein (FIG. 16A) and transferred onto a Polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) for probing. The blots are probed using serum obtained from a human control donor (B) or from a human obtained 10 days post TBI (C) and autoantibodies are detected using anti-IgG/IgM. Several intense bands are observed in the serum from the post-TBI subject indicating the presence of serum autoantibodies.

[00178] Human serum contains autoantibodies are observed at increasing levels following TBI. Serum from five individual human control and TBI subjects are probed for the presence of autoantibodies. Samples of solubilized human post-TBI brain lysate as described above are separated by SDS-PAGE and transferred to PVDF membranes by the iblot method. The blot is probed with either serum from normal control human subjects or from five TBI subjects where samples are obtained either at 72 hours post TBI or 30 days post-TBI. Following several washes with TBST, the PVDF membranes are removed from the multiscreen apparatus and washed three more times, then finally incubated with a AP-conjugated goat anti-human IgG+IgM or AP-conjugated donkey anti-human IgG diluted 1:10,000 for one hour followed by washing with TBST. Positive signals are visualized using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg,

MD, USA). FIG. 17 illustrates the presence of autoantibodies to several human brain proteins that are present following TBI. The amount of autoantibody is increased from day 72 to day 30 as illustrated by enhanced signal at the later timepoint.

[00179] The level of autoantibody increases with time following TBI. The serum from a single human subject obtained at admission and up to 30 days following TBI is used to probe human brain lysates loaded at equal concentrations in each lane of a gel and transferred to PVDF. The autoantigen is an IgG as demonstrated by detection using an anti-IgG secondary antibody (FIG. 18A). The rate at which autoantibody is produced is surprisingly fast with detectable levels appearing at day 5 and increasing out to day 30 (FIG. 18A). Confirmation that the autoantibodies are IgG specific is illustrated in FIG. 18B where blots are probed with secondary antibodies to particular immunoglobulin species.

[00180] The autoantibodies are directed to brain specific antigens. Lysates (30 μg of protein) from human brain, heart, kidney, lung, spleen, intestine, skin, muscle, and testes are separated by SDS-PAGE, transferred to PVDF and detected using western blot by probing with the serum of a human TBI subject at a dilution of 1:100. Any bound autoantibodies are detected by AP conjugated goat anti-human IgG/IgM at a dilution of 1:10,000. FIG. 19 illustrates that autoantibodies from TBI subjects recognize autoantigens migrating between 38 and 52 kDa from brain alone.

[00181] The autoantibodies are directed to a breakdown product as the result of calpain digestion. Naïve rat brain lysates are loaded onto a gel and probed with the serum from a human TBI subject. In the absence of calpain cleavage products, no antigen recognition is observed (data not shown). However, calpain digested rat brain lysate loaded at identical concentrations is recognized by human TBI patient autoantibody detectable beginning from day 5 (lane 12) and increasing to day 10 (lane 17) following TBI (FIG. 20).

[00182] To identify which antigens were recognized by autoantibodies in human serum post-TBI, purified GFAP, neurofacein, and MBP were loaded onto gels, separated by SDS-PAGE, transferred to PVDF and probed with serum from a TBI human subject obtained 10 days after injury followed by detection using an anti-IgG antibody. GFAP, neurofascin, and MBP are each detected as autoantigens recognized by autoantibodies generated in human serum following TBI (FIG. 21).

[00183] GFAP is confirmed as recognized by autoantibodies in the serum from a human subject obtained 10 days following TBI. Post-mortem human brain lysates are subjected to ion exchange chromatography and eluate fractions are loaded onto a gel and separated. Total protein