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(54) Title: BIO-ASSAY CAPTURE SURFACES WITH BLEACHED AUTOFLUORESCENCE

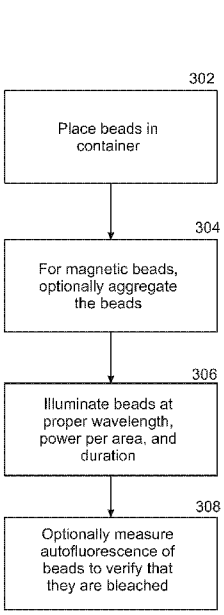

FIG. 3

(57) Abstract: A method of photobleaching polymer capture surface elements suitable to use for providing a capture surface in a fluorescent bio-assay, the method comprising: a) providing one or more of the capture surface elements having an autofluorescence excited by light in a peak range of excitation wavelengths, producing light predominantly in a peak range of emission wavelengths that may depend on the excitation wavelength; and b) exposing the capture surface elements to light from a light source used for bleaching, with suitable wavelength distribution and sufficient intensity and duration so that a level of the autofluorescence, for at least one excitation wavelength within the peak range of excitation wavelengths and at least one emission wavelength within the peak range of emission wavelengths for that excitation wavelength, is reduced by at least a factor of 2.

BIO-ASSAY CAPTURE SURFACES WITH BLEACHED AUTOFLUORESCENCE

RELATED APPLICATION

This application claims the benefit of priority under 35 USC §119(e) of U.S. Provisional Patent Application No. 62/482,302, with the title "BIO-ASSAY BEADS WITH QUENCHED AUTOFLUORESCENCE," filed April 6, 2017, the contents of which are incorporated herein by reference in their entirety.

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to capture surfaces used in fluorescence-based bio-assays and, more particularly, but not exclusively, to magnetic beads used in fluorescence-based bio-assays.

Bio-assays of biological samples are often performed by exposing the sample to a capture surface, coated with a ligand (for example, a capture antibody) that binds specifically to an analyte that may be found in the sample. Although the analyte may be referred to herein as an "analyte molecule" or "target molecule," it should be understood that what the ligand binds to may be a small particle that comprises more than one molecule, and references herein to an analyte, an analyte molecule, or a target molecule also include that case. Fluorescent reporter molecules (for example, fluorescently labeled antibodies) that bind specifically to the analyte are also added to the sample, and they indirectly bind to the capture surface through the analyte, when the analyte binds to the capture surface. It should be understood that particles comprising more than one molecule, such as fluorescent quantum dots, may also play the role of fluorescent reporter molecules, and are optionally included when fluorescent reporter molecules are referred to herein. The concentration of the analyte in the sample can be determined by measuring the intensity of fluorescence of the reporter molecules after exposing the capture surface to the sample, and after washing away any loose molecules of analyte and reporter molecules from the capture surface. It is common to use the surfaces of a large number of microscopic polymer beads, also referred to as particles, as the capture surface.

U.S. Patent No. 9,187,691 to Weng et al. describes coated magnetic polymer particles which exhibit low autofluorescence, processes for the preparation of such particles, and their uses in various medical and biochemical fields. Such particles may have utility in a variety of assays, in particular assays in which fluorescence is detected. The use of fluorescent entities as labels in both non-magnetic and magnetic polymer beads is described. Using magnetic beads has the advantage of enabling a combination of magnetic sample preparation and detection. They

find that the autofluorescence exhibited by a magnetic polymer particle can be minimized by the avoidance of conjugated delocalized electron systems (other than those found in benzene rings) in the matrix polymer, in the particle coating, and in any surface functionalities. Such conjugated delocalized electron systems had previously been considered essential to allow attraction of iron oxides in the magnetization step and polymer coating to occur successfully. Autofluorescence was measured for a variety of coated magnetic polymer particles prepared by the methods they describe, and compared to autofluorescence measurements of other coated magnetic polymer particles, and commercially available non-magnetic "Certified Blank" calibration particles with low autofluorescence, using excitation light and emitted light in various wavelength ranges.

U.S. patent 8,062,894 to Schwartz describes methods of producing microbead populations that mimic the fluorescence intensity profile distribution of fluorescent biological cells so that they may be used as a standard for flow cytometry. One method starts with a uniform population of microbeads which are fluorescently labeled with fluorescein. If different portions of the population are photobleached in a precise and predetermined manner at specific rates, the resulting intensity profile of the microbead population can be made to mimic the intensity profile of fluorescently labeled biological cells. This process is referred to as precision photobleaching.

U.S. patent 9,134,271 to Ward et al, states that too much light can often photobleach (or excite to non-radiative states) the fluorophores used to generate the signal, in a fluorescence-based bio-assay.

M. Nowakowska et al, "Fluorescence Quenching of Polystyrene by Oxygen," in *European Polymer Journal* **12**, 387-391 (1976), and K. Sienicki et al, "Fluorescence Quenching and Singlet Energy Migration in Polystyrene and its Copolymers," in *Polymer Photochemistry* **7**, 245-259 (1986), describe chemical quenching of fluorescence in polystyrene.

E. W. K. Young et al, "Assessment of Enhanced Autofluorescence and Impact on Cell Microscopy for Microfabricated Thermoplastic Devices," in *Analytical Chemistry* **85**, 44-49 (2013), note that thermoplastics such as polystyrene and cyclo-olefin polymer have become common materials for fabrication of microfluidic cell-based systems, but these thermoplastics are known to exhibit autofluorescence levels that may hinder their utility for cell-based and imaging applications. It is found that the autofluorescence of polystyrene increases after thermal treatment, at the range of pressures and temperatures used for polystyrene bonding. Lower autofluorescence is found if solvent bonding of polystyrene or thermal bonding of cyclo-olefin polymer is used instead of thermal bonding of polystyrene, for fabricating microfluidic systems.

U.S. Patent No. 8,557,604 to Song describes a membrane-based assay device for detecting the presence or quantity of an analyte residing in a test sample. The device utilizes phosphorescence to detect the signals generated by excited phosphorescent labels. The labels may have a long emission lifetime so that background interference from many sources, such as autofluorescence, is practically eliminated during detection.

B. Lu et al, "A study of the autofluorescence of parylene materials for μTAS applications," in *Lab Chip* **10**, 1826-1834 (2010), states that parylene-C has been widely used as a biocompatible material for microfluidics and micro total analysis system (μTAS) applications. However, its autofluorescence can be a major obstacle for parylene-C based devices used in applications requiring sensitive fluorescence detection. Parylene-HT, which exhibits low initial autofluorescence, decreasing autofluorescence behavior under UV excitation and higher UV stability, can be a promising alternative for μTAS applications with fluorescence detection.

U.S. Patent No. 8,988,679 to Natan describes Surface Enhanced Raman Scattering (SERS) nanotags, and states that they can be used in biological and chemical assays as replacements for fluorescent tags. SERS nanotags possess a number of characteristics that make them superior to optical tags based on fluorophores. For example, assays using fluorophore detection are commonly hampered by the presence of autofluorescence and other background effects. Quenching of fluorescent activity is observed when some fluorophores are conjugated to proteins. Finally, irreversible photodegradation resulting from the creation of a triplet or singlet excited state, followed by a non-reversible chemical reaction that permanently eliminates the excited state, puts a severe limitation on the sensitivity of fluorophore detection. By contrast, SERS nanotags cannot be photobleached or photodegraded, and they can be readily resolved from the background.

Loling Song et al, "Photobleaching Kinetics of Fluorescein in Quantitative Fluorescence Microscopy," in *Biophysical Journal* **68**, 2588-2600 (1995), describes an investigation of the photobleaching behavior of free and bound fluorescein, using theoretical analysis, mathematical simulation, and experimental means. Both the theoretical simulation and experimental data show that photobleaching of bound fluorescein in microscopy is, in general, not a single-exponential process.

K. Sienicki and C. Bojarski, "Fluorescence Quenching and Singlet Energy Migration in Polystyrene and its Copolymers," *Polymer Photochemistry* **7**, 245-259 (1986), describes a study of fluorescence quenching of polystyrene and statistic styrene copolymers by carbon tetrachloride in solution. The results of the study have been interpreted on the basis of a model

considering the existence of isolated chromophore groups, whose excitation does not participate in the energy migration process.

Khalid E. Al Ani and Azza M. Suleiman, "Substituent effect on the fluorescence quenching of polystyrene derivatives by polymeric plasticizers," *Journal of Photochemistry and Photobiology A: Chemistry* **188**, 177-184 (2007), and J.R. MacCallum and A.L. Rudkin, "Quenching of fluorescence of solutions of polystyrene and copolymers with methyl methacrylate," *European Polymer Journal* **17**, 953-956 (1981), also describe chemical quenching of fluorescence in polystyrene.

Xiaoshan Zhu et al, "Magnetic bead based assay for C-reactive protein using quantum-dot fluorescence labeling and immunoaffinity separation," *Analyst* **135**, 381-389 (2010), describes a magnetic bead based assay for C-reactive protein (CRP) in human serum, in which magnetic microbeads conjugated with monoclonal anti-CRP are used to capture CRP, then incubated with biotinylated monoclonal anti-CRP and streptavidin coated quantum dots (QDs) to form sandwich immunocomplexes. Immunoaffinity separation buffer is then used to release the QDs from the magnetic bead surface, and after separation from the magnetic beads, the fluorescence signal from the released QDs is measured to quantify CRP in the serum. By separating the QDs from the magnetic beads before measuring the fluorescence of the QDs, the assay avoids interference of magnetic bead autofluorescence in signal transduction, enhancing the detection resolution of the assay. A similar assay method is described by Chloe Kim and Peter C. Searson, "Magnetic bead-quantum dot assay for detection of a biomarker for traumatic brain injury," *Nanoscale* **7**, 17820-17826 (2015), to detect brain-specific biomarkers in the blood.

Amit Agrawal et al, "Single-Bead Immunoassays Using Magnetic Microparticles and Spectral-Shifting Quantum Dots" *J. Agric. Food Chem.* **55**, 3778-3782 (2007), describes a single-bead immunoassay method based on the combined use of magnetic microparticles (MMPs) for target capturing/enrichment and antibody-conjugated quantum dots (QDs) for fluorescence detection. A major problem in using magnetic beads for fluorescence immunoassays is that the bead's autofluorescence strongly interferes with the target detection signal. This spectral overlapping problem can be overcome by using semiconductor QDs as a new class of spectral-shifting labels. By shifting the QD emission signals away from the bead autofluorescence, it is possible to detect biomolecular antigens such as tumor necrosis factor at femtomolar (10^{-15} M) concentrations when the target molecules are captured and enriched on the magnetic bead surface.

Robert C. Leif et al, "Calibration beads containing luminescent lanthanide ion complexes," *Journal of Biomedical Optics* **14**, 024022 (2009), describes 0.5 µm, 3 µm, and 5 µm diameter beads containing a luminescent europium complex, for use in spectral calibration of microscopes equipped with a spectrograph, as test particles for time-delayed luminescence flow cytometers, and possibly as labels for macromolecules and cells. A study was made of the rate of UV irradiation-induced photobleaching of the luminescence of the beads. It was found that, for the 0.5 µm and 5µm diameter beads, the exposure time to UV necessary to obtain an image of the beads was much less than the exposure time to UV needed to bleach the luminescence intensity to half its initial value, so these beads were considered suitable for use.

Additional background art includes E. Abuin et al, "Polystyrene Fluorescence Spectra: Molecular Weight Dependence in Different Solvents," *European Polymer Journal* **20**, 105-107 (1984); Norio Hasegawa, "Quantitative Comparison of the Autofluorescence of Bacteria and Polystyrene Microspheres under Violet Wavelength Excitation for Verification of Fluorescence-based Bioaerosol Detector Results," *Biocontrol Science* **18**, 211-215 (2013); and published patent application US 2014/0077100 to Hasegawa.

SUMMARY OF THE INVENTION

An aspect of some embodiments of the invention concerns capture surfaces, such as polymer beads, used for fluorescence-based bio-assays, with a very low autofluorescence, and a method for preparing the capture surfaces using photobleaching to reduce their autofluorescence.

There is thus provided, in accordance with an exemplary embodiment of the invention, a method of photobleaching polymer capture surface elements suitable to use for providing a capture surface in a fluorescent bio-assay, the method comprising:

a) providing one or more of the capture surface elements having an autofluorescence excited by light in a peak range of excitation wavelengths, producing light predominantly in a peak range of emission wavelengths that may depend on the excitation wavelength; and

b) exposing the capture surface elements to light from a light source used for bleaching, with suitable wavelength distribution and sufficient intensity and duration so that a level of the autofluorescence, for at least one excitation wavelength within the peak range of excitation wavelengths and at least one emission wavelength within the peak range of emission wavelengths for that excitation wavelength, is reduced by at least a factor of 2.

Optionally, the one or more polymer capture surface elements comprise a plurality of polymer beads, between 0.3 and 10 micrometers in diameter, and the capture surface comprises outer surfaces of the polymer beads.

Optionally, exposing the capture surface elements to light from the light source used for bleaching comprises causing the beads to move along a micro-fluidic channel illuminated by the light source used for bleaching.

Optionally, the beads comprise one or more types of internally embedded fluorescent reporter molecules of sufficiently different peak range of excitation wavelengths from the autofluorescence of the beads, sufficiently low susceptibility to photobleaching, or both, so that said exposing the capture surface elements to light from the light source used for bleaching reduces a level of fluorescence of the internally embedded fluorescent reporter molecules by less than 10%, for at least one excitation wavelength within a peak range of excitation wavelengths for said fluorescence of the internally embedded fluorescent reporter molecules, for at least one emission wavelength within a peak range of emission wavelengths for that excitation wavelength.

Optionally, the method also comprises chemically bleaching the autofluorescence of the polymer beads.

Optionally, the light source used for bleaching comprises a laser.

Alternatively, the light source used for bleaching comprises a broadband light source.

A method according to claim 1, wherein the broadband light source is a flash lamp.

In an embodiment of the invention, the autofluorescence is sufficiently bleached so that, if the capture surface elements are exposed to 10^5 W/m^2 of light at peak excitation wavelength for the autofluorescence for at least 15 minutes, after the bleaching, then the level of autofluorescence of the capture surface elements will be reduced by less than a factor of 2, or there will be no measurable reduction in level of autofluorescence of the capture surface elements, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength.

Optionally, the light source used for bleaching produces an intensity of light at the capture surface of at least 500 W/m^2.

Optionally, the light source used for bleaching produces said intensity of light at the capture surface for at least 1 second.

Optionally, bleaching comprises exposing the capture surface elements to a light intensity integrated over time of at least 10^7 joules per square meter.

Optionally, bleaching the autofluorescence of the one of more capture surface elements also comprises exposing the capture surface elements to a chemical bleaching agent.

In an embodiment of the invention, the method also comprises performing a bio-assay for detecting an analyte in a biological sample, after exposing the capture surface elements to light from the light source used for bleaching, by:

a) exposing a capture surface of the capture surface elements to the biological sample, capturing molecules of the analyte;

b) binding the analyte molecules to fluorescent reporting molecules;

c) exposing the capture surface to excitation light from an excitation light source, the same as or different from the light source used for bleaching, after exposing the capture surface to the biological sample and binding the analyte molecules to the reporting molecules, the excitation light having a wavelength distribution that would excite the autofluorescence of the capture surface elements;

d) measuring a fluorescence signal emitted by the reporting molecules bound to the captured analyte molecules, in response to the excitation light, over a band of emission wavelengths; and

e) determining a presence of the analyte in the sample, a concentration of the analyte in the sample, or both, from the measured fluorescence signal.

In an embodiment of the invention, the method also comprises performing a bio-assay for detecting an analyte in a biological sample, after exposing the capture surface elements to light from the light source used for bleaching, by:

a) performing a preparatory procedure with the biological sample that results in reporter molecules being bound directly or indirectly to a capture surface of the capture surface elements, such that, when excited by excitation light of suitable wavelength range, the reporter molecules emit a fluorescent emission light of an intensity that depends on a concentration of the analyte in the biological sample;

b) exposing the capture surface to excitation light from an excitation light source, the same as or different from the light source used for bleaching, after performing the preparatory procedure, the excitation light having a wavelength distribution that would excite the autofluorescence of the capture surface elements;

c) measuring a signal of the fluorescent emission light emitted by the reporting molecules in response to the excitation light, over a band of emission wavelengths; and

d) determining a presence of the analyte in the sample, a concentration of the analyte in the sample, or both, from the measured fluorescence signal;

wherein the excitation light has a wavelength range that would excite the autofluorescence of the capture surface elements to emit light in the band of emission wavelengths.

Optionally, the bio-assay comprises a sandwich assay, the preparatory procedure comprising exposing the capture surface to the biological sample, capturing molecules of the analyte, and binding the analyte molecules to the fluorescent reporter molecules, resulting in a concentration of the fluorescent reporter molecules bound indirectly to the capture surface depending on the concentration of analyte molecules in the biological sample.

Optionally, the method also comprises attaching to the capture surface a ligand that binds specifically to the analyte, and capturing molecules of the analyte comprises binding the molecules of the analyte to the ligand that is attached to the capture surface.

Optionally, the ligand is attached to the capture surface before exposing the capture surface elements to light from the light source used for bleaching,

Alternatively, the ligand is attached to the capture surface after exposing the capture surface elements to light from the light source used for bleaching,

Optionally, the bio-assay comprises a fluorescence resonance energy transfer (FRET) bio-assay.

Optionally, a level of the analyte in the sample is such that the fluorescence signal emitted by the reporting molecules has higher power than an autofluorescence background from the capture surface elements, but lower power than the autofluorescence background would have been if the autofluorescence of the capture surface elements had not been bleached.

Optionally, the excitation light source is the light source used for bleaching.

Optionally, exposing the capture surface to the excitation light is done with the capture surface elements in a same location as exposing them to light from the light source used for bleaching.

Optionally, the excitation light source comprises a laser.

Optionally, measuring the fluorescence signal comprises integrating over a time longer than 1 second.

Optionally, measuring the fluorescence signal comprises integrating over a time longer than 5 seconds.

In an embodiment of the invention, performing the bio-assay comprises causing the beads to undergo an oscillatory motion relative to the excitation light while measuring the fluorescence signal, alternately illuminating the beads with higher and lower intensities of the excitation light, and measuring the fluorescence signal comprises measuring a time-varying component of the emitted fluorescence power that is synchronous with a time variation of the intensity of the excitation light that the beads are exposed to, due to the relative motion of the excitation light and the beads.

Optionally, the capture surface elements comprise a plurality of paramagnetic or superparamagnetic beads, and causing the beads to undergo an oscillatory motion relative to the excitation light comprises applying a magnetic field with time varying direction of gradient, that causes an oscillatory motion of the beads.

Alternatively or additionally, causing the beads to undergo an oscillatory motion relative to the excitation light comprises oscillating a position of a beam of the excitation light so that it alternately illuminates the beads at higher and lower intensities.

Optionally, exposing the capture surface to excitation light and measuring the fluorescence signal is done when the capture surface is immersed in water or a water solution, and the fluorescence signal has power less than a background signal from one or both of Raman scattering from the water or water solution, and fluorescence from unbound fluorescent molecules in the water or water solution.

Optionally, the reporter molecules have a peak excitation wavelength in the peak range of excitation wavelengths of the autofluorescence, and a peak emission wavelength in the peak range of emission wavelengths of the autofluorescence for a peak excitation wavelength of the autofluorescence.

In an embodiment of the invention, one or more capture surface elements are incorporated as one or more structural parts that provide a capture surface in a bio-assay system for performing the bio-assay, the bio-assay system configured so that the capture surface can be exposed to the sample and bind molecules of the analyte present in the sample, and so that the fluorescence signal can be produced from the capture surface and measured to determine a presence or concentration of the analyte in the sample.

Optionally, the autofluorescence of at least one capture surface element is bleached before the capture surface element is incorporated into the bio-assay system.

Alternatively or additionally, the autofluorescence of at least one capture surface element is bleached after the capture surface element is incorporated into the bio-assay system.

Optionally, the method also comprises manufacturing the bio-assay system, and incorporating the one or more capture surface elements into the bio-assay system is at least a part of the manufacturing.

Optionally, measuring the fluorescent signal comprises measuring light most of which has wavelength greater than a wavelength of the excitation light, and less than 700 nm.

Optionally, when the one or more capture surface elements are exposed to the light from the light source used for bleaching, they are comprised in one or more structural parts of a bio-

assay system for performing a bio-assay that uses the capture surface elements for providing a capture surface.

Optionally, the polymer capture surface elements comprise one or more of polystyrene, latex, methyl methacrylate, ethylene glycol dimethacrylate, and methacrylic acid.

Optionally, the method also comprises storing the capture surface elements for at least three days after bleaching the autofluorescence of the capture surface elements, substantially maintaining the reduced autofluorescence.

In an embodiment of the invention, the capture surface elements comprise a plurality of polymer beads, and exposing the capture surface elements comprises:

a) exposing a portion of the beads in the container to light of suitable wavelength and intensity for photobleaching the beads, while exposing the other beads in the container to a lower intensity of light of suitable wavelength for photobleaching, or to no light of suitable wavelength for photobleaching;

b) mixing at least some of the beads in the container that were exposed to the intensity of light suitable for photobleaching, with at least some of the beads that were exposed to a lower intensity of light or to no light, during or after exposing the portion of the beads to the light; and

c) exposing at least some of the mixed beads in the container to the light of suitable wavelength and intensity for photobleaching, including at least some of the beads that were exposed to a lower intensity of light or to no light before the mixing.

Optionally, exposing a portion of the beads to the light of an intensity suitable for photobleaching comprises exposing less than half of the beads to said light, the method also including repeating mixing the beads and exposing at least some of the mixed beads to the light of an intensity suitable for photobleaching, enough times, using enough of the beads for the mixing, doing the mixing thoroughly enough, and with a long enough total exposure time of the beads to the light, so that more than half of the beads are bleached to a level of autofluorescence at least a factor of 2 less than their level of autofluorescence before the photobleaching, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths.

Optionally, the beads are magnetic beads, and mixing at least some of the beads comprises introducing a strong enough magnetic field to the beads to magnetize them and cause them to aggregate into a clump, decreasing the magnet field to cause the clump to disperse, and again introducing a strong enough magnetic field to cause the beads to aggregate into a clump.

There is further provided, in accordance with an exemplary embodiment of the invention, a polymer bead product, suitable for use in bio-assays, with a measurable level of autofluorescence having a peak range of excitation wavelengths and a peak range of emission wavelengths for each excitation wavelength, the level of autofluorescence being reduced by photobleaching, on average, by at least a factor of 2 below what it would be for beads manufactured in a same way without the photobleaching, for at least one excitation wavelength in the peak range of excitation wavelengths and at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength.

Optionally, the level of autofluorescence is sufficiently reduced by the photobleaching so that exposure of the bead product to 10^5 W/m^2 of light at a peak excitation wavelength of the autofluorescence, directed for at least 15 minutes, results in a reduction of less than a factor of 2 in level of autofluorescence of the bead product, or results in no measurable reduction in level of autofluorescence of the bead product, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength.

Optionally, for the bead product, 532 nm is within the peak range of excitation wavelengths and 575 nm is within the peak range of emission wavelengths for an excitation wavelength of 532 nm, and the bead product has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 50% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

Optionally, the bead product has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 30% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

Optionally, the bead product has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 20% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

Optionally, for the bead product 532 nm is within the peak range of excitation wavelengths and 575 nm is within the peak range of emission wavelengths for an excitation wavelength of 532 nm, and the bead product has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 10 times a level of

Raman scattering from a column of pure water 8 mm long at that excitation wavelength and emission wavelength.

Optionally, the bead product has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 6 times a level of Raman scattering from a column of pure water 8 mm long at that excitation wavelength and emission wavelength.

Optionally, the bead product has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 4 times a level of Raman scattering from a column of pure water 8 mm long at that excitation wavelength and emission wavelength.

Optionally, the method produces a polymer bead product according to an exemplary embodiment of the invention.

Optionally, the bead product comprises paramagnetic or superparamagnetic beads.

Optionally, the method comprises using the bead product for the paramagnetic or superparamagnetic beads.

Optionally, the bead product is suitable for binding to analytes in bio-assays.

There if further provided, according to an exemplary embodiment of the invention, a heterogeneous bio-assay system for detecting an analyte in a biological sample, the system comprising capture surface elements comprising beads of a bead product according to an exemplary embodiment of the invention.

Optionally, the capture surface elements comprise a plurality of beads of a bead product according to an exemplary embodiment of the invention, and the capture surface comprises outer surfaces of the beads.

Optionally, the bead product is packaged in a package containing at least 1000 beads.

There is further provided, according to an exemplary embodiment of the invention, a polymer bead product, suitable for use in bio-assays, with a measurable level of autofluorescence for which 532 nm is in the peak range of excitation wavelengths and 575 nm is in the peak range of emission wavelengths for an excitation wavelength of 532 nm, wherein the level of autofluorescence, for an excitation wavelength of 532 nm and an emission wavelength of 575 nm, is less than 50% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

There is further provided, according to an exemplary embodiment of the invention, a heterogeneous bio-assay system for detecting an analyte in a biological sample, the system comprising a capture surface element comprising a polymer having an autofluorescence excited

by light in a peak range of excitation wavelengths, producing light predominantly in a peak range of emission wavelengths that may depend on the excitation wavelength, with a capture surface suitable for immobilizing ligand molecules that bind to molecules of the analyte when exposed to the sample, the level of autofluorescence of the capture surface element being reduced by photobleaching, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength, by at least a factor of 2 below what it would be for an unbleached capture surface element manufactured in a same way.

Optionally, the system also comprises:

a) an optical sub-system for measuring a concentration of bound analyte molecules on the capture surface by exciting and detecting fluorescent emission of reporter molecules whose emission power depends on how many analyte molecules are bound to the ligand molecules; and

b) a fluidic sub-system for exposing the capture surface, with the ligand molecules immobilized on it, to the biological sample.

Optionally, the capture surface element has its level of autofluorescence sufficiently reduced so that exposure of the capture surface element to 10^5 W/m^2 of light at peak excitation wavelength for the autofluorescence for at least 15 minutes, results in a reduction in level of autofluorescence of less than a factor of 2, or results in no measurable reduction in level of autofluorescence, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths.

There is further provided, according to an exemplary embodiment of the invention, a method of preparing one or more capture surface elements suitable to use for providing a capture surface in a fluorescent bio-assay, the method comprising:

a) providing the one or more capture surface elements of known composition, exhibiting autofluorescence;

b) measuring a level of autofluorescence of the capture surface elements, using an excitation light source with a distribution of one or more excitation wavelengths, and an optical detection sub-system with a distribution of sensitivity to emission wavelength;

c) determining from the measured level of autofluorescence, from the distribution of excitation wavelengths, from the distribution of sensitivity to emission wavelength, and from the known composition, whether and to what extent the autofluorescence of the capture surface elements is already bleached; and

d) photobleaching the autofluorescence of the capture surface elements if they are not already bleached by more than a predefined amount.

There is further provided, according to an exemplary embodiment of the invention, a system for bleaching bio-assay beads and using them to perform a bio-assay, comprising:

a) a fluidics subsystem comprising a container, the fluids subsystem configured to expose a biological sample to a quantity of bio-assay beads, in the container, that bind molecules of an analyte if they are found in the sample, to expose the beads, with any analyte that is bound to them, to fluorescent reporter molecules that bind to the analyte, and to remove any unbound reporter molecules and analyte molecules from the container;

b) an excitation light source that produces light of at least one wavelength suitable for exciting fluorescence in the reporter molecules;

c) an excitation optics subsystem configured to illuminate the beads in the container with light from the light source at an intensity suitable for exciting the reporter molecules and measuring their concentration during a measuring time, but low enough not to significantly bleach the reporter molecules during the measuring time;

d) a detection optical subsystem configured to measure an intensity of fluorescent emission light from any reporter molecules bound to the beads when the beads are illuminated by the excitation optics subsystem;

e) a photobleaching light source, the same as or different from the excitation light source, that produces light of at least one wavelength suitable for photobleaching the beads; and

f) a photobleaching optics subsystem, the same as or different from the excitation optics subsystem, configured to illuminate the beads in the container with light from the photobleaching light source at an intensity suitable for photobleaching the beads.

Optionally, the photobleaching light source is the same as the excitation light source, and the photobleaching optics subsystem is the same as the excitation optics subsystem.

There is further provided, according to an exemplary embodiment of the invention, a system for photobleaching bio-assay beads, comprising:

a) a container suitable for holding at least a minimum quantity of bio-assay beads;

b) a light source that produces light of at least one wavelength suitable for photobleaching the bio-assay beads;

c) an optical subsystem configured to illuminate a portion, but not all, of the minimum quantity of bio-assay beads in the container, with light from the light source, at an intensity suitable for photobleaching the beads, at a same time; and

d) a mixing subsystem configured to mix the minimum quantity of beads in the container, such that, after the mixing, the optical subsystem illuminates a different portion of the minimum quantity of bio-assay beads, than was illuminated before the mixing, with light from the light source at the intensity suitable for photobleaching the beads.

Optionally, the system is suitable for photobleaching magnetic bio-assay beads, and the mixing subsystem comprises a magnet that mixes the beads by causing them to move.

Optionally, the system is suitable for photobleaching magnetic bio-assay beads, and the mixing subsystem comprises:

a) a motor configured to bring the magnet successively closer to and further from a portion of the container, wherein the magnetic field and field gradient produced by the magnet at the portion of the container are sufficiently strong to magnetically aggregate the beads and attract them to the portion of the container, when the magnet is brought closer to the portion of the container, but not strong enough to aggregate or attract the beads to the portion of the container when the magnet is further from the portion of the container; and

b) a motor controller configured to control the motor to alternately bring the magnet closer to a portion of the container that the optical subsystem illuminates, for a long enough time to aggregate the beads and attract them there, and to bring the magnet further from the portion of the container for a long enough time so the beads are at least partially dispersed away from the portion of the container.

Alternatively or additionally, the mixing subsystem comprises a shaker that mixes the beads by shaking the container.

There is further provided, in accordance with an exemplary embodiment of the invention, a method of photobleaching polymer capture surface elements suitable to use for providing a capture surface in a chemiluminescent bio-assay that uses a chemiluminescence excitation agent, the method comprising:

a) providing one or more of the capture surface elements having a background chemiluminescence excited by the chemiluminescence excitation agent; and

b) exposing the capture surface elements to light from a light source used for bleaching, with suitable wavelength distribution and sufficient intensity and duration so that a level of the background chemiluminescence, for at least one emission wavelength within a peak range of emission wavelengths for that chemiluminescence excitation agent, is reduced by at least a factor of 2.

Optionally, the method also comprises performing a bio-assay for detecting an analyte in a biological sample, after exposing the capture surface elements to light from the light source used for bleaching, by:

a) performing a preparatory procedure with the biological sample that results in reporter molecules being bound directly or indirectly to a capture surface of the capture surface elements, such that, when excited by a chemiluminescence excitation agent, the reporter molecules emit a chemiluminescent emission light of an intensity that depends on a concentration of the analyte in the sample;

b) exposing the capture surface to the chemiluminescence excitation agent that would excite the reporting molecules, after performing the preparatory procedure;

c) measuring a signal of the chemiluminescence emission light emitted by the reporting molecules, in response to the chemiluminescence agent, over a band of emission wavelengths; and

d) determining a presence of the analyte in the sample, a concentration of the analyte in the sample, or both, from the measured chemiluminescence signal;

wherein the chemiluminescence excitation agent would excite the background chemiluminescence of the capture surface elements to emit light in the band of emission wavelengths.

There is further provided, in accordance with an exemplary embodiment of the invention, a method of photobleaching polymer capture surface elements suitable to use for providing a capture surface in an electro-chemiluminescent bio-assay, the method comprising:

a) providing one or more of the capture surface elements having a background electro-chemiluminescence; and

b) exposing the capture surface elements to light from a light source used for bleaching, with suitable wavelength distribution and sufficient intensity and duration so that a level of the background electro-chemiluminescence, for at least one emission wavelength within a peak range of emission wavelengths for the background electro-chemiluminescence, is reduced by at least a factor of 2.

Optionally, the method also comprises performing a bio-assay for detecting an analyte in a biological sample, after exposing the capture surface elements to light from the light source used for bleaching, by:

a) performing a preparatory procedure with the biological sample that results in reporter molecules being bound directly or indirectly to a capture surface of the capture surface elements, such that, when excited by an electric current, the reporter molecules emit an electro-

chemiluminescent emission light of an intensity that depends on a concentration of the analyte in the sample;

c) exposing the capture surface to an electric current sufficient to excite the reporting molecules to a detectable level of electro-chemiluminescent emission, after performing the preparatory procedure;

d) measuring a signal of the electro-chemiluminescence emission light emitted by the reporting molecules in response to the electric current, over a band of emission wavelengths; and

e) determining a presence of the analyte in the sample, a concentration of the analyte in the sample, or both, from the measured electro-chemiluminescence signal;

wherein the electric current would excite the background electro-chemiluminescence of the capture surface elements to emit light in the band of emission wavelengths.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control the interpretation of terms. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 schematically shows a system for photobleaching the autofluorescence of polymer beads used in fluorescence-based bio-assays, and for the performing a bio-assay with the photobleached beads, according to an exemplary embodiment of the invention;

FIG. 2 schematically shows a system for photobleaching the autofluorescence of magnetic polymer beads used in fluorescence-based bio-assays, and for performing a bio-assay with the photobleached magnetic beads, according to an exemplary embodiment of the invention;

FIG. 3 is a flowchart for a method of photobleaching bio-assay beads using the system of FIG. 1 or FIG. 2;

FIG. 4 is a bar graph showing the autofluorescence before and after photobleaching several different kinds of bio-assay beads, magnetic and nonmagnetic, using the method of FIG. 3;

FIG. 5 is a flowchart for performing a fluorescence-based bio-assay, for example with photobleached magnetic beads using the system of FIG. 2;

FIG. 6 is a graph showing bio-assay signal strength vs. concentration of analyte, in bio-assays performed according to the method of FIG. 5, with and without photobleaching the beads, showing the increased sensitivity and signal to noise ratio that can be achieved with photobleached beads;

FIG. 7 shows a bio-assay system similar to the system of FIG. 1, but using a single flat capture surface element made of an autofluorescent polymer, instead of autofluorescent polymer beads, according to an exemplary embodiment of the invention;

FIG. 8 schematically shows a system for mixing and photobleaching a relatively large quantity of magnetic beads in advance, before using the beads to perform a bio-assay, for example in an apparatus such as the system in FIG. 2, using a method such as the method shown in FIG. 5, according to an exemplary embodiment of the invention;

FIGS. 9A-9D schematically show a time sequence of the system shown in FIG. 8, illustrating how it operates to mix and photobleach magnetic beads, according to an exemplary embodiment of the invention; and

FIG. 10 schematically shows a system for photobleaching beads as they pass, one at a time, though a microfluidic channel, according to an exemplary embodiment of the invention.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to capture surface elements used in fluorescence-based bio-assays and, more particularly, but not exclusively, to magnetic beads and other polymer beads used in fluorescence-based bio-assays.

An aspect of some embodiments of the invention concerns a method of preparing polymer bio-assay capture surface elements, such as polymer beads, with reduced autofluorescence, by starting with a capture surface element that exhibits autofluorescence, and treating the capture surface element to bleach the autofluorescence, for example by photobleaching the capture surface element. Using a capture surface element with lower autofluorescence, in a fluorescence-based bio-assay, has the potential advantage that the

fluorescent signal from the bio-assay may be less subject to a background signal from the autofluorescence of the capture surface, allowing the assay to be more sensitive.

Definitions of Autofluorescence and Bleaching

As used herein, autofluorescence refers to fluorescence that is intrinsically emitted by the capture surface element, independent of the amount of an analyte that is being assayed. In particular, autofluorescence does not include the fluorescence of fluorescent reporter molecules used in the bio-assay to measure the quantity of the analyte present. Typically, autofluorescence is intrinsic to the material that the capture surface element is made out of, for example the autofluorescence of polystyrene and other polymers that bio-assay beads are made of. However, the level of autofluorescence can depend on what analyte-specific ligand molecules are bound to the capture surface element, possibly due to absorption of some of the autofluorescent emission light by the ligand molecules. For example, the inventors have found that the autofluorescence of M280 beads is different if the beads are coated with protein-A than if they are coated with avidin.

As used herein, autofluorescence does not include fluorescence due to fluorescent dyes, such as fluorescein, and other materials that are sometimes added to polymer beads for fluorescent labeling, but only the natural fluorescence of the unlabeled polymer, possibly as modified by analyte-specific ligand molecules bound to the capture surface. The natural fluorescence of polymers such as polystyrene is much weaker than the fluorescence of fluorescent labeling materials, which have a much higher cross-section for absorption of excitation light, and consequently are much more easily photobleached.

As used herein, bleaching autofluorescence means causing a permanent or long-term reduction in autofluorescence, by causing an irreversible chemical change in the material, for example induced by exposure to light or to a chemical agent. The autofluorescence continues to stay at a reduced level even after the exposure to the light or to the chemical agent has ended, for example for hours, days, or a longer time, though autofluorescence may slowly recover, partially or completely, over a period of time.

Characteristics of Bleached Polymers

A polymer of known composition, including surface treatment, will generally exhibit a particular level of autofluorescence before any bleaching is performed to reduce the autofluorescence, and it will generally have a minimum level of autofluorescence that can be achieved by bleaching, at least for a given method of bleaching such as photobleaching. It is

generally possible to tell whether a polymer capture surface element of known composition has been bleached, and to what extent, by measuring its autofluorescence, and seeing whether it is below the level expected for that composition of polymer before bleaching, and how close it is to the minimum level expected for that composition when it is fully bleached.

Another way to tell whether a polymer capture surface element has been almost fully bleached or not, even if its composition is not known, is to expose it to light of an intensity, duration, and wavelength, that would reduce the autofluorescence by a large factor, for example much greater than 2, if it hadn't been bleached. If the capture surface element has previously been almost fully bleached, then this attempt to bleach it again may reduce its autofluorescence by a much smaller factor, for example less than a factor of 2. For example, in the case of some polymers such as polystyrene, light of intensity at least 10^5 W/m^2, at the peak excitation wavelength or at a wavelength in a peak range of excitation wavelengths for that autofluorescence, for example 532 nm in the case of polystyrene, for at least 15 minutes, reduces the autofluorescence by a large factor, in tests done by the inventors. In these tests, the autofluorescence is excited by light at the same wavelength that was used for bleaching, for example, and the autofluorescent emission is measured at a wavelength in a peak range of emission wavelengths for that autofluorescence, for example 560 nm or 575 nm in the case of polystyrene. Alternatively, the capture surface element is exposed to a higher intensity light for a shorter time, for example from a pulsed light source such as a pulsed laser, or a lower intensity light for a longer time, that would reduce the autofluorescence by a large factor, for example much greater than 2, if it hadn't been bleached. If the total energy per area that the capture surface is exposed to is at least 10^8 J/m^2, about the same energy per area as 10^5 W/m^2 for 15 minutes, or even at least 10^7 J/m^2, then a capture surface element that has never been bleached might be expected to have its autofluorescence reduced by a large factor, at least for intensities over some range that includes the range the inventors have tested, between 5×10^2 and 3×10^5 W/m^2. But if the capture surface element had already been almost fully bleached, even if the autofluorescence has recovered somewhat since bleaching, then this attempt to further bleach the capture surface element, by either a steady state or pulsed light source, will generally only reduce its level of autofluorescence by a small amount, for example by less than a factor of 2.

For some materials, a higher intensity of light and/or a longer exposure time may be needed to fully or substantially bleach the unbleached capture surface element, for example an intensity 2, 5, 10, or 20 times greater than 10^5 W/m^2, and/or an exposure time 2, 5, 10, or 20 times greater than 15 minutes, and for other materials a lower intensity and/or a shorter exposure time may be sufficient to fully or substantially bleach the unbleached capture surface element,

for example 2, 5, 10 or 20 times less than 10^5 W/m^2, and/or 2, 5, 10 or 20 times shorter than 15 minutes. In all these cases, the same intensity and exposure time may be expected to reduce the level of autofluorescence by only a small amount, for example by less than a factor of 2, if the capture surface element has already been fully or substantially photobleached.

Furthermore, if the capture surface element has previously been almost fully bleached, then the initial rate of bleaching with a steady state light source, expressed as percent decrease in autofluorescence per second, has been observed by the inventors to be less than for a capture surface element that has never been bleached, for the same intensity of light. However, there is some evidence that the initial rate of bleaching does not go down as much as the total relative change in autofluorescence, for a capture surface element that has already been bleached, so the saturation time may be less for a capture surface element that has been previously bleached, than for a capture surface element that has never been bleached.

Bio-assay beads and other capture surface elements

It is common in bio-assays to use small polymer beads, a few micrometers or a few hundreds of nanometers in diameter, as capture surface elements. In the description herein, the capture surface elements will often be referred to as beads. But it should be understood that the invention can also be used with other capture surface elements, for example with a single capture surface that is part of a cell used in a microfluidics system, made of a polymer or another material that exhibits autofluorescence.

In some embodiments of the invention, the bleaching comprises photobleaching. Alternatively or additionally, chemical bleaching is used. A combination of photobleaching and chemical bleaching may be more effective at reducing autofluorescence in some materials than either photobleaching or chemical bleaching alone. In some embodiments, magnetic beads are used. Alternatively, non-magnetic beads are used. Some magnetic beads have relatively high autofluorescence, because of the way they are prepared, and the method may be particularly useful for those beads.

Performing bio-assays with bleached beads

In some embodiments of the invention, the method also includes performing a fluorescence-based bio-assay with the bleached beads. Optionally, the bio-assay is performed using magnetic beads, according to the magnetic modulation biosensing (MMB) methods described in Danielli, A., Arie, A., Porat, N. and Ehrlich, M., "Detection of fluorescent-labeled probes at subpicomolar concentrations by magnetic modulation," *Optics Express* **16**, 19253-

19259 (2008); in Danielli, A., Porat, N., Arie, A. and Ehrlich, M., "Rapid homogenous detection of the Ibaraki virus NS3 cDNA at picomolar concentrations by magnetic modulation," *Biosensors & Bioelectronics* **25**, 858-863, doi:10.1016/j.bios.2009.08.047 (2009); and in Verbarg, J., Hadass, O., Olivo, P. D. and Danielli, A., "High sensitivity detection of a protein biomarker Interleukin-8 utilizing a magnetic modulation biosensing system," *Sensor and Actuators B: Chemical* **241**, 614-618 (2017). In this type of bio-assay, a magnetic field aggregates the beads, and varying the gradient of the magnetic field over time causes the position of the aggregated beads to vary in time, passing into and out of the beam of the excitation light, for example periodically. At the same time, a component of the bio-assay signal, that varies in time synchronously with the magnetic field gradient, is measured. This type of bio-assay provides particularly high sensitivity, because it makes it possible to distinguish the fluorescent signal emitted by the beads, which is modulated over time, from a background signal coming from the surrounding solution, for example from Raman scattering from the surrounding water molecules, and from any unbound fluorescent molecules in the solution, which is not modulated over time. To take full advantage of the sensitivity to very small quantities of analyte that this assay method allows, it is particularly useful to use beads with very low autofluorescence, and thereby ensure that the signal is not masked by autofluorescence of the beads. In some embodiments, the fluorescent signal from reporter molecules in the bio-assay is integrated over a time of at least 1 second, or 2 seconds, or 5 seconds, or 10 seconds, or 20 seconds, or 50 seconds. In some embodiments, the fluorescent signal from the bio-assay is weaker than 100 times, 50 times, 20 times, 10 times, 5 times, or 2 times the Raman scattering signal, or weaker than the Raman scattering signal, or 2 times, 5 times, 10 times, 20 times weaker than the Raman scattering signal, from the surrounding water, for example for a column of water 8 mm long in the direction of the light beam, for at least some wavelengths, for example at a wavelength where the Raman scattering signal is strongest, or at a wavelength where the fluorescent signal is strongest. In some embodiments, the fluorescent emission of the bio-assay is in a wavelength range that significantly overlaps the wavelength range of the autofluorescence, and the bleaching significantly reduces the autofluorescence in that wavelength range.

Alternatively, instead of performing the bio-assay as an MMB bio-assay by magnetically aggregating the beads and using a time-varying magnetic field gradient to move them into and out of a beam of excitation light, the bio-assay is performed using magnetic beads according to the methods described by commercially available systems, such as the Luminex-100, Luminex-200, MagPix (made by Luminex Corp.), BioPlex-200 (sold by Bio-Rad), and SIMOA (made by Quanterix), or by other systems that use a capture surface and fluorescent reporter molecules.

In some embodiments of the invention, bleaching of the beads is done using an apparatus that is also used for performing the bio-assay. For example the beads are photobleached using a light source, for example a continuous and/or pulsed laser, and/or other optical components that are also used for excitation of the beads during the bio-assay. In other embodiments of the invention, in particular if the beads are bleached well in advance of being used in any bio-assay, and the bleached beads are sold to end users or stored for later use, a different apparatus is used for bleaching the beads. Using a different apparatus for bleaching the beads has the potential advantage that in the apparatus used for a bio-assay, the beads may be confined to a very small container in order to perform the bio-assay with a very small sample, and it may be difficult to remove the beads from such a small container, for later use.

In some embodiments of the invention, fluorescent reporter molecules may be attached directly to the beads, for example in order to test the sensitivity of the optical system to low levels of fluorescence. In this case, the bleaching is optionally done before the reporter molecules are attached to the beads. This has the potential advantage that the bleaching process will not also reduce the fluorescent emission of the reporter molecules. Alternatively, the bleaching is done after fluorescent reporter molecules are attached to the beads, but the bleaching is performed in a way that substantially does not reduce the fluorescent emission of the reporter molecules, or reduces it proportionately less than the reduction in autofluorescence of the beads, for example because a wavelength is used for the bleaching that does not excite the reporter molecules, even if there is some overlap in the emission wavelengths of the autofluorescence and the reporter molecules. In some embodiments of the invention, fluorescent reporter molecules are embedded directly inside the beads, for example to label the beads according to the quantity of one or more types of fluorescent reporter molecules that are embedded in them. In this case, the bleaching is done after the fluorescent reporter molecules are embedded in the beads, but here also the bleaching is optionally performed in a way that substantially does not reduce the fluorescent emission of the embedded reporter molecules, or reduces it proportionately less than the reduction in autofluorescence of the beads. For example, the bleaching reduces a level of fluorescence of the embedded fluorescent reporter molecules by less than 10%, or less than 20%, or less than 50%, for a least one excitation wavelength within a peak range of excitation wavelengths for that fluorescence of the embedded fluorescent reporter molecules, for at least one emission wavelength within a peak range of emission wavelengths for that excitation wavelength. This may occur, for example, because the embedded reporter molecules have a molecular structure such that they are less susceptible in general to photobleaching than the autofluorescence of the bead material, and/or because the peak range of

excitation wavelengths for the embedded reporter molecules is sufficiently different from the peak range of excitation wavelengths for the autofluorescence. Tests by the inventors have shown that commercially available bio-assay beads with embedded fluorescent reporter molecules, sold by Luminex and Bio-Rad, can have their autofluorescence greatly reduced by the bleaching methods described here, without appreciably reducing the level of fluorescence of the embedded fluorescent reporter molecules.

Optionally, the bio-assay comprises attaching analyte-specific ligand molecules to the beads, for example the bio-assay comprises a "sandwich" bio-assay in which fluorescent reporter molecules, attached to their own analyte-specific ligand molecules, become attached indirectly to analyte molecules that are attached indirectly to the beads. In this case, the beads are optionally bleached before the analyte-specific ligand molecules are attached to them. These analyte-specific ligands can include any of proteins, oligonucleotides, and antibodies, for example. Bleaching the beads before attaching the ligand molecules has the potential advantage that the functionality of the ligand molecules will not be adversely affected by the bleaching. Alternatively, the beads are bleached after attaching the ligand molecules. Tests by the inventors have shown that the functionality of many types of attached analyte-specific ligand molecules is not adversely affected by bleaching the beads they are attached to, though avidin and streptavidin can be adversely affected.

Bleaching the beads before attaching reporter molecules, or before attaching analyte-specific ligand molecules, also has the potential advantage that even if the reporter molecules or the ligand molecules have to be attached shortly before the assay is performed, for example by the end user of the beads, the bleaching can be done in advance, possibly by the manufacturer of the beads before the beads are sold. Optionally, the reduction in autofluorescence produced by the bleaching, or at least a large part of the reduction in autofluorescence, lasts indefinitely, or lasts a long enough time, for example at least several days or several weeks, so that the bleaching can be done well in advance of performing a bio-assay with the beads, and the bleached beads can be sold with a reasonably long shelf life.

Definitions of level of autofluorescence for beads

In some embodiments of the invention, the bleaching reduces the level of autofluorescence of the beads by at least 20%, or by at least a factor of 1.5, or by at least a factor of 2, immediately after the bleaching is done, with the level of autofluorescence defined in the same way before and after the bleaching. Optionally, this is true for at least one excitation wavelength within the peak range of excitation wavelengths for the autofluorescence, for at least

one emission wavelength within the peak range of emission wavelengths for the autofluorescence. Optionally, the level of autofluorescence is reduced by at least a factor of 5, 10, 20, 50, or 100. Optionally, the autofluorescence remains reduced by at least 20%, or by at least a factor of 1.5, or by at least a factor of 2, 5, 10, 20, 50, or 100, for at least a day, or at least 3 days, or at least a week, or at least a month, or at least two months, or at least 6 months, or at least a year, after the bleaching is done. The inventors have found that some bleached beads have a level of autofluorescence that is only 11% of the unbleached level, even two months after bleaching.

For purposes of this statement, and other statements herein about level of autofluorescence, and about the peak range of excitation wavelengths and the peak range of emission wavelengths, the following definitions are used, although it should be understood that there are alternative ways to measure autofluorescence, and other definitions could be used:

1) The autofluorescence is measured using excitation light with an intensity and an exposure time low enough to cause very little bleaching of the beads, for example much less than 1 second at an intensity between 10^3 W/m^2 and 10^5 W/m^2. Using a higher intensity excitation light to measure the autofluorescence has the potential advantage that the emission light will be stronger, and easier to measure precisely. Although the excitation light intensity should not be so high that the autofluorescent emission power starts to saturate, and is no longer proportional to the excitation power, the inventors have not seen any such saturation effect even at intensities of 10^5 W/m^2.
2) For a given autofluorescence, in a given material, there is a spectrum of absorption cross-section, for exciting the fluorescent state, as a function of excitation wavelength. Generally there is a peak excitation wavelength, where the autofluorescence is most easily excited, and a peak range of excitation wavelengths around the peak, which is defined herein as the range where the absorption cross-section is at least 50% of its value at the peak. When specifying the level of autofluorescence, the excitation wavelength, or distribution of excitation wavelengths, is specified. Often an excitation wavelength is chosen which is within the peak range of excitation wavelengths, for example 488 nm or 532 nm for the beads listed in FIG. 4. A given material may have different fluorescent states excited by light in different wavelength ranges. In that case, a statement about the level of autofluorescence of the material, as used herein, generally refers only to the autofluorescence in one wavelength range, and does not necessarily apply to the other autofluorescence of that material. In general, the various fluorescent reporter molecules used in bio-assays may have excitation wavelengths ranging from 250 nm to 800 nm, and it may be advantageous to photobleach any autofluorescence, in

beads or other capture surface elements used in bio-assays, with excitation wavelengths in this range.

3) For a given autofluorescence, in a given material, there is an autofluorescence emission spectrum S(λ), proportional to the emitted power as a function of wavelength λ. Generally there is a peak emission wavelength, and a peak range of emission wavelengths around the peak, which is defined herein as the range where the emission spectrum S(λ) is at least 50% of its peak value. In general, the emission spectrum, and the peak range of emission wavelengths, may be a function of the excitation wavelength. The emitted autofluorescence power is measured over a wavelength range that is narrow compared to the characteristic range of wavelengths for the emitted light, by using a narrow bandwidth emission filter, for example a flat-top filter with a bandwidth of 25 nm, centered at an emission wavelength of interest. When specifying the level of autofluorescence, the emission wavelength is specified. For narrow enough bandwidth, the detected power is nearly proportional to the bandwidth of the emission filter, and the "level of autofluorescence" as used herein is normalized to the bandwidth of the emission filter, and it is not necessary to specify the bandwidth of the emission filter, only the central emission wavelength, when specifying such a normalized level of autofluorescence. The emission wavelength is often chosen to be within the peak range of emission wavelengths, for the excitation wavelength used. For example, for the beads listed in FIG. 4, an emission wavelength of 560 nm or 575 nm or 679 nm may be used, in the case of an excitation wavelength of 532 nm or 488 nm, or an emission wavelength of 520 nm may be used, in the case of an excitation wavelength of 488 nm. Any statement made herein about "level of autofluorescence," without specifying the excitation wavelength and emission wavelength, and when the excitation wavelength and emission wavelength are not clear from context, means "for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength," with the peak range of excitation wavelengths and the peak range of emission wavelengths referring to the kind of autofluorescence being considered.

4) The excitation light is projected onto a layer of beads, for example in a direction normal to the layer, the layer being much broader and thicker than a bead diameter, and at least moderately well packed together, for example by settling under the influence of gravity or by magnetic attraction. The beam is much wider than a bead diameter, but much narrower than the width of the layer in the directions perpendicular to the direction of the beam, and the layer is much thicker, in the direction along the beam, than the penetration depth of the light into the layer.

In these circumstances, the excitation light typically penetrates only through the first few layers of beads, for example the first 5 or 10 layers of beads, before becoming substantially attenuated by scattering and/or absorption, and the emission power is insensitive to how much thicker the layer of beads is, and how much broader the layer of beads is. In practice, autofluorescence levels are sometimes measured using a layer of beads that is less than or comparable to the penetration depth in thickness, and/or is not much broader than the width of the beam, and correction factors may be used to estimate what the emission signal would be from a much thicker and broader layer of beads.

5) Although the volume of the beads illuminated by the excitation light is much greater in diameter than a bead, it is much smaller than the distance to the detection optics, for example a microscope objective lens that is collecting the emitted light and sending it to a detector. The emitted light is generally emitted isotropically from the beads, and the detection optics has only a small light collection efficiency, but detects emission integrated over the whole illuminated volume.

The detector signal, for example the output voltage of a photomultiplier tube, when measuring emitted light from the beads, is given by:

$$Output = \sigma \cdot \sum_i I_i \cdot \frac{\lambda_{ex}}{\lambda_{em}} \cdot QY \cdot S(\lambda_{em}) \cdot \Delta\lambda_{em} \cdot \eta \cdot G \quad (1)$$

Here σ is the absorption cross-section of one fluorophore molecule, the sum is over all the fluorophore molecules i that are exposed to the excitation light, and I_i is the intensity of the excitation light at fluorophore molecule i. λ_{ex} is the wavelength of the excitation light. The emission light is assumed to be measured with a flat-top filter centered at wavelength λ_{em} with bandwidth $\Delta\lambda_{em}$. The autofluorescence emission spectrum $S(\lambda)$, which may depend on λ_{ex}, is assumed to be much broader than $\Delta\lambda_{em}$ and to be normalized with $\int d\lambda\, S(\lambda) = 1$. QY is the quantum yield of the fluorescent emission process for that value of λ_{ex}. η is the collection efficiency of emission light, within the emission bandwidth, received by the detector, taking into account the numerical aperture of the detection optics and any attenuation of the received light due to optical elements such as filters or dichroic mirrors. G is the gain of the detector in output volts per watt of received light, which in general depends on λ_{em}. The excitation light beam is assumed to have an intensity profile, for example a Gaussian intensity profile, with a characteristic radius r_{ex} that is small compared to the lateral extent of the layer of beads, and to penetrate into the layer of beads a characteristic distance D, the penetration depth, that is small

compared to the thickness of the layer of beads. For fluorophores that are well within the illumination volume, a cylinder of radius r_{ex} and depth equal to the penetration depth, the intensity I_i is close to the peak intensity I of the excitation light in the center of the beam at the front of the layer of beads. For fluorophores that are well outside the illumination volume, the intensity I_i is much less than the peak intensity I. Then, to good approximation, $\sum_i I_i \approx N \cdot I$, where N is the number of fluorophores within the illumination volume, so the output voltage may be written

$$Output = \sigma \cdot N \cdot I \cdot \frac{\lambda_{ex}}{\lambda_{em}} \cdot QY \cdot S(\lambda_{em}) \cdot \Delta\lambda_{em} \cdot \eta \cdot G \quad (2)$$

It should be noted that some of the light emitted by fluorophores located behind the front layer of beads may be absorbed before it gets outside the layer of beads where it can be collected, so those fluorophores may be given a reduced weighting in the sum over fluorophores. But the penetration depth for the emitted light is likely to be comparable to or greater than the penetration depth for the excitation light, so this effect can be taken into account by replacing the penetration depth of the excitation light by a slightly reduced (less than a factor of 2) effective penetration depth, and accordingly replacing the illumination volume by a slightly smaller effective illumination volume.

The relative level of autofluorescence of different types of beads, as used herein, means the relative output voltage of the detector, for measurements of the different types of beads, using the same optical system (and hence the same light collection efficiency η), the same detector (and hence the same gain G), the same excitation wavelength λ_{ex}, the same central detected emission wavelength λ_{em} and emission bandwidth $\Delta\lambda_{em}$, the same excitation light power, and the same cross-sectional area of the beam of excitation light, with the beam of excitation light impinging on a layer of beads that is much wider than the width of the beam and much thicker than the penetration depth D of the excitation light in the layer of beads.

Normalized levels of autofluorescence for beads

It may also be of interest to define one or more normalized levels of autofluorescence, which depend only on characteristics of the beads, and on λ_{ex} and λ_{em}, but not on characteristics of the optical system, the filter (other than the value of λ_{em}), the detector, and the excitation beam used to measure the autofluorescence. Optionally, such a normalized signal is also independent of the packing density of beads in the layer of beads that is measured. If the output signal of the

detector is normalized by the detector gain G, the excitation light intensity I, the emission bandwidth $\Delta\lambda_{em}$, and the ratio $\lambda_{ex}/\lambda_{em}$, then the resulting normalized signal can still be used to compare the level of autofluorescence of different types of beads, because none of the normalization factors depend on the type of bead. This normalized signal, which does not depend on the detector, the emission bandwidth, or the excitation light intensity, is used herein for giving the results of measuring autofluorescence in different types of beads in FIG. 4. It is given by:

$$Normalized \quad Signal \quad = \sigma \cdot N \cdot QY \cdot S(\lambda_{em}) \cdot \eta \tag{3}$$

However, this normalized signal still depends on the collection efficiency η of the optical system, and on the cross-sectional area $A=\pi r_{ex}^2$ of the beam of excitation light. For the data shown in FIG. 4, the beam had a circular cross-section with diameter 0.15 mm at the front of the layer of beads, so $A = 1.8 \times 10^{-2}$ mm^2. The optical system collecting the light had a numerical aperture of approximately 0.25, so the collection efficiency due to the numerical aperture would be $(0.25)^2/4 = 0.016$. In addition, there were two filters, and a dichroic mirror, that each admitted a fraction 0.9 of the light within the emission bandwidth, 25 nm centered at 575 nm, so the total collection efficiency η is given by $0.016 \times 0.9^3 = 0.012$.

If the normalized signal given by Eq. (3) is further normalized, by the collection efficiency η, and by the cross-sectional area A of the beam, then the resulting fully normalized signal will depend only on properties of the beads, and possibly on the packing density of the beads. This fully normalized autofluorescence level can be found from Eq. (3), and from the fact that the total number of fluorophores N in the illumination volume is equal to n, the number of fluorophores per volume in a bead, times the volume packing fraction f of beads in the illumination volume, times the illumination volume, which is the beam area A times the penetration depth D. Then the fully normalized autofluorescence level is

$$Autofluore \quad scence \quad = \sigma \cdot n \cdot f \cdot D \cdot QY \cdot S(\lambda_{em}) \tag{4}$$

The penetration depth D, for a given excitation wavelength and emission wavelength, depends only on properties of the type of bead, and on the packing fraction f. Indeed, it seems likely that, in many cases, for a given type of bead the penetration depth D scales like $1/f$, so $f \cdot D$ will be independent of the packing fraction f, and will depend only on characteristics of the bead. This may be true particularly if the penetration depth is due mostly to scattering and absorption of

light by the beads, rather than by the solution surrounding the beads. The other quantities on the right hand side of Eq. (4) also depend only on characteristics of the bead, and on λ_{ex} and λ_{em}. So the fully normalized autofluorescence level defined by Eq. (4) would depend only on characteristics of the bead, and on λ_{ex} and λ_{em}. Also, because it is normalized only by quantities that do not depend on the type of bead, this normalized autofluorescence level will have the same ratio, for different types of beads, as the raw output signal given by Eq. (2), or the normalized signal given by Eq. (3) and used in FIG. 4.

Definitions of level of autofluorescence for other capture surface elements

Definitions of level of autofluorescence, or normalized level of autofluorescence, similar to the measures defined by Eq. (2) and (4), may be used for larger capture surface elements, for example polymer structural parts of microfluidic systems, except that the requirement that the measurement be made with a thick layer of beads need not apply in this case. In a capture surface element that is much greater in dimensions than a bead, made of polymer of uniform density, without magnetic material, the penetration depth may be much greater than for a layer of beads. If the penetration depth of excitation light is not much smaller than the thickness of such a capture surface element, then the level of autofluorescence may depend on its thickness. Optionally, for large capture surface elements, the autofluorescence level is defined using an excitation light beam that is wider than the distance to which it penetrates (either the penetration depth or the thickness of the element), and is fairly uniform in width throughout that distance, so that the excitation light intensity is easy to calculate as a function of position over the illumination volume.

If the beads are to be used in an assay in which the fluorescence is measured one bead at a time, similar to flow cytometry measurements, then the autofluorescence per bead, or the autofluorescence per surface area of bead, might be more relevant than some of the measures of autofluorescence defined above.

In some embodiments of the inventions, statements herein about level of autofluorescence, or emitted autofluorescence intensity, are true using a different definition of level of autofluorescence, for example with different excitation wavelength, or a different peak wavelength of the emission filter, or with different assumptions about the bandwidth of the emission filter, or about the configuration of the beads and the excitation light beam. In practice, even if some of the assumptions about the configuration of the beads and the excitation light beam are not very well satisfied when the autofluorescence level is measured, for example if the layer of beads is not very thick or not much wider than the excitation light beam, corrections for

these effects may be made, for example by using optical modeling, so that the level of autofluorescence may be determined according to the definition given above. Approximate correction factors may also be found empirically, by measuring autofluorescence emission power from different configurations of beads.

It should be understood that, wherever a comparison is made herein between level of autofluorescence before and after a change in level of autofluorescence, for example a relative change in level of autofluorescence before and after the bleaching, the same parameters are to be used for defining the level of autofluorescence, before and after the change. For example, the same excitation wavelength and emission wavelength are used. In this way, it is meaningful to compare level of autofluorescence before and after the change. Generally also the same excitation light intensity is used, as well as the same configuration of excitation light beam and beads (or other capture surface element), and the same detector and detector optics, which may help to ensure that differences in measured level of autofluorescence are not due to failure to correctly take into account all of these things in measuring the level of autofluorescence.

Definitions for bleached beads

An aspect of some embodiments of the invention comprises a polymer bead product, suitable for use in bio-assays, with a measurable level of autofluorescence, but with the autofluorescence bleached below the original unbleached level for polymer beads manufactured in the same way, for example below the unbleached level by at least a factor of 2, for at least one excitation wavelength in the peak range of excitation wavelengths for the autofluorescence, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength. It should be understood that often, photobleaching reduces the level of autofluorescence by roughly the same factor for all excitation wavelengths and emission wavelengths, at least in the peak ranges of excitation and emission wavelengths, so it may be enough to measure the level of autofluorescence for a typical excitation wavelength and emission wavelength in the peak ranges. It should be understood that here “manufactured in the same way” does not include any procedures of bleaching.

In some embodiments of the invention, the autofluorescence is sufficiently bleached so that further exposure of the beads to light, of an intensity, duration and wavelength that with unbleached beads would result in a large reduction of autofluorescence by photobleaching, for example light of 10^5 W/m^2 at the peak excitation wavelength for a duration of 15 minutes, does not cause a further measurable reduction in level of autofluorescence, or only causes a relatively small further reduction in level of autofluorescence, for example by less than a factor of 2, or

less than a factor of 1.5, or less than a 20% reduction. The further exposure to light is done, for example, in the same device used to measure the autofluorescence of the beads, using the same light source and optics to apply the bleaching light as are used to apply the excitation light to measure the autofluorescence, but at much higher intensity, and/or for much longer duration, and applying the light, for example, in a beam normal to a broad, thick, settled layer of the beads, for example a layer like the layer described above for purposes of defining how level of autofluorescence is measured. Optionally, the beads are mixed, for example repeatedly, during or between exposing them to the bleaching light. Thoroughly mixing the beads has the potential advantage that all or almost all of the beads are exposed to the light, with similar average exposure intensity and duration, during the bleaching process, so that all or almost all of the beads are fully bleached. Otherwise, beads that are too deeply buried below the surface of the layer, and beads that are located outside the width of the beam of bleaching light, may not be adequately bleached. Alternatively or additionally, a thin enough layer of beads and a broad enough light beam are used so that all or almost all of the beads are exposed to the requisite light intensity at the same time, and there is no need to mix the beads.

As used herein, the level of autofluorescence of a collection of beads means an average level of autofluorescence of all the beads in the collection, for example a mean value of the level of autofluorescence of the individual beads. Similarly, the level of autofluorescence of a bead product manufactured by a specified method means an average level of autofluorescence that would result for beads manufactured by that method. When a collection of beads is photobleached to reduce its autofluorescence, there may be differences in the intensity and duration of light applied to different beads, so that the resulting bleached beads may have a range of values of level of autofluorescence, even if their level of autofluorescence was uniform before bleaching.

As used herein, a "bead product" means a single bead or a plurality of similar beads, whose properties are typically measured collectively using a large number of the beads. In some embodiments of the invention, the beads are nominally identical, manufactured in a way that gives them the same properties, within a manufacturing tolerance. Alternatively, the different beads are intentionally manufactured with a distribution of values of one or more properties, but are intended to be used together in a bio-assay. The beads are typically sold in a buffer solution, for example in a package of 1000 beads, or 10,000 beads, or 100,000 beads, or 1 million beads, or a smaller, larger or intermediate number of beads.

As used herein, beads "suitable for bio-assays" refers to beads with the size, shape, material, and mechanical properties, of beads that are typically used for binding analytes in bio-

assays. For example, the beads are approximately spherical, between 0.3 and 10 micrometers in diameter, and made of one or more polymers, such as any of polystyrene, latex, methyl methacrylate, ethylene glycol dimethacrylate, and methacrylic acid. Optionally, the beads have surface properties that allow them to immobilize molecules that bind specifically to analytes in a bio-assay. For example, the surfaces of the beads have functional groups that can be joined to ligands that specifically bind analytes of interest, the ligands including any of streptavidin, avidin, tosyl, and protein A. However, "suitable for bio-assays," as used herein, also includes beads that have surface properties that do not allow them to immobilize such molecules, as long as the beads are usable for purposes such as calibrating bioassays, for example by measuring a background signal using beads that are known not to have any of the analyte bound to them, or that are known not to be emitting any fluorescence, or to be emitting only a known low level of fluorescence.

As used herein, a measurable level of autofluorescence, or a measurable change in level of autofluorescence, means a level or a change in level of more than 1.25% of the level of autofluorescence exhibited by Certified Blank beads 890, made by Bangs Laboratories, for an excitation wavelength of 532 nm and an emission wavelength of 575 nm, with level of autofluorescence defined in the way stated above. These beads are polystyrene, 7.65 μm in diameter. In tests done by the inventors, the level of autofluorescence of Certified Blank beads, with an excitation wavelength of 532 nm, and an emission bandpass filter with wavelength centered at 575 nm, 25 nm wide, was measured to be about 7 nm, using the normalized signal defined by Eq. (3), with the collection efficiency η approximately equal to 0.012, and the excitation light beam having a circular cross-section 0.15 mm in diameter, and with a broad layer of beads about 5 bead diameters thick. If a layer much thicker than that were used, the normalized signal would be expected to be slightly greater, perhaps about 8 nm. The lowest measurable level or change in level of autofluorescence, defined as 1.25% of that, would correspond to a normalized signal of 0.1 nm, with that collection efficiency and beam diameter. It should also be noted that, in practice, the inventors have found that it is possible to measure the level of autofluorescence of all the commercially available polymer bio-assay beads that they have tested, bleached or unbleached, even in layers that are not much thicker than a bead diameter, and to measure the level of autofluorescence well enough to be able to determine if the level of autofluorescence decreases by at least a factor of 2 when the beads are exposed to a level of light that would substantially photobleach the autofluorescence of unbleached beads.

Bio-assay system with bleached capture surface element

An aspect of some embodiments of the invention concerns a bio-assay system, usable for bio-assays employing fluorescent reporter molecules, for measuring or detecting an analyte in a biological sample, with a capture surface element made of a polymer that exhibits a measurable level of autofluorescence. The capture surface element has its autofluorescence bleached below its original unbleached level of autofluorescence, or below the level of autofluorescence for a capture surface element manufactured in the same way and never bleached. For example, the level of autofluorescence is reduced below the unbleached level by 20%, or by a factor of 1.5, 2, 3, 5, 10, 20, 50, or 100, or by a smaller, larger, or intermediate factor. In some embodiments of the invention, the capture surface element is sufficiently bleached so that further exposure of the capture surface element to light, of an intensity, duration and wavelength that with unbleached polymer would result in a large reduction of autofluorescence by photobleaching, for example light of 10^5 W/m^2 at the peak excitation wavelength for a duration of 15 minutes, does not cause a further measurable reduction in level of autofluorescence, or only causes a relatively small further reduction in level of autofluorescence, for example by less than a factor of 2, or less than a factor of 1.5, or less than a 20% reduction. The capture surface is used to immobilize ligand molecules that bind to molecules of the analyte when they are exposed to the biological sample. The ligand molecules are labeled by the reporter molecules in such a way that a fluorescent signal from the reporter molecules will be greater, the more analyte molecules are bound to the ligand molecules. Bleaching the autofluorescence of the capture surface element has the potential advantage that it will reduce a background fluorescent signal from the capture surface, potentially improving the signal to noise ratio, for noise due to autofluorescence, for a fully bound assay, and potentially allowing the bio-assay to report lower levels of analyte bound to the ligand molecules of the capture surface, and hence to be sensitive to lower levels of analyte in the biological sample.

An aspect of some embodiments of the invention concerns a method of preparing one or more polymer capture surface elements of known composition, for example polymer beads, for use in a fluorescence-based bio-assay. A level of autofluorescence of the capture surface elements is measured, and is compared to the level of autofluorescence that would be expected for capture surface elements of that composition, if their autofluorescence were not bleached. From this comparison, it is determined whether and to what extent autofluorescence of the surface capture elements has been and remains bleached. If it is determined that the capture surface elements do not have their level of autofluorescence reduced by more than a predefined amount, for example 0%, 50%, or 75% of their unbleached level of autofluorescence, or 50%,

75%, 90%, or 98% of an expected maximum possible reduction in their level of autofluorescence, then the capture surface elements are bleached, for example by exposing them to light of a suitable intensity, wavelength, and duration. The bleached surface capture elements may then be used in a bio-assay of potentially increased sensitivity, as described above.

Criteria for bleached beads

An aspect of some embodiments of the invention concerns a polymer bead product that has a level of autofluorescence less than 50% of the level of autofluorescence of by Certified Blank beads 890, made by Bangs Laboratories, or less than 30% or less than 20% of the level of autofluorescence of these Certified Blank beads, with "level of autofluorescence" defined according to any of the definitions given above, for example with excitation wavelength of 532 nm and central emission wavelength of 575 nm, both for the polymer bead product, and for the Certified Blank beads. Optionally, this aspect only concerns a bead product, for example a polystyrene bead product, with a measurable level of autofluorescence for which 532 nm is within the peak range of excitation wavelengths, and 575 nm is within the peak range of emission wavelengths for an excitation wavelength of 532 nm. It would not make much sense to define level of autofluorescence at these values of excitation and emission wavelength, if the bead were made of an autofluorescent material with a very different peak range of excitation and emission wavelength, since for such beads the autofluorescence might be extremely small at an excitation wavelength of 532 nm and an emission wavelength of 575 nm, even without any bleaching. In some embodiments of the invention, the beads are paramagnetic beads, for example superparamagnetic beads, and the level of autofluorescence is less than that of Certified Blank beads, or less than 50%, 30%, or 20% of the level of autofluorescence of Certified Blank beads, using any of the definitions given above for "level of autofluorescence." As noted above, tests by the inventors showed that these Certified Blank beads, without any bleaching, have a normalized autofluorescence signal, defined by Eq. (3), of about 8 nm, with an excitation wavelength of 532 nm and measuring the emission at a wavelength centered at 575 nm, with a light collection efficiency of 0.012 and an excitation beam of circular cross-section with diameter 0.15 mm. So this aspect of some embodiments of the invention concerns beads with a normalized autofluorescence signal, defined in this way, of less than 4 nm, or 2.4 nm, or 1.6 nm. In some embodiments of the invention, this low level of autofluorescence is achieved by bleaching the autofluorescence of the beads, for example by photobleaching.

An aspect of some embodiments of the invention concerns a polymer bead product that has a level of autofluorescence less than 10 times the level of Raman scattering of a beam of

light at 532 nm, in a column of water 8 mm long, the scattered light measured at 575 nm, for at least one excitation wavelength of 532 nm and an emission wavelength of 575 nm. Again, the beads are assumed to have a measurable level of autofluorescence for which 532 nm is in the peak range of excitation wavelengths, and for which 575 nm is in the peak range of emission wavelengths for an excitation wavelength of 532 nm. In tests done by the inventors, the Raman scattering signal from an 8 mm long column of pure (doubly distilled) water, excited at 532 nm, normalized as defined in Eq. (3), with a light collection efficiency of 0.012 and using an excitation light beam of circular cross-section 0.15 mm in diameter, was about 0.5 nm at 560 nm emission wavelength, and about 0.4 nm at 575 nm emission wavelength, respectively about 6% and 5% of the autofluorescent emission signal from unbleached Certified Blank beads, illuminated with an excitation beam at 532 nm of the same intensity and diameter, and detected at an emission wavelength of 575 nm with the same detection optics and detector. Optionally, in these circumstances, the level of autofluorescence is weaker than 10 times the level of Raman scattering from 8 mm of water, or weaker than 6 times or 4 times or 2 times the level of Raman scattering, or weaker than the level of Raman scattering, or weaker than a greater, smaller, or intermediate multiple of the level of Raman scattering. These levels correspond to a normalized autofluorescence signal, by this definition, of less than 4 nm, or 2.4 nm, or 1.6 nm, or 0.8 nm, or 0.4 nm. It should be understood that, when comparing the level of autofluorescence to the level of Raman scattering, the autofluorescent emission power is found using low enough excitation light power and/or a short enough exposure time that the beads are not bleached, and the emission power is proportional to the excitation light power. The Raman scattering power is also proportional to the excitation light power, so in this limit the ratio of autofluorescent emission power to Raman scattering power will not depend on the excitation light power. Only if the excitation power were so high that the autofluorescent emission power starts to saturate, would the ratio of autofluorescent emission power to Raman scattering power differ very much from this value. In practice, the inventors have not seen any saturation in the autofluorescent emission power at the highest excitation powers used, with the beads they have tested.

Bio-assay system that can photobleach beads

An aspect of some embodiments of the invention concerns a system for performing fluorescence bio-assays using polymer beads, in which the beads can be photobleached in the same location where they are located during the bio-assay, before the bio-assay is performed. Optionally, a light source and optics that are used for illuminating the beads with excitation light during the bio-assay are also used for photobleaching the beads. Optionally, the system is used

for further photobleaching beads immediately before using them in a bio-assay, even if the beads had previously been photobleached, for example as part of their manufacturing process before they were sold. Further photobleaching such beads immediately before using them in a bio-assay has the potential advantage that if their level of autofluorescence has partly recovered since they were manufactured, it can be partly or fully restored to their level of autofluorescence when they were newly manufactured, or reduced to an even lower level.

System for mixing and photobleaching beads

An aspect of some embodiments of the invention concerns a system and method for photobleaching bio-assay beads, in which only a portion of the beads are exposed to an intensity of light suitable for photobleaching, at a given time, but a mixing subsystem repeatedly or continuously mixes the beads, allowing different portions of the beads to be exposed to the light, until all or almost all of the beads are photobleached to about the same level of autofluorescence. In some embodiments of the invention, the system photobleaches magnetic beads, and the mixing is performed by alternatively bringing a magnet closer to and further away from the beads. When the magnet is brought closer to the beads, the magnetic field and field gradient are strong enough to magnetize the beads, so that they aggregate together into a clump, and the clump is attracted to a location adjacent to the magnet. At that location, some of the beads, for example the ones on an outer surface of the clump facing the light, are exposed to the light for photobleaching. When the magnet moves further away, the beads become demagnetized, and gradually disperse away from the location they were attracted to. Repeatedly aggregating and dispersing the beads mixes them, so that different beads are on the outside of the clump and exposed to the light for photobleaching each time the clump re-forms. In some embodiments of the invention, the mixing is done with two magnets that are alternatively brought closer to and further away from different locations in a container holding the beads. In some embodiments of the invention, the mixing is done by alternately magnetizing and demagnetizing an electromagnet, or two electromagnets, positioned close to a container holding the beads.

Photobleaching beads used in chemoluminescence assays

In some embodiments of the invention, bio-assay beads are photobleached using any of the methods described herein, but the bleached beads are then used for a chemoluminescent assay, or for an electro-chemiluminescent assay, rather than for a fluorescent assay. The assay may be a “sandwich” assay or a FRET assay, as described below for fluorescence assays in the description of FIG. 5, or any other type of assay known in the art that uses beads or other capture

surface elements. Optionally, the beads are designed specifically for chemiluminescent or electro-chemiluminescent assays. Alternatively, the same beads can be used either for fluorescent assays or for chemiluminescent or electro-chemiluminescent assays. There are theoretical reasons for believing that the same photobleaching process that reduces the autofluorescence of beads used in fluorescent assays will also reduce the background chemiluminescence or electro-chemiluminescence of beads used in chemiluminescent or electro-chemiluminescent assays, since in all these cases, it is expected that the photobleaching process would cause a long-term change in the electronic energy levels of the bead material, to eliminate metastable energy levels that are associated with fluorescence or with chemiluminescence or electro-chemiluminescence. A further reason for believing that photobleaching bio-assay beads may reduce their background chemiluminescence as well as their autofluorescence, is that Bangs Laboratories, Inc., which sells Certified Blank Beads 890 that have very low autofluorescence, also sells a bio-assay bead product for chemiluminescence assays, ProMag® HP Magnetic Microspheres, described as having very low levels of background chemilumiscence in a brochure "Product Data Sheet 743," downloaded from www(dot)bangslabs(dot)com/sites/default/files/imce/docs/PDS%20743%20Web.pdf> on March 27, 2018. However, the inventors do not know whether Bangs Laboratories uses a similar procedure for preparing the two bead products, or what that procedure is.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Bio-assay and bleaching system that can be used for non-magnetic beads

Referring now to the drawings, FIG. 1 illustrates a system 100 which can be used for one or more of 1) performing a bio-assay to determine a concentration of an analyte in a biological sample, using fluorescent reporter molecules that bind indirectly to polymer beads, whose fluorescent emission is a measure of the concentration of analyte molecules present in the sample; 2) measuring a level of autofluorescence of the beads even in the absence of any analyte molecules in the sample, which sets a limit on the sensitivity of the bio-assay; 3) photobleaching the polymer beads before performing the bio-assay, in order to reduce the level of autofluorescence of the beads, and hence increase the sensitivity of the bio-assay. System 100 may be used for either magnetic or non-magnetic beads, as opposed to system 200 shown in FIG.

2, which performs the same functions but only using paramagnetic or superparamagnetic beads, often referred to as magnetic beads.

System 100, as well as system 200 in FIG. 2, and system 700 in FIG. 7, can be used to perform various types of fluorescent bio-assays in which fluorescent reporter molecules, attached indirectly to beads, or to another capture surface element, produce a fluorescent emission signal that depends on the concentration of an analyte in a biological sample. Examples of such assays include "sandwich" assays, fluorescence resonance energy transfer (FRET) assays, other types of energy transfer assays in which the emission light is quenched or enhanced for example by colloidal gold or silver, competition assays in which either of two analytes can bind to a ligand, and direct assays in which an analyte is linked directly to a bead via a cross-linker and only the reporter molecule has an analyte-specific ligand. The description of system 100, system 200, and system 700 will refer to a sandwich assay, as an example, but it should be understood that these systems can also be used for other types of assay, known in the art, in which a fluorescent emission signal from beads or other capture surface elements depends on the concentration of analyte in a biological sample. A FRET assay will be described below in the description of FIG. 5.

System 100 includes a container 102 made of an optically transparent material, for example a glass cuvette, capable of holding and confining a quantity of polymer beads 104. Optionally, beads 104 settle on a bottom surface of container 102, due to gravity, and form a somewhat concentrated layer comprising a large number of beads. If system 100 is used for performing a sandwich bio-assay to measure a concentration of an analyte in a biological sample, then beads 104 in container 102 are exposed to the sample, after the beads are coated with a ligand that binds specifically to the analyte, and fluorescent reporter molecules are used to measure how much analyte is bound to the ligand. If it is desired to use system 100 for performing a bio-assay on a relatively small sample, then it is potentially advantageous to make container 102 relatively small, and to use a relatively small number of beads 104 that will fit in container 102, so that analyte molecules from the sample will bind to the beads with as high a surface density as possible, making it easier to detect a given quantity of analyte in the sample. For example, in tests done by the inventors, container 102 consisted of a glass plate 4 mm in diameter surrounded by walls 17 mm high, and about 5 layers of beads, each bead about 7 μm in diameter, covering the entire bottom were placed in the container.

A light source 106, for example a laser, or a broadband light source such as a flash lamp, produces a beam of light 108, that optionally reflects, at least partially, from a dichroic mirror 110, or a polarization beam splitter, and illuminates beads 104 in container 102. For example,

beam 108 optionally enters container 102 and illuminates beads 104 through a transparent bottom of container 102, as shown in FIG. 1. When system 100 is used to perform a bio-assay, or to measure a level of autofluorescence of beads 104, then beam 108 excites the beads to emit a fluorescent emission light 112, produced by the reporter molecules and/or by the autofluorescence of the beads themselves. If beam 108 is used to excite the beads in a bio-assay, then it is potentially advantageous if light source 106 is a monochromatic light source such as a laser, or at least has a narrow enough range of wavelengths so that little or none of the spectral distribution of beam 108 overlaps the band of wavelengths being detected from emission light 112, to minimize interference from scattered excitation light. In general, fluorescent emission light 112 will be of a longer wavelength than beam 108 which excites the emission. Light 112 is detected by a light detector 114, after passing through dichroic mirror 110, which substantially reflects the shorter wavelength light of beam 108, but transmits the longer wavelength light 112, at least a substantial portion of it that detector 114 will detect. In some embodiments of the invention, a polarization beam splitter is used instead of dichroic mirror 110. In this case, the excitation light is polarized, and is essentially all reflected from the beam splitter, but the fluorescent emission light will not be polarized, and half of it will pass through the beam splitter, and will reach detector 114. Any excitation light that is reflected from the beads or the container will still be polarized, and will not pass through the beam splitter, so will not reach detector 114. A filter 116, optionally placed in front of detector 114, preferentially passes light of a particular wavelength range of emitted light 112, while substantially blocking stray light of other wavelengths, including the wavelength of beam 108. For example, in tests done by the inventors to measure the autofluorescence of non-magnetic beads, light source 106 was a 532 nm laser diode module (ThorLabs, CPS532), dichroic mirror 110 was a Semrock BrightLine Di02-R532 mirror, and emitted light 112 was measured, after passing through the dichroic mirror and the filter, in a band 25 nm wide around 575 nm.

Optionally, a lens 118 gathers emitted light 112 over a relatively wide range of angles, and, optionally together with another lens 120 located closer to the detector, concentrates the emitted light on a relatively small area of detector 114. Optionally, the function of lens 118 and/or lens 120 is served by multiple lenses and/or other optical elements. For example, lenses 118 and 120 may focus an image of beads 104 on detector 114. Using such an optical imaging system is particularly advantageous if, as is often the case, container 102 is small compared to the distance between container 102 and detector 114, so that lenses 118 and 120 greatly concentrate emitted light 112 on detector 114, making the detection of light 112 more efficient and more sensitive. Optionally, a pinhole in front of detector 114, not shown in FIG. 1, further

blocks undesired stray light from entering detector 114, while allowing light 112 to enter. For example, the pinhole only admits light at the location of an image of beads 104 focused on the pinhole.

Optionally, an additional lens 122, or multiple lenses and/or other optical elements, together with lens 118, changes the diameter and/or the angular spread of beam 108. For example, lenses 118 and 122 may concentrate light beam 108 on container 102, if light beam 108 initially has a diameter greater than the diameter of container 102, or if light beam 108 would have a greater diameter than container 102, when it reached container 102, in the absence of lens 122. Since lens 118 is designed to collect the wide-angle emitted light 112, it may have an undesired effect on light beam 108, which also passes through lens 118, for example making it too wide or focusing it at an undesired location, and lens 122 can be designed to counter such an undesired effect of lens 118 on light beam 108.

A controller 124, for example a personal computer or another general purpose computer, or dedicated electronic circuitry, optionally controls light source 106, and receives data from detector 114. The data received from detector 114 is optionally used by controller 124 to calculate a concentration of the analyte in the biological sample, in the case of a bio-assay, or to calculate a level of autofluorescence of beads 104, in the case where there is no analyte binding to the beads, or no fluorescent reporter molecules attached to the beads. Controller 124 optionally calculates the concentration of analyte or the level of autofluorescence by comparing the detected signal from detector 114 with calibration signals generated by samples with known concentrations of analyte, and/or by beads with known levels of autofluorescence. Controller 124 may also be used, for example, to increase the sensitivity of detector 114 by modulating light source 106, and looking for a component of a time-dependent signal from detector 114 that has a same modulation as light source 106. Optionally, controller 124 is connected to a user interface 126, for example comprising one or more of a monitor, keyboard, touch screen, and mouse. A user may use interface 126 to set parameters for a task being performed by system 100, and/or to obtain calculated values of analyte concentration in a sample, or autofluorescence level in beads 104.

When system 100 is used to photobleach beads 104, then beam 108 from light source 106, or light from a different light source, optionally illuminates beads 104, at an intensity, duration, and wavelength appropriate for bleaching the autofluorescence of beads 104 down to a desired level. For example, in tests on photobleaching of non-magnetic beads done by the inventors, a light beam from a 532 nm laser diode was used, for a duration of 15 minutes, with a light intensity at the beads of 10^5 W/m^2. In this case, detector 114, filter 116, and lens 120 are

optionally replaced by a camera, for example a CCD camera, to monitor the progress of the bleaching process. Optionally, beads can instead be photobleached in a system that does not have a detector and associated optical elements, but only a light source and optical elements for illuminating the beads, which might also make it possible to place the light source closer to the beads, and to simplify the optics for the light source. But a potential advantage of using system 100 for photobleaching beads is that it is possible, during or after bleaching a sample of beads, to measure the autofluorescence of the bleached beads.

Bio-assay and bleaching system for magnetic beads

Figure 2 shows a system 200, which can perform any of the three functions performed by system 100, but only using paramagnetic beads or superparamagnetic beads. Such beads comprise a small core of ferromagnetic material, which is, however, too small to become permanently magnetized, but is only magnetized when a magnetic field is applied to it. This property of the beads allows them to magnetically attract each other and clump together when a magnetic field is applied to them, and also allows such a clump of beads to be manipulated, moving it around in response to magnetic field gradients applied to it.

System 200 comprises a container 202, with two magnets 204 and 206, optionally electromagnets, on opposite sides of the container, for example above and below the container as shown in FIG. 2. It should be understood that FIG. 2 shows a top view of the system used by the inventors, and magnets 204 and 206 are actually located to the sides of container 202, though the orientation of system 200 with respect to gravity is relatively unimportant if the magnetic force on the beads is much greater than the force of gravity on the beads. Magnet 204 has a small sharply curved pole piece 208, adjacent to container 202 on one side, and magnet 206 has a similar pole piece 210, adjacent to container 202 on the other side. Each pole piece, when its associated electromagnet is turned on, produces a high enough magnetic field in the container to magnetize the beads and make them clump together. For example a field of 0.6 tesla works well for the magnetic beads tested by the inventors, listed below in the description of FIG. 4. When one of the electromagnets is turned on and the other one is turned off, then its associated pole piece also produces a large field gradient, for example 800 T/m measured at the pole tip, to produce a big enough magnetic force on a clump 212 of the magnetic beads, so that if the clump starts on the opposite side of the container, it will move across the container, toward the electromagnet that is turned on, in a short time, for example in about 0.5 seconds. For example, if container 202 is 0.4 mm wide in the direction between the magnets, and if it contains a clump of between 1000 and 200,000 magnetic beads, each bead between 1 and 8 μm in diameter and

with volume magnetization of about 14,000 A/m when it is fully magnetized, and if the container is filled with water, with viscosity 8.9×10^{-4} Pa-sec, then a field gradient of 800 T/m is expected to produce a force on the clump of between 0.1 and 100 pN, depending on the size of the clump, which should be enough to move it the 0.4 mm across the container in about 0.1 to 2 seconds. For a given magnetic field and field gradient, larger clumps cross the container more quickly, because their magnetic moment, and hence magnetic force, increases with clump diameter faster than the viscous force would increase with clump diameter at a given speed through the water. These numbers are in agreement with what the inventors have observed.

Pole pieces 208 and 210 need not be identical in shape, and the magnetic field gradient, and resulting force on the beads, need not be the same when clump 212 moves in one direction, as when it moves in the other direction. If the two pole pieces are not the same shape, or if magnets 204 and 206 are not the same strength, then clump 212 might move faster in one direction than in the other direction.

Optionally, pole pieces 208 and 210 are located slightly to the front of container 202, to the right as seen in FIG. 2. This has the potential advantage that the field gradients of the pole pieces will attract clump 212 to the front of container 202, so the clump presses against the front side of container 202 as it moves back and forth. Having the clump stay right against the front side of container 202 may make it easier to keep the illuminating light beam in focus on the clump, and to keep the clump in focus when detecting the emitting fluorescent light from the clump, as will be explained below. Also, if the clump is pressed against the front side of container 202, there will be very little water between the clump and the front wall, which can minimize interference from Raman scattering from the water, and from any unbound fluorescent molecules in the water.

A light source 214, for example a laser diode or another type of laser, or a broadband light source such as a flash lamp, produces a light beam 216. As in system 100, if system 200 is used to perform a bio-assay, then it is potentially advantageous if light source 214 is a monochromatic light source such as a laser, or has a narrow enough spectral distribution so that it has little or no overlap in wavelength with the emission light that is being detected. Light beam 216 optionally reflects off a dichroic mirror 218, and optionally reaches container 202 on a side of the container, directed perpendicular to the direction of the magnetic forces that act on clump 212. In FIG. 2, the magnetic forces are in the direction shown as vertical in the figure, and beam 216 is directed in the direction shown as horizontal when it reaches container 202. When light beam 216 reaches container 202, it is narrower than the width of the container between the two magnetic poles, and consequently, if the clump of beads travels from one side of the container to

the other, then it will only be illuminated by beam 216 over part of its path, for only part of its travel time. Optionally, the magnetic poles are positioned so that clump 212 forms close to the front of container 202, so that beam 216 has to travel through very little water in order to reach clump 212. Also, the clump is optionally much wider than beam 216, and optionally the clump is thick enough to absorb or scatter most of the light in beam 216. This has the potential advantage that there will be very little Raman scattering of beam 216 from the water in container 202, when the clump is in the path of the beam. However, in some tests performed by the inventors with a system similar to system 200, described below, the clump of beads was not much wider than the beam, and was not thick enough to absorb or scatter most of the light in beam 216.

As in system 100, when the beads in clump 212 are illuminated by beam 216, they emit fluorescent light 220, which is optionally collected by a lens 222, and concentrated by lens 222 and a lens 224 on a detector 226. Optionally a filter 228 is located in front of detector 226, that preferentially admits light of a particular wavelength range of emitted light 220, while blocking other wavelengths, for example the wavelength of beam 216. Optionally, as with detector 114 in FIG. 1, a pinhole in front of detector 226, not shown in FIG. 2, helps to keep out stray light, not coming from fluorescent emission of the beads. Optionally a lens 230, together with lens 222, changes the width and/or angular spread of beam 216, making it somewhat narrower than the width of container 202 in the direction of the magnetic forces, but optionally not too much narrower. Optionally, any of lenses 222, 224, and 230 are replaced by multiple lenses and/or other optical elements that perform similar functions.

In an exemplary magnetic modulation biosensing (MMB) system, similar to system 200, used by the inventors for tests with magnetic beads, the light source is a 532 nm laser diode module (ThorLabs, CPS532), working at 5 mW with a 3.5 mm beam diameter. The beam diameter is reduced using two lenses (ThorLabs, 200 mm and 100 mm focal lengths) and diverted using a dichroic beam splitter (Semrock, BrightLine Di02-R532) into an objective lens (Newport, M-10X, with a numerical aperture NA = 0.25). The beam exiting the objective lens is focused on a rectangular, borosilicate sample cell, which contains the magnetic beads and has inner dimensions of 8 mm x 0.4 mm x 70 mm (Vitrocom, W2540). Two electromagnets, located on each side of the sample cell, magnetize the magnetic beads, causing them to form a clump. The magnetic poles are optionally located near the front of the cell, or slightly in front of the cell, so the clump stays near the front of the cell, and the excitation beam does not go through very much water in front of the clump of beads, which helps to reduce the Raman scattering signal from the water, and interference from any unbound fluorescent molecules in the water. The emitted fluorescence is collected using the same objective lens, and then passes through the

dichroic beam splitter and two identical emission filters. According to the experiment, the emission filters' central wavelength is 560 nm (Semrock, FF01-560/25-25), 575 nm (Semrock, FF03-575/25-25), or 679 nm (Semrock, FF01-679/41-25). The fluorescence is then spatially filtered by a pinhole (ThorLabs, 500um, P500S) and detected by a photomultiplier tube (PMT, Hamamatsu, H10722-20). The electrical signal from the PMT is measured over time and sent to an oscilloscope (Tektronix MDO3054) for analysis. A similar system was used by the inventors for tests with non-magnetic beads, but with the sample cell replaced by an open glass container, like container 102 in FIG. 1, and with the system rotated so that the excitation light beam is oriented vertically, and enters the glass container through the bottom, where the beads settle due to gravity.

When system 200 is used to perform a bio-assay, or to measure autofluorescence of the beads, then, if magnets 204 and 206 are electromagnets, they are alternately magnetized, for example for 0.5 seconds each, alternately producing a stronger magnet field and field gradient on the side of container 202 adjacent to magnet 204 and on the side adjacent to magnet 206, and a weaker magnetic field and field gradient on the other side of the container. Alternatively, magnets 204 and 206 are permanent magnets, for example rare earth magnets, that are rotating synchronously, or alternately moving towards and away from container 202 synchronously, for example every 0.5 seconds, so that there is a stronger magnetic field and field gradient alternately on the side of the container adjacent to magnet 204 and on the side of the container adjacent to magnet 206, and a weaker magnetic field and field gradient on the other side of the container. The stronger magnetic field is optionally great enough to magnetize the beads and cause them to stick together in a clump, and the stronger field gradient is optionally great enough to cause the clump to move from one side of container 202 to the other side, in the direction shown as vertical in FIG. 2, in a transit time of about 0.5 seconds, or whatever the timing is for each magnet to be magnetized or to be rotated or moved back and forth. As a result, magnetic clump 212 will go back and forth fairly continuously, and will be illuminated by light beam 216 only for a limited time in its path, on each transit. The transit time is much longer than the fluorescent emission lifetime for the fluorescent emission induced by light beam 216, which is typically in a range of nanoseconds, so the fluorescent emission, whether it is due to reporter molecules or to autofluorescence of the beads, will be significantly modulated at the same frequency that clump 212 goes into and out of beam 216, for example at 2 Hz if the electromagnets are alternately activated every 0.5 seconds.

Alternatively, instead of or in addition to moving magnetic clump 212 into and out of light beam 216, light beam 216 is moved so that it is alternately aimed at magnetic clump 212,

and aimed to the side of magnetic clump 212. This can be done even without moving magnetic clump 212, and it can have the same effect as moving clump 212, that clump 212 alternately is illuminated and not illuminated by light beam 216, or alternately is illuminated by light beam 216 at a higher and lower intensity. Moving light beam 216 can be done, for example, by moving and/or changing the orientation of light source 214, and/or by moving and/or changing the orientation of one or more optical components on the path of light beam 216, for example one or more of dichroic mirror 218, lens 222, and lens 230. Moving or rotating light source 214 or one or more of the optical components on the path of the light beam is done, for example, by a stepper motor, not shown in FIG. 2. Moving light beam 216, instead of or in addition to moving clump 212, has the potential advantage that it might be possible to do more rapidly than moving clump 212, for example at 10 Hz rather than 2 Hz, thereby modulating the illumination of clump 212 more rapidly. Modulating the illumination of the beads, by moving the light beam illuminating the beads, can also be done in a system such as 100 in FIG. 1, in which the beads do not move, by moving light beam 108 so that beads 104 are alternately illuminated and not illuminated, or alternately illuminated at a higher and lower intensity, and this can be done even using non-magnetic beads.

A controller 232 receives signals of fluorescent emission as a function of time, generated by detector 226. Controller 232 also optionally controls the switching on and off of magnets 204 and 206 if they are electromagnets, or their motion or rotation if they are permanent magnets, or the motion of a stepper motor that moves the position of light beam 216. Or at least controller 232 has information about when each magnet is switched on and off, or about how it is oriented or where it is located, or about the position of light beam 216 if it is changing. By looking at a component of the detector signal that is modulated at the frequency at which light beam 216 is moving relative to the beads, system 200 can potentially reject background noise, in particular the very significant noise caused by unbound fluorescent molecules in the surrounding water, excited by light beam 216, or by Raman scattering of light beam 216 by the surrounding water. As a result, system 200, or system 100 if it moves the light beam, can potentially detect very low levels of fluorescent emission of the beads, possibly making a bio-assay performed with system 200, using magnetic beads, much more sensitive than a bio-assay performed with system 100 without moving the light beam. But this increased sensitivity also means that a bio-assay performed with system 200, or with system 100 moving the light beam, may be more subject to interference from low levels of autofluorescence of the beads, and it may be especially important to reduce autofluorescence of the beads, in order to be able to take full advantage of that increased sensitivity.

Controller 232 optionally calculates a concentration of analyte in the biological sample, or a level of autofluorescence in the beads, using the modulated component of the signal from detector 226. Controller 232 optionally also controls light source 214. Controller 232 is optionally connected to a user interface 234, similar to user interface 126 of system 100, allowing a user to obtain information on the calculated concentration of analyte of level of autofluorescence, and/or to control the functions performed by system 200, and the parameters to be used.

When system 200 is used to photobleach beads, one of the magnets, if electromagnets are used, is optionally left turned on for an extended time while the other magnet is left off, producing a magnetic field gradient in container 202 that forces clump 212 of beads to rest against one of the walls of the container. If permanent magnets are used instead, then the same result is optionally achieved by leaving the magnets in a position and orientation to produce such a magnetic field gradient in container 202 on one side. The position and/or width of light beam 216 in container 202 is then optionally adjusted so that it illuminates the immobilized clump, for example by moving container 202 slightly, or by moving light source 214 slightly, or by adjusting one or more of lenses 222, 230, and dichroic mirror 218. The clump then remains illuminated at a high enough intensity of light, for a long enough time, and with an appropriate wavelength of light, so that the beads are photobleached down to a desired level of autofluorescence. Optionally, during the photobleaching, the clump is occasionally broken apart and reformed, by turning the magnet off and then on again, or moving it back and forth or rotating it, in order to allow different beads to be located on the outside and the interior of the clump, allowing all the beads to be exposed adequately to light beam 216. Additionally or alternatively, the beads are occasionally mixed mechanically, by pipetting the water out of and back into container 202, with the magnets turned off or moved away, which also helps to ensure that all of the beads are adequately exposed to light beam 216.

Although many parts of system 200 are not needed for photobleaching beads, a potential advantage of using system 200 for photobleaching, as with system 100, is that system 200 can be used to measure autofluorescence of a clump of beads, as soon as it is photobleached. A potential advantage of using a separate system for photobleaching beads is that if container 202 is too small, for example 0.4 mm across as in the system used by the inventors, then it may be difficult to remove photobleached beads from the container, to store them or sell them for later use in bio-assays. But in a separate system, used only for photobleaching beads and not for performing bio-assays, a wider container can be used.

Method of photobleaching beads

Figure 3 shows a flowchart 300 for an exemplary method of photobleaching beads, using for example a system similar to system 100 for photobleaching magnetic or non-magnetic beads, or a system similar to system 200 for photobleaching magnetic (that is, superparamagnetic) beads.

At 302, beads are placed in a container where they can be exposed to light, for example a transparent container, such as container 102 in system 100 or container 202 in system 200, where the beads can be exposed to light through the sides of the container. Alternatively, the container is open on top, even if it is not transparent, and the beads are illuminated through the open top.

At 304, in the case of magnetic beads being photobleached in a system like system 200, the beads are optionally aggregated by applying a magnetic field to them, and are optionally held immobilized on one side of the container by activating one of the electromagnets and not the other one. Although beads may be photobleached without aggregating them, aggregating magnetic beads may make it possible to photobleach a larger quantity of beads at one time. In the case of beads, magnetic or non-magnetic, that are being photobleached in a system like system 100, the beads are kept in place by having them rest on the bottom of the container, for example.

In some embodiments of the invention, for example as described below in FIG. 10, the beads are photobleached one at a time, as they pass through the channel of a microfluidics device, that is illuminated by a sufficiently strong light source, with the beads moving slowly enough, and the illuminated part of the channel long enough, so that the beads are exposed to the light for a long enough time to bleach them.

At 306, the beads are illuminated with a light source, at the proper wavelength, and with great enough intensity and for a long enough duration, so that the beads become photobleached to a desired degree. If using a system like system 100 or system 200, then the light beam is optionally given a position and width so that it illuminates all or most of the beads simultaneously. Alternatively, the light source is placed next to the beads, so that it illuminates the beads at a great enough intensity. For example, light at a wavelength of 488 nm or 532 nm, with an intensity of at least 10^5 W/m^2, and a duration of at least 15 minutes, has been found by the inventors to be sufficient for reducing the level of autofluorescence of several kinds of polymer beads by a large factor. For example, depending on the kind of beads used, the level of autofluorescence can be reduced by a factor of 2, 5, 10, 20, 50, 100, or a larger number, or by any intermediate number. Tests done by the inventors have shown that, for light intensities between 2×10^4 W/m^2 and 1.7×10^5 W/m^2, exposing M280 streptavidin beads to a total of $1.2 \times$

10^7 J/m^2 integrated over time is enough to reduce the autofluorescence to about 10% of its initial value, with bleaching and excitation light at 532 nm and emission measured at 560 nm. But an initial much steeper rate of decrease of autofluorescence, down to about 50% of the initial value, depends more weakly on the light intensity, varying from 0.33/sec to 0.56/sec over this range of intensities.

At 308, the level of autofluorescence of the beads is optionally measured, after or during bleaching, to verify that it has been reduced as much as expected. This can be done, for example, using the method described in FIG. 5 for performing a bio-assay, but using a sample without any of the analyte or fluorescently labeled probes present, or using beads without any ligand attached to them, or without any reporter molecules, so that the emitted fluorescence comes entirely or at least largely from autofluorescence.

Beads can be photobleached, for example using the method of FIG. 3, at any stage in the manufacturing process, or at any time after manufacturing, including by the end user at least up until the beads are used in a bio-assay. Optionally, the beads are bleached a year before being used in a bio-assay, or 6 months before being used, or 2 months before, or 1 month before, or 1 week before, or 3 days before, or 1 day before being used. Optionally, the beads are sold with a label indicating the date of bleaching, and/or with a recommended "use by" date, and/or with an indication of an expected level of autofluorescence at one or more different dates.

Even before the polymer is formed into beads, batches of the polymer can be photobleached in a similar way. If photobleaching is done as part of the manufacturing process, either to batches of beads or batches of unformed polymer, optionally a scaled-up version of an apparatus such as that of FIG. 1 is used, capable of photobleaching larger quantities of beads or polymer than the quantities of beads used in a bio-assay. If the capture surface element is a structural part of a bio-assay system, for example a microfluidics system or another system similar to system 700 in FIG. 7, then the capture surface element is optionally photobleached after it has been incorporated into the bio-assay system, for example as part of the manufacturing process of the bio-assay system, or by an end user before using the bio-assay system to perform an assay. Alternatively, the capture surface element is photobleached before it is incorporated into the bio-assay system. As used herein, the "manufacturing process" of the bio-assay system may comprise assembling the system from separate previously manufactured parts, optionally including the capture surface element, or producing a part or all of the system directly in a single piece, optionally including the capture surface element, for example by molding a polymer that the system is made from, or it may comprise some of both processes.

As used herein, a capture surface element is suitable or configured to use for providing a capture surface for a bio-assay system, if it is a bio-assay bead, or if it is incorporated as a structural part of a bio-assay system, or if it is shaped specifically for assembling into a bio-assay system to provide the capture surface. A plain piece of polymer, not already part of a bio-assay system and not shaped specifically for assembling into a bio-assay system, is not considered to be a piece suitable or configured for use for providing a capture surface for a bio-assay system, even though it could be fashioned into such a piece.

Measured levels of autofluorescence of bleached and unbleached beads

Figure 4 shows a bar graph 400, comparing the level of autofluorescence measured before and after photobleaching for five types of commercially available bio-assay beads. Four of these beads are magnetic: PA-D (Protein-A Dynabeads, made by Thermo Fisher, 2.8 μm in diameter), M280 (Streptavidin Dynabeads, 2.8 μm in diameter), MagPlex (carboxylated Microspheres, made by Luminex, 6.5 μm in diameter), and PA-B (Protein-A Surebeads, made by Bio-Rad, 2.4-3.4 μm in diameter), and they were bleached, and their autofluorescence was measured before and after bleaching, using a system similar to system 200 shown in FIG. 2. The excitation wavelength was 532 nm, and the emission was measured in a narrow band around 560 nm. The fifth type of bead, Certified Blanks (Certified Blank beads 890, made by Bangs Laboratories), is non-magnetic, and it was bleached, and its autofluorescence was measured before and after bleaching, using a system similar to system 100 shown in FIG. 1. In order to compare the autofluorescence of the Certified Blanks before bleaching, with the autofluorescence of the magnetic beads, the Magplex beads were also bleached, and had their autofluorescence measured before and after bleaching, using the same system used for the Certified Blank beads.

The levels of autofluorescence measured for these beads have been corrected using an estimated correction factor to take into account that the beads are configured in a layer that is not very much thicker than a bead diameter, and, in the case of the magnetic beads measured in a system similar to system 200, the layer is also not much wider than the diameter of excitation light beam 216. To estimate the correction factor for the magnetic beads measured in the system similar to system 200, a measurement was also made of autofluorescent emission from one of the types of magnetic beads, M280 beads, in a system similar to system 100, using broad flat layers of beads with different known values of thickness, which can be controlled in a system like system 100, in contrast to system 200 where it is difficult to determine the thickness of a clump of magnetized beads. Measurements of emission were made with a layer that was 1 bead

diameter thick, 2 bead diameters thick, 3 bead diameters thick, 5 bead diameters thick, 10 bead diameters thick, and 20 bead diameters thick. The same optical system and illumination beam diameter were used for these measurements, and for the measurements made in a system like system 200, so it should be meaningful to compare the normalized levels of autofluorescence, defined by Eq. (3), seen in the two cases. It was found that the normalized level of autofluorescence seen in the system like system 200 was about 180 nm, which is close to the level seen in the system like system 100 when the layer of beads was only 1 bead diameter thick. The emission level in the system like system 100 was proportional to the thickness of the layer up to a thickness of about 5 bead diameters, and then saturated, increasing very slowly for thicker layers. For a layer 20 bead diameters thick, the signal was about 5.5 times as great as for a layer 1 bead diameter thick. Assuming that this ratio would apply also for the other types of magnetic beads, the normalized levels of emission measured for the different magnetic beads in the system like system 200 were multiplied by a correction factor of 5.5.

For the measurements of fluorescent emission made in the system like system 100 for the Certified Blank beads, a correction factor was estimated by comparing the normalized level of autofluorescence found in the system like system 100 for Magplex beads, with the level found in the system like system 200 for Magplex beads. The Magplex beads have similar bead diameter to the Certified Blank beads, and were in a layer of about the same thickness as the Certified Blank beads when the measurements were made in a system like system 100, so it was assumed that approximately the same correction factor would apply to the Certified Blank beads and to the Magplex beads. The normalized level of autofluorescence of the Magplex beads in the system like system 100 was about 4.5 times as great as the normalized level of autofluorescence of the Magplex beads in the system like system 200. Assuming that the effective thickness of the layer in the system like system 200 was about 1 bead diameter, the thickness of the layer in the system like system 100 would have been about 5 bead diameters, and a correction factor of 1.19 should be applied to find the normalized level of autofluorescence that would be measured for a much thicker layer, such as 20 bead diameters thick. This correction factor was also assumed to apply to the Certified Blank beads whose normalized level of autofluorescence was measured in the system like system 100.

It should be noted that all of these beads had their autofluorescence reduced by at least a factor of 4, even those beads, like the Certified Blanks, that had very low autofluorescence to begin with, and some of the magnetic beads had their autofluorescence reduced by a factor of more than 100. On the other hand, the inventors have found that, once beads are fully photobleached, further exposure to light of similar intensity, wavelength, and duration, will have

much less effect in reducing the autofluorescence, even if the autofluorescence recovers somewhat several days after bleaching. For example, the further attempt at photobleaching typically only reduces the autofluorescence by less than 20% or 30%. This difference, incidentally, may provide a way to distinguish photobleached beads from beads that were initially manufactured with low autofluorescence, even if the past history of the beads is not known. A photobleached bead could be defined, for example, as a bead whose autofluorescence is not reduced by more than a factor of 2, when it is exposed to light of a wavelength, intensity and duration that would normally produce a reduction in autofluorescence much greater than that, when applied to never bleached beads.

Method of performing a bio-assay using bleached magnetic beads

Figure 5 shows a flowchart 500, for an exemplary method of performing a "sandwich" bio-assay using photobleached magnetic beads, using a magnetic modulation bio-sensing (MMB) system to measure a fluorescent signal from the beads. As noted above in the section headed "Performing bio-assays with bleached beads," MMB bio-assays may have particularly high sensitivity, but other types of bio-assays can also be performed with photobleached beads, magnetic or non-magnetic, or with other types of photobleached capture surface elements. At 502, bleached beads are prepared, with an attached ligand that binds to the analyte that the bio-assay is measuring. The ligand may be attached before or after bleaching the autofluorescence of the beads. Although attaching the ligand after photobleaching has the potential advantage that the photobleaching process will not adversely affect the ligand, some tests done by the inventors have shown that the ligand need not be affected by the photobleaching. Attaching the ligand after photobleaching also has the potential advantage that a large number of beads can be photobleached in advance and stored for later use, or even sold commercially after being photobleached by the manufacturer. Attaching the ligand before photobleaching has the potential advantage that the photobleaching can be done immediately before the assay, avoiding possible recovery of the autofluorescence after photobleaching. However, tests by the inventors have shown relatively little recovery of autofluorescence even several days or weeks after photobleaching, for the beads tested. In some cases, apparent recovery of autofluorescence may be due to not exposing all beads equally to light, when they were being photobleached.

At 504, the beads are exposed to the sample, so that molecules of the analyte, if present in the sample, bind to the ligand that is attached to the surface of the beads.

At 506, the remaining sample, including all or almost all unbound molecules of the analyte, is optionally washed away. At 508, the beads are exposed to fluorescent reporter

molecules, which ideally only bind to beads that have the analyte bound to them via the ligand. Reporter molecules can include fluorescent dyes such as rhodamine, fluorescein, phycobiliproteins, nitrobenzoxadiazole, acridines, BODIPY and cyanine compounds or their derivates. Fluorescent quantum dots and lanthanide chelates can also be used as reporter molecules. Typically, the reporter molecules are attached to their own reporter ligand molecules that bind specifically to the molecules of analyte, and thereby bind indirectly to the beads. At 510, excess unbound reporter molecules are optionally washed away.

At 512, electric current is alternately applied to the electromagnets on the two sides of the container, so that the beads are magnetized and form a clump, which travels back and forth across the container, attracted by the magnetic field gradient to whichever electromagnet has current applied to it at a given time. For example, the current applied to each electromagnet is a square wave going between zero and the maximum current, with the square wave 180 degrees out of phase in the two electromagnets. The frequency of the square wave is, for example, 1 Hz. As noted above, a similar effect can be achieved by alternately moving magnets, for example permanent magnets, closer and further away from each side of the container, or by rotating magnets, for example permanent magnets, to alternately produce a stronger and weaker magnetic field gradient on each side of the container.

At 514, a part of the container is illuminated with fluorescent excitation light, and optionally a part of the container is not, or is illuminated less strongly. This will result in a modulation of the fluorescent emission light at the frequency of modulation of the current of the electromagnets, or at twice the frequency of modulation of the current, depending on whether the beads are illuminated in the middle of their range of motion, or at one end of the their range of motion. Alternatively, even if the container is illuminated uniformly, the fluorescent emission light may be significantly modulated at the frequency of modulation of the current of the electromagnets, or at twice that frequency, if the field of view of the detector is narrower than the width of the container, and the beads are moving into and out of the field of view of the detector.

At 516, the modulated fluorescent emission light is recorded as a function of time. At 518, the component of fluorescent emission at the expected modulation frequency is optionally calculated, and used to calculate the concentration of analyte in the sample. Alternatively, the motion of the beads is more complicated as a function of time, and the modulation of the fluorescent emission is more complicated as a function of time, involving more than one modulation frequency, possibly with specific phase relations between them, and components of the fluorescent emission signal at these multiple frequencies, possibly including their relative

phases, are used to calculate the concentration of analyte in the sample. Optionally, the concentration of analyte in the sample is calculated using results from calibration samples with known quantities of analyte.

The method of flowchart 500 is called a "sandwich" bio-assay, because a capture ligand attaches the analyte molecules to the beads, and fluorescent reporter molecules are then attached to the analyte molecules by a reporter ligand, sandwiching the analyte molecules between the beads and the reporter molecules. As noted above, system 100 or system 200 can also be used to perform other types of bio-assays, in which fluorescent reporter molecules, attached indirectly to beads, emit a fluorescent emission signal with an intensity that depends on the concentration of analyte in the biological sample. For example, in another embodiment of the invention, instead of using a "sandwich" bio-assay, a fluorescence resonance energy transfer (FRET) bio-assay is used, optionally also using a magnetic modulation biosensing (MMB) system to measure the fluorescent signal from the beads. Both "sandwich" and FRET bio-assays, with an MMB system to measure the fluorescent signal, are described, for example, by A. Danielli, N. Porat, M. Ehrlich and A. Arie, "Magnetic Modulation Biosensing for Rapid and Homogeneous Detection of Biological Targets at Low Concentrations," *Current Pharmaceutical Biotechnology* **11**, 128-137 (2010). In a FRET bio-assay, used for detecting a specific target DNA sequence, the probe is a strand of DNA, complementary to the target DNA, with a biotin molecule, for example, and a fluorescent reporter molecule attached to one end, and with a quencher molecule attached to the other end. As long as the quencher molecule is in such close proximity to the fluorescent reporter molecule, fluorescent emission from the reporter molecule is suppressed. The biological sample is exposed to this probe, and if the target DNA sequence is present in the sample, then the complementary DNA in the probe binds to the target DNA. The sample is then exposed to Taq polymerase, which breaks apart the complementary strand of DNA that is bound to the target DNA, separating the quencher from the fluorescent reporter molecule, but keeping the biotin molecule bound to the reporter molecule. A ligand on a photobleached bio-assay capture surface, for example a photobleached magnetic bio-assay bead, then captures the biotin molecule, so that the biotin molecule, with the reporter molecule bound to it, is attached to the surface of the bead. Measuring a fluorescent emission signal from the beads then provides a measure of the concentration of the target DNA sequence in the sample, because in the absence of the target DNA, the Taq polymerase will not break apart the complementary DNA strand of the probe, and the quencher will remain in the vicinity of the fluorescent reporter molecule, suppressing its fluorescent emission. Once the fluorescent reporter molecules are bound to the beads, the method optionally proceeds as in flowchart 500, starting at 512, as described above. In some

embodiments of the invention, for "sandwich" assays, FRET assays, or other types of assays, chemiluminescent or electro-chemiluminescent reporter molecules are used, instead of fluorescent reporter molecules, and the chemiluminescence or electro-chemiluminescence of the reporter molecules is excited and measured.

In another embodiment of the invention, a bio-assay for an analyte in a biological sample is performed using magnetic or non-magnetic beads. Bleached beads are prepared with an attached ligand, as in 502, and the prepared beads are exposed to the sample so that molecules of the analyte, if present in the sample, bind to the ligand, as in 504. The sample is optionally washed away, as in 506, the beads are exposed to fluorescent reporter molecules which bind indirectly to the beads only through binding to the analyte molecules that are bound to the ligand, as in 508, and excess reporter molecules are optionally washed away, as in 510. Alternatively, instead of performing a "sandwich" assay as in 502 through 510, a FRET assay is performed as described above, but in either case the end result will be beads with unquenched reporter molecules bound to them in a concentration that depends on the concentration of analyte in the sample. The beads, with the reporter molecules bound to them, are optionally placed in a container, such as container 102 of FIG. 1, in an optical system similar to system 100 of FIG. 1, or the beads may already be in such a container in such a system, They are illuminated with excitation light, of a wavelength that would produce fluorescent emission from the reporter molecules, for example from a light source similar to light source 106 of FIG. 1. The optical system is used to illuminate the beads with the excitation light, and to measure the fluorescent emission from the beads, as described above for FIG. 1, detecting the emission light with a light detector similar to detector 114. In this method, in contrast to the method of flowchart 500 of FIG. 5, the fluorescent emission light is not modulated, and as a result the bio-assay may be more subject to interference, and less sensitive, than the method of flowchart 500. On the other hand, this method does not require the use of magnetized beads and controllable magnets.

As noted above, a modulated bio-assay can also be performed, using a system similar to system 200 or system 100, by moving the light beam back and forth, while the beads remain in the fixed position, so that the light beam alternately illuminates the beads and does not illuminate the beads, or alternately illuminating the beads with a higher and lower intensity of light.

Results of bio-assays using bleached and unbleached beads

Figure 6 shows a plot 600 of the fluorescent emission signal vs. analyte concentration, using photobleached magnetic beads, and a similar plot 602 using unbleached magnetic beads. In both cases, the calibration samples were used, with different concentrations of IL-8, including 0,

0.5, 5, and 50 picograms per milliliter. In plot 602, the beads have relatively high autofluorescence, which contributes significantly to the fluorescent emission signal even at a concentration of 5 pg/mL, and is the overwhelmingly dominant contribution of the fluorescent emission signal at 0.5 pg/mL and below. At 5 pg/mL, the signal to noise ratio, the ratio of the fluorescent emission signal to the autofluorescence noise level, is about 1. It is estimated that, due to this autofluorescence of the unbleached beads, the assay cannot detect concentrations of IL-8 lower than about 1.5 pg/mL. In plot 600, the autofluorescence of the beads is much lower, and is a very small contribution to the fluorescent emission signal even at 0.5 pg/mL. The signal to noise ratio at 5 pg/mL is about 50, much higher than for the unbleached beads. It is estimated that with these photobleached beads, the assay could detect concentrations of IL-8 as low as 0.04 pg/mL.

Bio-assay system with bleached structural capture surface element

Figure 7 shows an exemplary bio-assay system 700, similar to bio-assay system 100 in FIG. 1, but instead of using polymer beads 104 in container 102 as a capture surface, there is a cell 702 with a single flat polymer capture surface 704 incorporated into the cell. Capture surface 704 may be made of any of the polymers that may be used for beads 104, and possibly of other polymers as well, for example any of PMMA, polycarbonate, Topas (Cyclo Olefin Copolymer), and Zeonor (Cyclo Olefin Polymer), and like the beads, capture surface 704 exhibits autofluorescence. Optionally, cell 702 is part of a microfluidics system, including an input channel 706 for bringing a biological sample in contact with surface 704, and an exit channel 708 for removing the sample after molecules of analyte in the sample are bound to a ligand on surface 704. Input channel 706 and exit channel 708 are also optionally used for any of the steps used in preparing a heterogeneous bio-assay, for example introducing a ligand, specific to the analyte, for binding to the capture surface, introducing fluorescent reporter molecules that bind to the ligand when it is bound to the analyte, and removing excess ligand and reporter molecules that are not bound to the capture surface. The other parts of system 700 shown in FIG. 7 are similar to the parts with the same numbers shown in FIG. 1, and play a similar role in performing bio-assays. In particular, beam 108 from light source 106 is optionally used to photobleach capture surface 704, by illuminating it with light of a duration, intensity, and wavelength suitable for substantially reducing the autofluorescence of capture surface 704, just as beam 108 from light source 106 is optionally used in system 100 for photobleaching beads 104. Reducing the autofluorescence of capture surface 704, like reducing the autofluorescence of beads 104, has the

potential advantage of reducing background interference with the fluorescent signal of the bio-assay, possibly making the bio-assay sensitive to lower concentrations of the analyte.

Optionally, if system 700 is used repeatedly over several days or several weeks, to perform bio-assays, capture surface 704 is periodically photobleached, for example once a day or once a week, to bring its autofluorescence down to a low level, since for some polymers, autofluorescence may partially recover over a period of days after photobleaching.

Alternatively, rather than photobleaching the capture surface periodically, a measurement is made of the autofluorescence of the capture surface 704, periodically, or before each bio-assay is performed, and if the measured autofluorescence is higher than a predefined value, then the capture surface is photobleached. Optionally, a similar procedure is used in system 100, particularly if beads are used that have been quenched earlier, for example by the manufacturer, but their autofluorescence may have recovered, partially or fully, since they were bleached. The autofluorescence of the beads is optionally measured before performing each bio-assay, and the beads are photobleached if their autofluorescence is too high. The determination of whether or not the capture surface, or the beads, should be photobleached, is optionally made based on the composition of the polymer. A table is optionally provided, listing the expected level of autofluorescence, for each type of polymer, before any bleaching, and immediately after bleaching, as shown for example in FIG. 4. If the measured autofluorescence has gone too far back toward its value before bleaching, for example more than 2%, 5%, 10%, 20%, or 50% of the way from its value immediately after bleaching to its original unbleached value, or if the level of autofluorescence has increased by more than a factor of 1.2, 1.5, 2, or 5 from its value immediately after bleaching, then the beads or capture surface are optionally photobleached to bring the level autofluorescence back down again. It should be noted that the expected levels of autofluorescence may depend not only on the bulk composition of the polymer, but also on details of how the beads or the capture surface element are manufactured, chemically treated, etc., and the table may take those details into account in the list of expected levels of autofluorescence.

System for mixing and photobleaching magnetic beads

Figure 8 schematically shows a system 800 for mixing and photobleaching magnetic beads, according to an exemplary embodiment of the invention. System 800 may be especially useful for preparing a large quantity of photobleached magnetic beads in advance, a much larger quantity than would be used in one bio-assay. The photobleached beads can then be saved for later use in bio-assays, or sold to end users. Although system 200 in FIG. 2 can also be used for

photobleaching magnetic beads, the inventors have found some potential advantages in using separate systems for photobleaching magnetic beads and performing bio-assays with the beads. For example, cuvette 202 in system 200 may have very small dimensions, so that it can be used to perform a bio-assay on a very small sample, and so that the clump of beads does not have to move very far when it is pulled from one side of the cuvette to the other side by the magnets. For example, in a system used by the inventors, similar to system 200, cuvette 202 is 0.4 mm thick in the direction of the magnetic field (the vertical direction as seen in FIG. 2). With such a small cuvette, the inventors have found that it can be very difficult to remove beads from the cuvette once they are bleached, so such a system is best used to photobleach only a small quantity of beads immediately before they are used in a bio-assay. If a batch of beads must be photobleached before each bio-assay, this can greatly increase the time required to perform a bio-assay, including the time required for the photobleaching. System 800 has a much larger cuvette, for example 4 mm thick in the direction of the magnetic field, and it is possible to photobleach a large quantity of magnetic beads in advance, using system 800, and then to remove the beads from system 800, and to store them for later use in bio-assays. When photobleaching such a large quantity of beads, it may be advantageous to thoroughly mix the beads during the photobleaching process, so that the beads are photobleached fairly uniformly, and system 800 provides a method for mixing the beads. It should be noted that system 800 is configured specifically for using a magnetic method of mixing, suitable only for magnetic beads. But the method of photobleaching a large quantity of beads by exposing only some of the beads to a suitable intensity of light at a given time, and mixing the beads so that different beads are successively exposed to the suitable intensity of light, can be implemented also using a different kind of mixing system, for example using mechanical shaking or stirring to mix the beads, and in that case the method can be used also for photobleaching non-magnetic beads.

System 800 comprises a container 802, such as a glass cuvette, which is filled with a batch of magnetic beads to be photobleached, suspended in a fluid 804, such as water or a buffer solution. It should be noted that, in the exemplary method described here, the magnetic beads are superparamagnetic beads, small enough so that they do not become permanently magnetized, but are only magnetized when in the presence of an external magnetic field. In the absence of a strong enough external magnetic field, the beads do not clump together, but remain suspended separately in fluid 804, and may slowly fall to the bottom of container 802 if they are not disturbed. A light source 806, for example a laser, an LED, or any other known light source, produces a light beam 807 that is used for photobleaching the beads, for example a light beam of 488 nm wavelength, or 532 nm, or any other wavelength or mixture of wavelengths used for

photobleaching magnetic polymer beads. Light beam 807 is optionally focused by a lens 808, for example a convex lens, or a plurality of lenses, with a focal length of 100 mm or 50 mm, with the light source and the lens positioned so that beam 807 is focused, or almost focused, at a location in the container 802 where fluid 804 is located. Alternatively, light beam 807 illuminates container 802 without being focused, but focusing light beam 807 has the potential advantage that the intensity of the light on the beads can be increased, and the beam power can be concentrated more on the beads, where it is useful. A stepper motor 810 is controlled by a control board 812, for example one of the control boards and motor control shields made by Arduino, and the control board and stepper motor are controlled by a power supply 814, or by two separate power supplies. Stepper motor 810 has an arm 816 mounted on it, and can rotate the arm back and forth around an axis of motor 810. Arm 816 has two cylindrical magnets 818 and 820 mounted on it, held in place by hollow cylindrical magnet holders. The operation of system 800 for mixing and photobleaching magnetic beads is described in FIGS. 9A-9D.

Figure 9A shows a schematic view of stepper motor 810 as seen along its axis, together with container 802, and magnetic beads suspended in water 804 in the container. Arm 816 is shown initially at its lowest position, with magnets 818 and 820 nowhere near container 802 and the magnetic beads. Because the magnetic field and field gradient in container 802 are very low when arm 816 is in this position, the magnetic beads are essentially not magnetized and there are essentially no magnetic forces on them. The beads remain suspended separately in the water, and may slowly settle toward the bottom of container 802.

In FIG. 9B, the stepper motor has rotated arm 816 so that magnet 818 is positioned directly against container 802, and the cylindrical axis of the magnet coincides with the axis of light beam 807. Magnet 818 produces a strong magnetic field and field gradient inside container 802, magnetizing the magnetic beads and causing them to attract each other and form a clump 902. Because of the magnetic field gradient produced by magnet 818, the clump is attracted to the wall of the container closest to magnet 818, and remains on that wall. However, magnet 818 blocks light beam 807 from reaching clump 902.

Stepper motor 810, under the control of control board 812, keeps arm 816 in the position shown in FIG. 9B for a short period of time, long enough for clump 902 to form, for example for 5 seconds. Then, stepper motor 810 moves arm 816 clockwise, moving magnet 818 away from container 802, optionally until arm 816 is at its lowest point, as shown in FIG. 9C, or at least until magnet 818 is no longer blocking light beam 807 from reaching clump 902. Stepper motor 810 remains in this position for a period of time, for example two minutes, during which light beam 807 illuminates clump 902. During this time, clump 902 gradually starts to break apart and

to slide down the wall of container 802 toward the bottom of the container, but parts of clump 902 remain illuminated by light beam 807 during most of this time period.

Even in the portion of the clump that is directly illuminated by light beam 807, the light beam generally becomes less intense as it penetrates through several layers of beads, since it may be absorbed to some extent by the beads, particularly by their magnetic cores, and it may spread out laterally due to reflection, refraction, and diffraction from the beads. In addition, clump 902 may be wider than light beam 807, especially as it starts to break up and slide down the wall of the container. As a result, only a fraction of the beads in clump 902, typically only a small fraction, are exposed to the full intensity of focused light beam 807, and the other beads in clump 902 are exposed to a much lower intensity of light, or to hardly any of the light from light beam 807, and undergo much less bleaching during that time than the beads that are exposed to the full intensity of focused light beam 807. However, as will be described, the mixing process eventually brings these other beads to a location where they will be exposed to the full intensity of focused light beam 807, and will also undergo bleaching.

Stepper motor 810 again moves arm 816 clockwise, until the other magnet, magnet 820, is directly in contact with container 802 on the side opposite light source 806, as shown in FIG. 9D. The magnetic beads again become magnetized, this time due to the magnetic field of magnet 820, and form a new clump 904, which is attracted by the magnetic field gradient to the other side of container 802, adjacent to magnet 820, centered on the axis of magnet 820. Clump 904, or at least a portion of clump 904, is now illuminated by light beam 807. Stepper motor 810, under the control of control board 814, keeps arm 816 in the position shown in FIG. 9D, with magnet 820 adjacent to container 802, for a period of time, for example two minutes, optionally the same period of time that arm 816 was kept in the position shown in FIG. 9C while clump 902 was illuminated. The beads in clump 904 that are directly illuminated by light beam 807, down to some depth, receive the full intensity, or a large part of the full intensity, of light beam 807, but the rest of the beads in clump 904 are exposed to a much lower intensity of light, or to almost no light. Because the beads in clump 902 separate and settle down toward the bottom of container 802 at different rates, after magnet 818 moves away from container 802, the beads are mixed to some extent between the break up of clump 902 and the formation of clump 904, and in general different beads are exposed to the full intensity of light beam 807 in FIG. 9D, than were exposed to the full intensity of light beam 807 in FIG. 9C.

Stepper motor 810 then moves arm 816 counter-clockwise, removing magnet 820 from container 802, swinging arm 816 past the bottom position shown in FIG. 9A, and returning it to the position shown in FIG. 9B, with magnet 818 adjacent to container 802. This cycle then

repeats many times, re-forming a clump from the beads each time one of the magnets is brought adjacent to container 802, and exposing each clump to light beam 807. Because the beads are mixed to some extent each time a clump breaks up and is re-formed, over a sufficient number of cycles almost every bead will be exposed to the full intensity or nearly the full intensity of light beam 807 for approximately the same amount of time, a long enough total time to substantially photobleach the beads. It should be noted, though, that the time needed to photobleach a bead down to a given level of autofluorescence, at a given light intensity, may be longer if the bead is exposed to that light intensity at a low duty cycle, as in system 800, rather than continuously. A small sample of the beads can then be removed from container 802, and tested elsewhere, for example in a system similar to system 200, to measure their autofluorescence. Or, if system 800 is equipped with a light source, light detector and optics suitable for measuring autofluorescence of the beads in container 802, similar to system 100 or system 200, then their autofluorescence can be measured without removing them. If they have not reached the desired level of autofluorescence, then the cycle shown in FIGS. 9B-9D can be continued for a period of time, for example for another hour, and another small sample of beads can be removed for testing, and the process can be repeated, if necessary, until the desired level of autofluorescence is reached.

In some embodiments of the invention, permanent magnets 818 and 820 are replaced by electromagnets, which need not be moved back and forth by a stepper motor, but optionally can instead be alternately turned on and off. However, using permanent magnets has the potential advantage that, in a system of the small dimensions described here for system 800, and in particular for the small dimensions of magnets 818 and 820, much stronger magnetic fields can be achieved with permanent magnets than with electromagnets.

Although magnets 818 and 820 are shown as cylinders with flat end surfaces in FIGS. 8 and 9A-9D, magnets of other shapes may be used. In particular, the inventors have found that better results may be achieved if one or both of magnets 818 and 820 has a protruding curved or pointed end on the side that is adjacent to the beads. The shapes of the protruding ends need not be the same for the two magnets. Such a protruding end may make the magnetic field gradient greater for a given energy product of the permanent magnet material and a given ratio of length to diameter of the magnets. The higher magnetic field gradient may result in better mixing of the beads when the beads are moved from one side of the cuvette to the other.

In some embodiments of the invention, instead of two magnets that are alternately brought adjacent to opposite sides of the container, a single magnet swings back and forth, alternately brought adjacent to opposite sides of the container. In other embodiments of the invention, there is only a single magnet alternately brought adjacent to, and away from, one side

of the container, and the beads are illuminated on that side of the container, while the magnet is adjacent to the container, and/or after the magnet has moved away from the container. But the inventors have found that the system shown in FIGS. 8 and 9A-9D, with the clump of bead alternately formed on opposite sides of the container, is effective for mixing the beads and photobleaching almost all of them. It seems possible that the beads, in travelling from one side of the container to the other side and re-forming the clump in a different location, will undergo more mixing than if the clump were re-formed each time at the same location in the container.

In a test of such a system, similar to system 800, done by the inventors, the inside of container 802 is 4.5 mm thick in the direction of the light beam, the magnets are DH28 cylindrical neodymium magnets, 0.1 inches in diameter and 0.5 inches long, the fluid is a 1XPBSTB buffer solution, and as many as 150,000 Magplex beads were bleached at a time. The Magplex beads are each 6.5 μm in diameter, and they form a flat pancake-like clump that is about 1 mm in diameter. A 4.5 mW laser, of wavelength 532 nm, is used as the light source, with a beam diameter that is 3.5 mm initially, and is reduced to about 1 mm where it reaches the beads, by the lens, so that the light intensity at the beads is about 6 kW/m^2. In these circumstances, the level of autofluorescence of the beads was reduced to 5% of its initial value, after continuing the cycle shown in FIGS. 9B-9D for four hours. More time was required to photobleach a similar volume of M-280 beads, only 2.8 μm in diameter, perhaps because, with more closely spaced layers of beads, the light beam does not penetrate as far into the clump, and more cycles of dispersing and re-forming the clump are needed, on average, before a bead is found close enough to the outside of the clump to be fully exposed to the light beam.

Alternatively, container 802 and magnets 818 and 820, and light beam 807 when it comes out of the light source and when it is focused, have different dimensions, for example any of their dimensions are smaller or larger by a factor of 1.2, 1.5, 2, 3, 5, 7, 10, 15, or 20, than the dimensions described above. Alternatively, stronger or weaker permanent magnets are used, for example with energy product smaller or larger by a factor of 1.2, 1.5, 2, 3, 5, 7, or 10, up to the maximum energy product available, or with coercivity greater or less than that of DH28 neodymium magnets, by any of those factors, up to the maximum coercivity available. However, if substantially lower coercivity magnets are used, then it is potentially advantageous for the magnets to have a greater ratio of length to diameter, so they remain fully or almost fully magnetized. Using magnets made of high coercivity permanent magnet materials, such as neodymium magnets, has the potential advantage that the magnets can be made shorter relative to their diameter, potentially allowing a greater area on the wall of the container where a clump can form, and allowing the clump to be spread out, and exposed to the light beam, over a larger

area. If it is desired to photobleach a larger volume of beads, then it is potentially advantageous to use larger dimensions for the container, the magnets, and the light beam, so that a larger area of beads can be exposed to the light at a given time. A light source of lower or higher power is optionally used, for example lower or higher by a factor of 1.2, 1.5, 2, 3, 5, 7, 10, 15, 20, 30, or 50. Using a higher power light source, for the same diameter of the light beam where it illuminates the beads, has the potential advantage that the beads may be bleached more quickly, but if the power per area is too high, the beads may be damaged. A higher power light source can also illuminate a greater area of beads with the same intensity, potentially allowing more beads to be photobleached at a time.

Figure 10 shows an alternative system 1000 for photobleaching beads, in which beads are each exposed directly to the light, instead of exposing an optically thick clump of beads to the light, so it is not necessary to mix the beads and expose them to the light repeatedly, in order to ensure that they are all exposed to the light. System 1000 is a microfluidic system, comprising a source reservoir 1002, a microfluidic channel 1004, and a destination reservoir 1006. A microfluidic pump, not shown in FIG. 10, causes fluid, with beads suspended in it, to flow from source reservoir 1002, through channel 1004, to destination reservoir 1006. A light source 1008, for example a laser, illuminates channel 1004, and the beads flowing through it, with sufficient light intensity of suitable wavelength or range of wavelengths, and for a long enough exposure time, to photobleach the beads to a desired level of autofluorescence. The exposure time is the width of the light beam, along channel 1004, divided by the speed of flow of the fluid and beads in the channel. Optionally, a level of autofluorescence of the bleached beads is measured after bleaching them, while they are still in the channel, to verify that they are being bleached to the desired level of autofluorescence, and optionally adjustments are made in the intensity of light and/or the exposure time, if the beads are not being bleached to the expected level.

A potential advantage of system 1000, over systems such as system 800 where a thick layer of beads is exposed to light for bleaching, is that it may be possible to better control the resulting level of autofluorescence, and to make the level of autofluorescence more consistent among different beads. A potential advantage of a system such as system 800, is that it may have a greater throughput of beads, because so many beads are bleached at one time, even though it may take longer to bleach each bead.

Alternatively, instead of using a microfluidic channel to bleach beads individually, beads are bleached in a system like system 100, but with only a single layer of beads in container 102. Optionally in this case, container 102 is made wider so that more beads can be bleached at one time, while still keeping the beads only one layer thick. Using a wider container may be

especially practical if the system is used only for bleaching beads, and not used also for bio-assays. However, configuring the system so that it can also be used for bio-assays, or at least can be used to measure the autofluorescence of the beads, has the potential advantage that the level of autofluorescence of the beads can be checked *in situ* after the beads are bleached. Optionally, container 102 is made much wider than light beam 108 used for photobleaching the beads, and the light beam is scanned over the container, to photobleach all the beads in it. Such a system, like system 1000, has the potential advantage over a mixing system that it may be easier to control the resulting level of autofluorescence of the beads, and to make it consistent for all the beads being bleached, though system 1000 may be even more effective for doing that. The system where beads are bleached in a single layer in container 102 has the potential advantage over system 1000 that it may have greater throughput than system 1000. But a system such as system 800, that bleaches a thick clump of beads and repeatedly mixes it, has the potential advantage that it may have still greater throughput.

It is expected that during the life of a patent maturing from this application many relevant fluorescence-based bio-assays and bio-assay capture surfaces, using different polymers, will be developed, and the scope of the terms fluorescence-based bio-assay and bio-assay capture surface is intended to include all such new technologies *a priori*.

As used herein the term “about” refers to ±10%.

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates mean "including but not limited to".

The term “consisting of” means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically

disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. A method of photobleaching polymer capture surface elements suitable to use for providing a capture surface in a fluorescent bio-assay, the method comprising:

a) providing one or more of the capture surface elements having an autofluorescence excited by light in a peak range of excitation wavelengths, producing light predominantly in a peak range of emission wavelengths that may depend on the excitation wavelength; and

b) exposing the capture surface elements to light from a light source used for bleaching, with suitable wavelength distribution and sufficient intensity and duration so that a level of the autofluorescence, for at least one excitation wavelength within the peak range of excitation wavelengths and at least one emission wavelength within the peak range of emission wavelengths for that excitation wavelength, is reduced by at least a factor of 2.

2. A method according to claim 1, wherein the one or more polymer capture surface elements comprise a plurality of polymer beads, between 0.3 and 10 micrometers in diameter, and the capture surface comprises outer surfaces of the polymer beads.

3. A method according to claim 2, wherein exposing the capture surface elements to light from the light source used for bleaching comprises causing the beads to move along a micro-fluidic channel illuminated by the light source used for bleaching.

4. A method according to claim 2, wherein the beads comprise one or more types of internally embedded fluorescent reporter molecules of sufficiently different peak range of excitation wavelengths from the autofluorescence of the beads, sufficiently low susceptibility to photobleaching, or both, so that said exposing the capture surface elements to light from the light source used for bleaching reduces a level of fluorescence of the internally embedded fluorescent reporter molecules by less than 10%, for at least one excitation wavelength within a peak range of excitation wavelengths for said fluorescence of the internally embedded fluorescent reporter molecules, for at least one emission wavelength within a peak range of emission wavelengths for that excitation wavelength.

5. A method according to claim 1, also comprising chemically bleaching the autofluorescence of the capture surface elements.

6. A method according to claim 1, wherein the light source used for bleaching comprises a laser.

7. A method according to claim 1, wherein the light source used for bleaching comprises a broadband light source.

8. A method according to claim 1, wherein the broadband light source is a flash lamp.

9. A method according to claim 1, wherein the autofluorescence is sufficiently bleached so that, if the capture surface elements are exposed to 10^5 W/m^2 of light at peak excitation wavelength for the autofluorescence for at least 15 minutes, after the bleaching, then the level of autofluorescence of the capture surface elements will be reduced by less than a factor of 2, or there will be no measurable reduction in level of autofluorescence of the capture surface elements, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength.

10. A method according to claim 1, wherein the light source used for bleaching produces an intensity of light at the capture surface of at least 500 W/m^2.

11. A method according to claim 10, wherein the light source used for bleaching produces said intensity of light at the capture surface for at least 1 second.

12. A method according to claim 1, wherein bleaching comprises exposing the capture surface elements to a light intensity integrated over time of at least 10^7 joules per square meter.

13. A method according to claim 1, wherein bleaching the autofluorescence of the one of more capture surface elements also comprises exposing the capture surface elements to a chemical bleaching agent.

14. A method according to claim 1, also comprising performing a bio-assay for detecting an analyte in a biological sample, after exposing the capture surface elements to light from the light source used for bleaching, by:

a) performing a preparatory procedure with the biological sample that results in reporter molecules being bound directly or indirectly to a capture surface of the capture surface elements, such that, when excited by excitation light of suitable wavelength range, the reporter molecules emit a fluorescent emission light of an intensity that depends on a concentration of the analyte in the biological sample;

b) exposing the capture surface to excitation light from an excitation light source, the same as or different from the light source used for bleaching, after performing the preparatory procedure, the excitation light having a wavelength distribution that would excite the autofluorescence of the capture surface elements;

c) measuring a signal of the fluorescent emission light emitted by the reporting molecules in response to the excitation light, over a band of emission wavelengths; and

d) determining a presence of the analyte in the sample, a concentration of the analyte in the sample, or both, from the measured fluorescence signal;

wherein the excitation light has a wavelength range that would excite the autofluorescence of the capture surface elements to emit light in the band of emission wavelengths.

15. A method according to claim 14, wherein the bio-assay comprises a sandwich assay, the preparatory procedure comprising exposing the capture surface to the biological sample, capturing molecules of the analyte, and binding the analyte molecules to the fluorescent reporter molecules, resulting in a concentration of the fluorescent reporter molecules bound indirectly to the capture surface depending on the concentration of analyte molecules in the biological sample.

16. A method according to claim 15, also comprising attaching to the capture surface a ligand that binds specifically to the analyte, wherein capturing molecules of the analyte comprises binding the molecules of the analyte to the ligand that is attached to the capture surface.

17. A method according to claim 16, wherein the ligand is attached to the capture surface before exposing the capture surface elements to light from the light source used for bleaching.

18. A method according to claim 16, wherein the ligand is attached to the capture surface after exposing the capture surface elements to light from the light source used for bleaching.

19. A method according to claim 14, wherein the bio-assay comprises a fluorescence resonance energy transfer (FRET) bio-assay.

20. A method according to claim 14, wherein a level of the analyte in the sample is such that the fluorescence signal emitted by the reporting molecules has higher power than an autofluorescence background from the capture surface elements, but lower power than the autofluorescence background would have been if the autofluorescence of the capture surface elements had not been bleached.

21. A method according to claim 14, wherein the excitation light source is the light source used for bleaching.

22. A method according to claim 21, wherein exposing the capture surface to the excitation light is done with the capture surface elements in a same location as exposing them to light from the light source used for bleaching.

23. A method according to claim 14, wherein the excitation light source comprises a laser.

24. A method according to claim 14, wherein measuring the fluorescence signal comprises integrating over a time longer than 1 second.

25. A method according to claim 24, wherein measuring the fluorescence signal comprises integrating over a time longer than 5 seconds.

26. A method according to claim 14, wherein performing the bio-assay comprises causing the capture surface elements to undergo an oscillatory motion relative to the excitation light while measuring the fluorescence signal, alternately illuminating the beads with higher and lower intensities of the excitation light, and measuring the fluorescence signal comprises measuring a time-varying component of the emitted fluorescence power that is synchronous with

a time variation of the intensity of the excitation light that the beads are exposed to, due to the relative motion of the excitation light and the beads.

27. A method according to claim 26, wherein the capture surface elements comprise a plurality of paramagnetic or superparamagnetic beads, and causing the capture surface elements to undergo an oscillatory motion relative to the excitation light comprises applying a magnetic field with time varying direction of gradient, that causes an oscillatory motion of the beads.

28. A method according to claim 26, wherein causing the capture surface elements to undergo an oscillatory motion relative to the excitation light comprises oscillating a position of a beam of the excitation light so that it alternately illuminates the capture surface elements at higher and lower intensities.

29. A method according to claim 14, wherein exposing the capture surface to excitation light and measuring the fluorescence signal is done when the capture surface is immersed in water or a water solution, and the fluorescence signal has power less than a background signal from one or both of Raman scattering from the water or water solution, and fluorescence from unbound fluorescent molecules in the water or water solution.

30. A method according to claim 14, wherein the reporter molecules have a peak excitation wavelength in the peak range of excitation wavelengths of the autofluorescence, and a peak emission wavelength in the peak range of emission wavelengths of the autofluorescence for a peak excitation wavelength of the autofluorescence.

31. A method according to claim 14, wherein the one or more capture surface elements are incorporated as one or more structural parts that provide the capture surface in a bio-assay system for performing the bio-assay.

32. A method according to claim 31, wherein the autofluorescence of at least one capture surface element is bleached before the capture surface element is incorporated into the bio-assay system.

33. A method according to claim 31, wherein the autofluorescence of at least one capture surface element is bleached after the capture surface element is incorporated into the bio-assay system.

34. A method according to claim 31, also comprising manufacturing the bio-assay system, and incorporating the one or more capture surface elements into the bio-assay system is at least a part of the manufacturing.

35. A method according to claim 14, wherein measuring the fluorescent signal comprises measuring light most of which has wavelength greater than a wavelength of the excitation light, and less than 700 nm.

36. A method according to claim 1, wherein, when the one or more capture surface elements are exposed to the light from the light source used for bleaching, they are comprised in one or more structural parts of a bio-assay system for performing a bio-assay that uses the capture surface elements for providing a capture surface.

37. A method according to claim 1, wherein the polymer capture surface elements comprise one or more of polystyrene, latex, methyl methacrylate, ethylene glycol dimethacrylate, and methacrylic acid.

38. A method according to claim 1, also comprising storing the capture surface elements for at least three days after bleaching the autofluorescence of the capture surface elements, substantially maintaining the reduced autofluorescence.

39. A method according to claim 1, wherein the capture surface elements comprise a plurality of polymer beads, and exposing the capture surface elements comprises:

a) exposing a portion of the beads in the container to light of suitable wavelength and intensity for photobleaching the beads, while exposing the other beads in the container to a lower intensity of light of suitable wavelength for photobleaching, or to no light of suitable wavelength for photobleaching;

b) mixing at least some of the beads in the container that were exposed to the intensity of light suitable for photobleaching, with at least some of the beads that were exposed to a lower intensity of light or to no light, during or after exposing the portion of the beads to the light; and

c) exposing at least some of the mixed beads in the container to the light of suitable wavelength and intensity for photobleaching, including at least some of the beads that were exposed to a lower intensity of light or to no light before the mixing.

40. A method according to claim 39, wherein exposing a portion of the beads to the light of an intensity suitable for photobleaching comprises exposing less than half of the beads to said light, the method also including repeating mixing the beads and exposing at least some of the mixed beads to the light of an intensity suitable for photobleaching, enough times, using enough of the beads for the mixing, doing the mixing thoroughly enough, and with a long enough total exposure time of the beads to the light, so that more than half of the beads are bleached to a level of autofluorescence at least a factor of 2 less than their level of autofluorescence before the photobleaching, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths.

41. A method according to claim 39, wherein the beads are magnetic beads, and mixing at least some of the beads comprises introducing a strong enough magnetic field to the beads to magnetize them and cause them to aggregate into a clump, decreasing the magnet field to cause the clump to disperse, and again introducing a strong enough magnetic field to cause the beads to aggregate into a clump.

42. A polymer bead product, suitable for use in bio-assays, with a measurable level of autofluorescence having a peak range of excitation wavelengths and a peak range of emission wavelengths for each excitation wavelength, the level of autofluorescence being reduced by photobleaching, on average, by at least a factor of 2 below what it would be for beads manufactured in a same way without the photobleaching, for at least one excitation wavelength in the peak range of excitation wavelengths and at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength.

43. A polymer bead product according to claim 42, the level of autofluorescence being sufficiently reduced by the photobleaching so that exposure of the bead product to 10^5 W/m^2 of light at a peak excitation wavelength of the autofluorescence, directed for at least 15 minutes, results in a reduction of less than a factor of 2 in level of autofluorescence of the bead product, or results in no measurable reduction in level of autofluorescence of the bead product,

for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength.

44. A polymer bead product according to claim 42, for which 532 nm is within the peak range of excitation wavelengths and 575 nm is within the peak range of emission wavelengths for an excitation wavelength of 532 nm, that has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 50% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

45. A polymer bead product according to claim 44 that has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 30% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

46. A polymer bead product according to claim 45 that has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 20% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

47. A polymer bead product according to claim 42, for which 532 nm is within the peak range of excitation wavelengths and 575 nm is within the peak range of emission wavelengths for an excitation wavelength of 532 nm, that has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 10 times a level of Raman scattering from a column of pure water 8 mm long at that excitation wavelength and emission wavelength.

48. A polymer bead product according to claim 47 that has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 6 times a level of Raman scattering from a column of pure water 8 mm long at that excitation wavelength and emission wavelength.

49. A polymer bead product according to claim 48 that has a level of autofluorescence for an excitation wavelength of 532 nm and an emission wavelength of 575 nm that is less than 4 times a level of Raman scattering from a column of pure water 8 mm long at that excitation wavelength and emission wavelength.

50. A method according to claim 2, that produces a polymer bead product according to claim 42.

51. A bead product according to claim 42, comprising paramagnetic or superparamagnetic beads.

52. A method according to claim 27, using the bead product of claim 51 for the paramagnetic or superparamagnetic beads.

53. A bead product according to claim 42, suitable for binding to analytes in bio-assays.

54. A heterogeneous bio-assay system for detecting an analyte in a biological sample, the system comprising capture surface elements comprising beads of a bead product according to claim 53.

55. A method according to claim 14, wherein the capture surface elements comprise a plurality of beads of a bead product according to claim 42, and the capture surface comprises outer surfaces of the beads.

56. A bead product according to claim 42, packaged in a package containing at least 1000 beads.

57. A polymer bead product, suitable for use in bio-assays, with a measurable level of autofluorescence for which 532 nm is in the peak range of excitation wavelengths and 575 nm is in the peak range of emission wavelengths for an excitation wavelength of 532 nm, wherein the level of autofluorescence, for an excitation wavelength of 532 nm and an emission wavelength of 575 nm, is less than 50% of the level of autofluorescence of Certified Blank beads 890 at that excitation wavelength and emission wavelength.

58. A heterogeneous bio-assay system for detecting an analyte in a biological sample, the system comprising a capture surface element comprising a polymer having an autofluorescence excited by light in a peak range of excitation wavelengths, producing light predominantly in a peak range of emission wavelengths that may depend on the excitation wavelength, with a capture surface suitable for immobilizing ligand molecules that bind to molecules of the analyte when exposed to the sample, the level of autofluorescence of the capture surface element being reduced by photobleaching, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths for that excitation wavelength, by at least a factor of 2 below what it would be for an unbleached capture surface element manufactured in a same way.

59. A system according to claim 58, also comprising:

a) an optical sub-system for measuring a concentration of bound analyte molecules on the capture surface by exciting and detecting fluorescent emission of reporter molecules whose emission power depends on how many analyte molecules are bound to the ligand molecules; and

b) a fluidic sub-system for exposing the capture surface, with the ligand molecules immobilized on it, to the biological sample.

60. A bio-assay system according to claim 58, wherein the capture surface element has its level of autofluorescence sufficiently reduced so that exposure of the capture surface element to 10^5 W/m^2 of light at peak excitation wavelength for the autofluorescence for at least 15 minutes, results in a reduction in level of autofluorescence of less than a factor of 2, or results in no measurable reduction in level of autofluorescence, for at least one excitation wavelength in the peak range of excitation wavelengths, for at least one emission wavelength in the peak range of emission wavelengths.

61. A method of preparing one or more capture surface elements suitable to use for providing a capture surface in a fluorescent bio-assay, the method comprising:

a) providing the one or more capture surface elements of known composition, exhibiting autofluorescence;

b) measuring a level of autofluorescence of the capture surface elements, using an excitation light source with a distribution of one or more excitation wavelengths, and an optical detection sub-system with a distribution of sensitivity to emission wavelength;

c) determining from the measured level of autofluorescence, from the distribution of excitation wavelengths, from the distribution of sensitivity to emission wavelength, and from the known composition, whether and to what extent the autofluorescence of the capture surface elements is already bleached; and

d) photobleaching the autofluorescence of the capture surface elements if they are not already bleached by more than a predefined amount.

62. A system for bleaching bio-assay beads and using them to perform a bio-assay, comprising:

a) a fluidics subsystem comprising a container, the fluids subsystem configured to expose a biological sample to a quantity of bio-assay beads, in the container, that bind molecules of an analyte if they are found in the sample, to expose the beads, with any analyte that is bound to them, to fluorescent reporter molecules that bind to the analyte, and to remove any unbound reporter molecules and analyte molecules from the container;

b) an excitation light source that produces light of at least one wavelength suitable for exciting fluorescence in the reporter molecules;

c) an excitation optics subsystem configured to illuminate the beads in the container with light from the light source at an intensity suitable for exciting the reporter molecules and measuring their concentration during a measuring time, but low enough not to significantly bleach the reporter molecules during the measuring time;

d) a detection optical subsystem configured to measure an intensity of fluorescent emission light from any reporter molecules bound to the beads when the beads are illuminated by the excitation optics subsystem;

e) a photobleaching light source, the same as or different from the excitation light source, that produces light of at least one wavelength suitable for photobleaching the beads; and

f) a photobleaching optics subsystem, the same as or different from the excitation optics subsystem, configured to illuminate the beads in the container with light from the photobleaching light source at an intensity suitable for photobleaching the beads.

63. A system according to claim 62, wherein the photobleaching light source is the same as the excitation light source, and the photobleaching optics subsystem is the same as the excitation optics subsystem.

64. A system for photobleaching bio-assay beads, comprising:

a) a container suitable for holding at least a minimum quantity of bio-assay beads;

b) a light source that produces light of at least one wavelength suitable for photobleaching the bio-assay beads;

c) an optical subsystem configured to illuminate a portion, but not all, of the minimum quantity of bio-assay beads in the container, with light from the light source, at an intensity suitable for photobleaching the beads, at a same time; and

d) a mixing subsystem configured to mix the minimum quantity of beads in the container, such that, after the mixing, the optical subsystem illuminates a different portion of the minimum quantity of bio-assay beads, than was illuminated before the mixing, with light from the light source at the intensity suitable for photobleaching the beads.

65. A system according to claim 64, suitable for photobleaching magnetic bio-assay beads, wherein the mixing subsystem comprises a magnet that mixes the beads by causing them to move.

66. A system according to claim 65, suitable for photobleaching magnetic bio-assay beads, wherein the mixing subsystem comprises:

a) a motor configured to bring the magnet successively closer to and further from a portion of the container, wherein the magnetic field and field gradient produced by the magnet at the portion of the container are sufficiently strong to magnetically aggregate the beads and attract them to the portion of the container, when the magnet is brought closer to the portion of the container, but not strong enough to aggregate or attract the beads to the portion of the container when the magnet is further from the portion of the container; and

b) a motor controller configured to control the motor to alternately bring the magnet closer to a portion of the container that the optical subsystem illuminates, for a long enough time to aggregate the beads and attract them there, and to bring the magnet further from the portion of the container for a long enough time so the beads are at least partially dispersed away from the portion of the container.

67. A system according to claim 64, wherein the mixing subsystem comprises a shaker that mixes the beads by shaking the container.

68. A method of photobleaching polymer capture surface elements suitable to use for providing a capture surface in a chemiluminescent bio-assay that uses a chemiluminescence excitation agent, the method comprising:

a) providing one or more of the capture surface elements having a background chemiluminescence excited by the chemiluminescence excitation agent; and

b) exposing the capture surface elements to light from a light source used for bleaching, with suitable wavelength distribution and sufficient intensity and duration so that a level of the background chemiluminescence, for at least one emission wavelength within a peak range of emission wavelengths for that chemiluminescence excitation agent, is reduced by at least a factor of 2.

69. A method according to claim 68, also comprising performing a bio-assay for detecting an analyte in a biological sample, after exposing the capture surface elements to light from the light source used for bleaching, by:

a) performing a preparatory procedure with the biological sample that results in reporter molecules being bound directly or indirectly to a capture surface of the capture surface elements, such that, when excited by a chemiluminescence excitation agent, the reporter molecules emit a chemiluminescent emission light of an intensity that depends on a concentration of the analyte in the sample;

b) exposing the capture surface to the chemiluminescence excitation agent that would excite the reporting molecules, after performing the preparatory procedure;

c) measuring a signal of the chemiluminescence emission light emitted by the reporting molecules, in response to the chemiluminescence agent, over a band of emission wavelengths; and

d) determining a presence of the analyte in the sample, a concentration of the analyte in the sample, or both, from the measured chemiluminescence signal;

wherein the chemiluminescence excitation agent would excite the background chemiluminescence of the capture surface elements to emit light in the band of emission wavelengths.

70. A method of photobleaching polymer capture surface elements suitable to use for providing a capture surface in an electro-chemiluminescent bio-assay, the method comprising:

a) providing one or more of the capture surface elements having a background electro-chemiluminescence; and

b) exposing the capture surface elements to light from a light source used for bleaching, with suitable wavelength distribution and sufficient intensity and duration so that a level of the background electro-chemiluminescence, for at least one emission wavelength within a peak range of emission wavelengths for the background electro-chemiluminescence, is reduced by at least a factor of 2.

71. A method according to claim 70, also comprising performing a bio-assay for detecting an analyte in a biological sample, after exposing the capture surface elements to light from the light source used for bleaching, by:

a) performing a preparatory procedure with the biological sample that results in reporter molecules being bound directly or indirectly to a capture surface of the capture surface elements, such that, when excited by an electric current, the reporter molecules emit an electro-chemiluminescent emission light of an intensity that depends on a concentration of the analyte in the sample;

c) exposing the capture surface to an electric current sufficient to excite the reporting molecules to a detectable level of electro-chemiluminescent emission, after performing the preparatory procedure;

d) measuring a signal of the electro-chemiluminescence emission light emitted by the reporting molecules in response to the electric current, over a band of emission wavelengths; and

e) determining a presence of the analyte in the sample, a concentration of the analyte in the sample, or both, from the measured electro-chemiluminescence signal;

wherein the electric current would excite the background electro-chemiluminescence of the capture surface elements to emit light in the band of emission wavelengths.

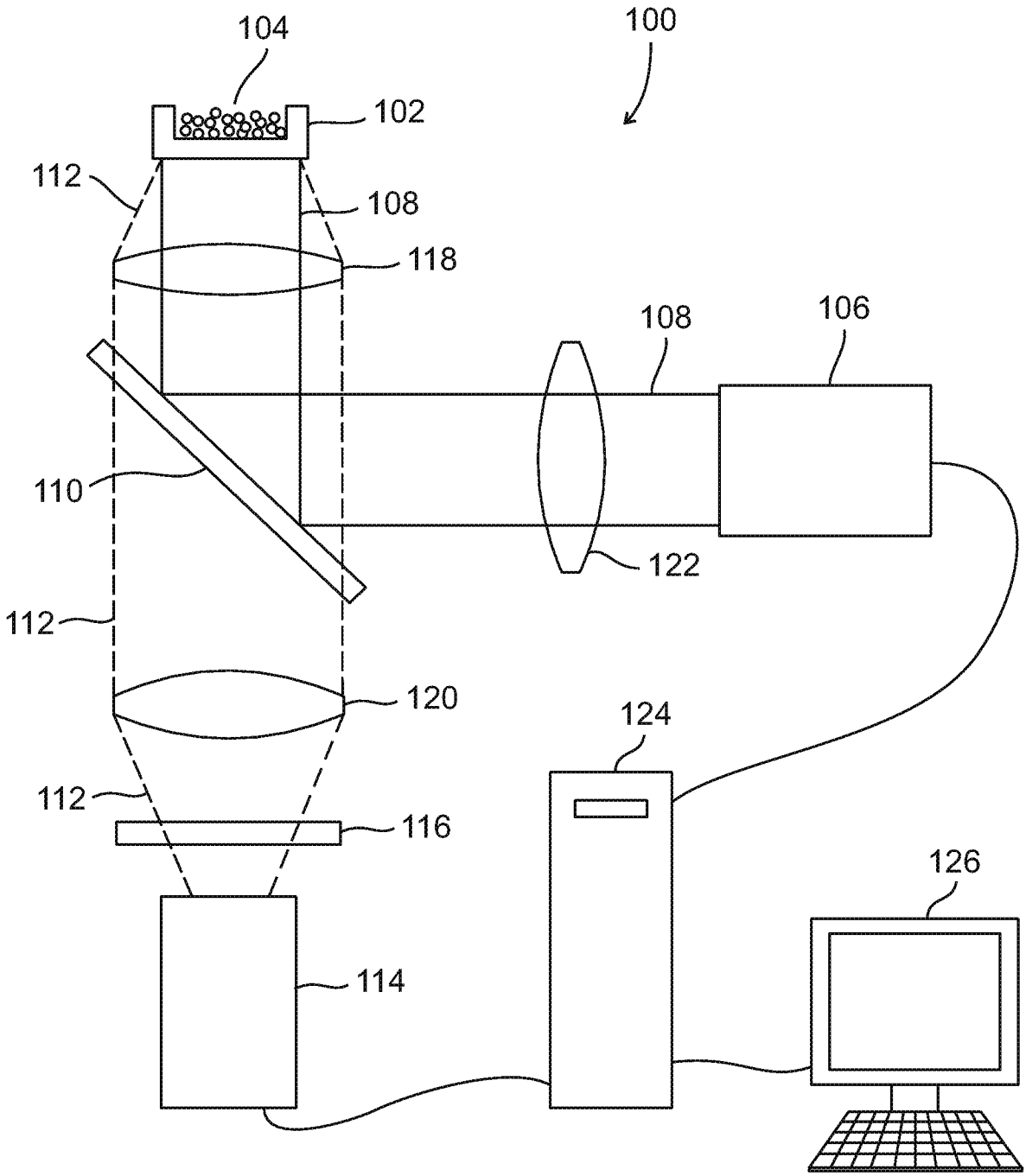
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FIG. 1

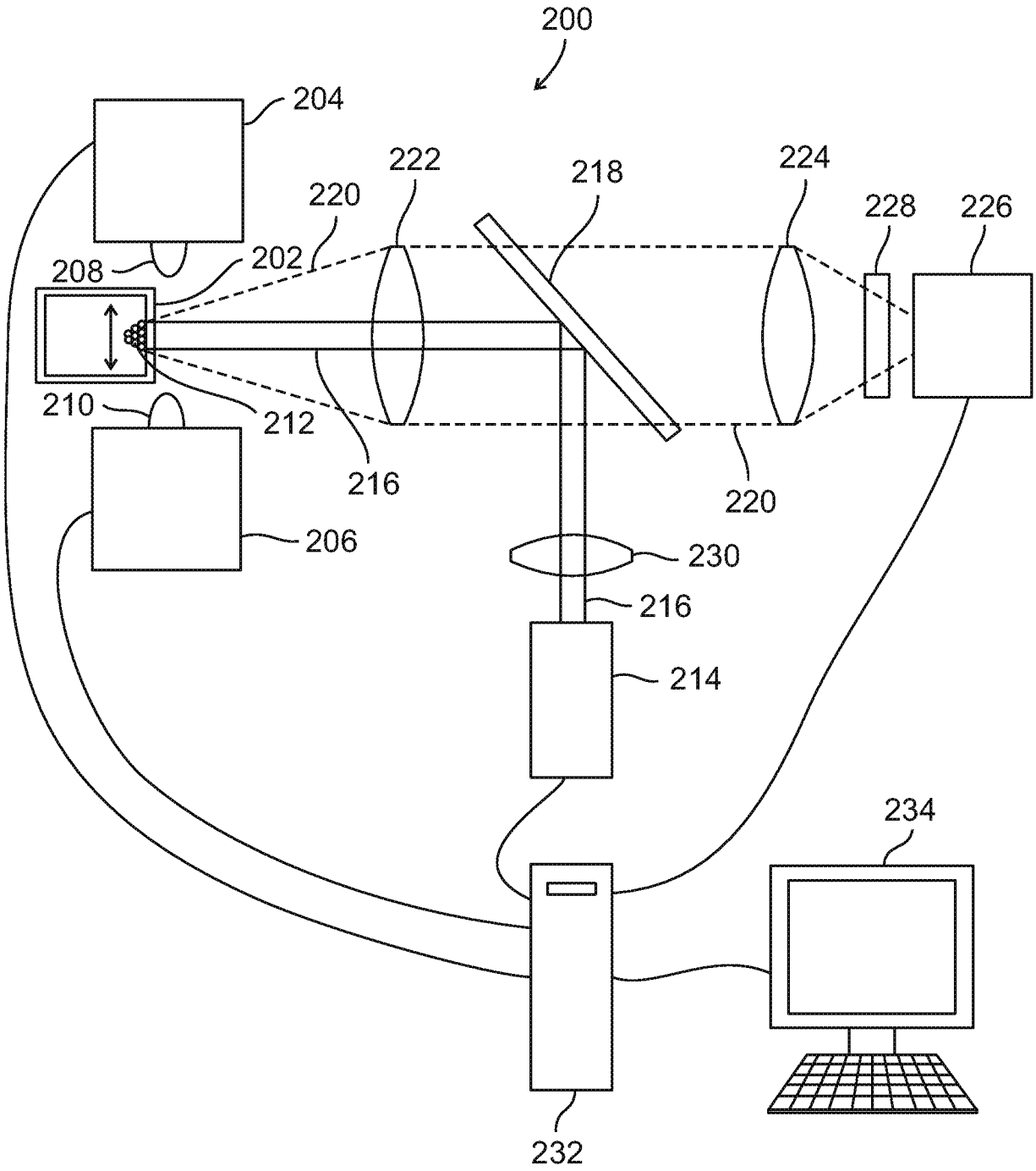
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FIG. 2

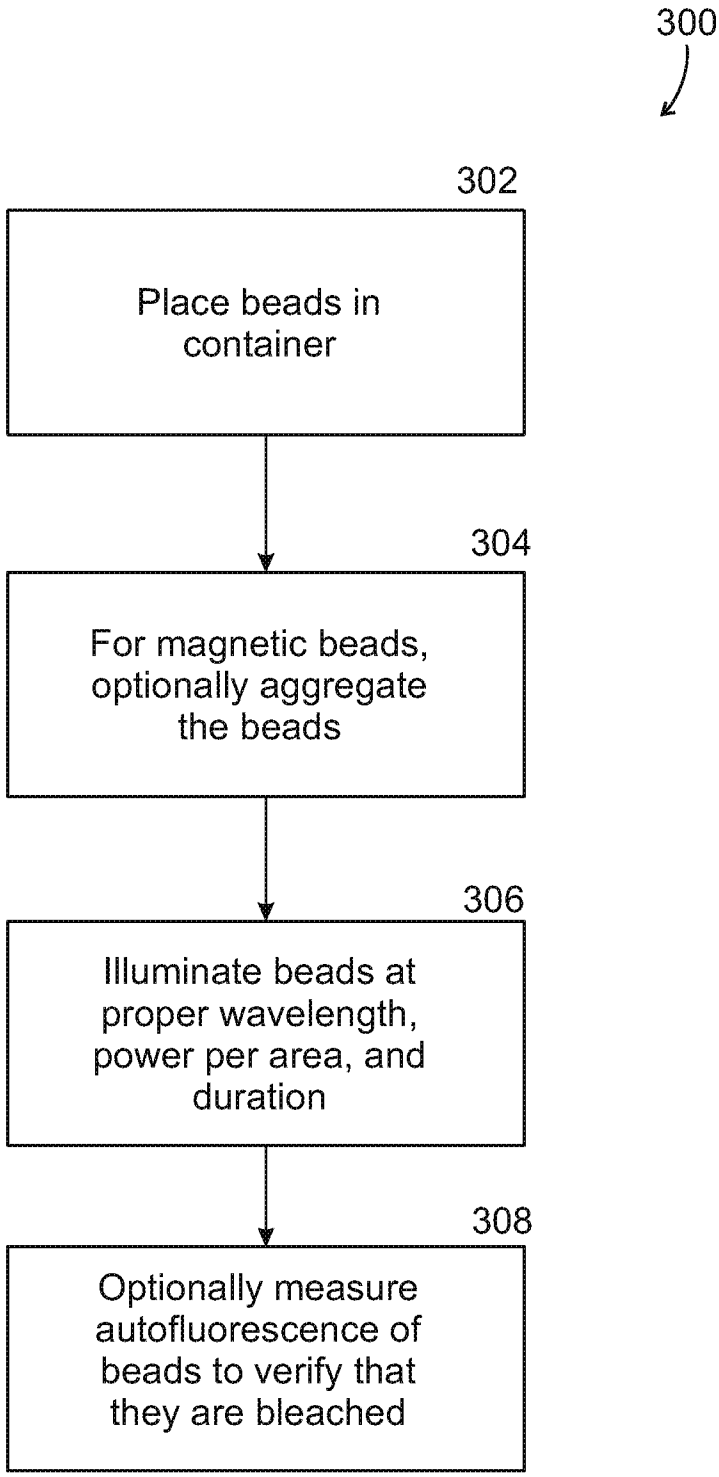

300
302
Place beads in container
304
For magnetic beads, optionally aggregate the beads
306
Illuminate beads at proper wavelength, power per area, and duration
308
Optionally measure autofluorescence of beads to verify that they are bleached

FIG. 3

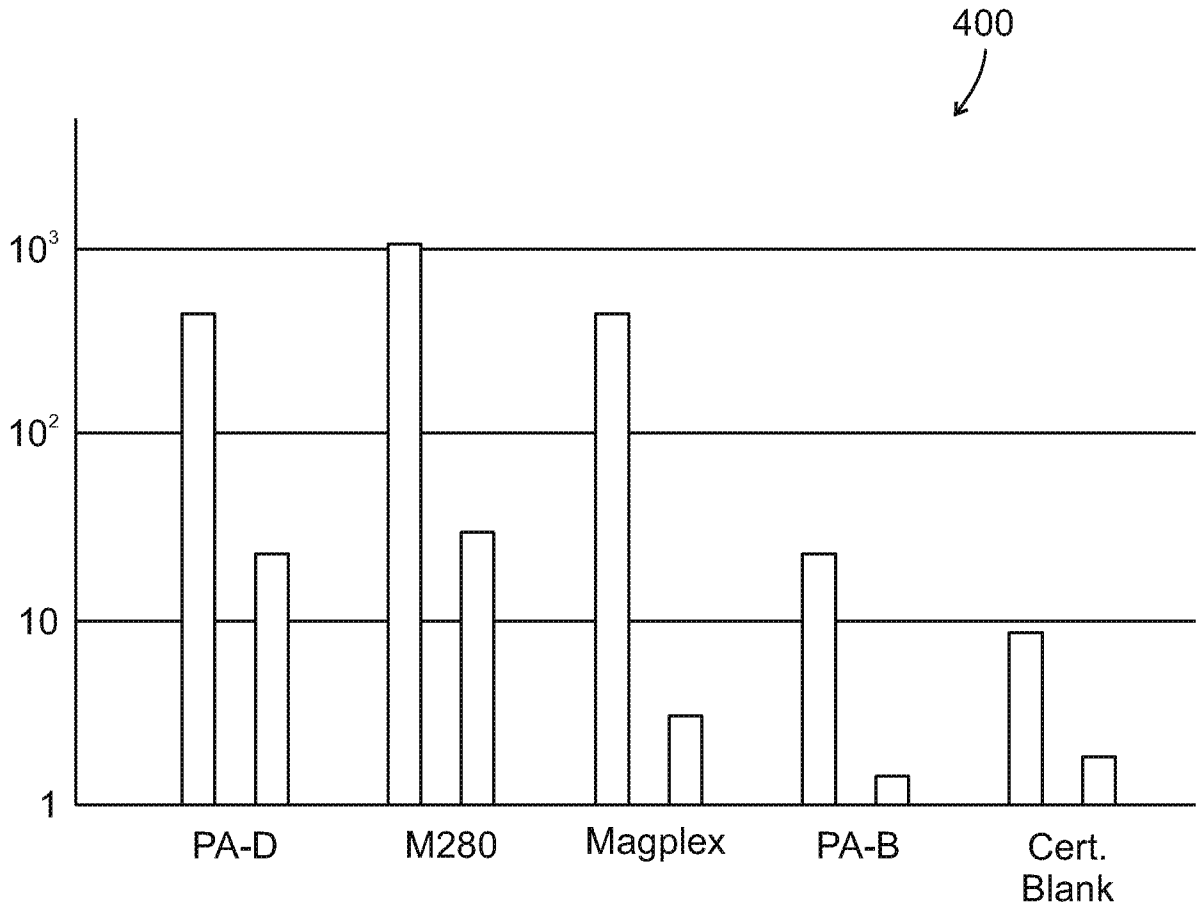


FIG. 4

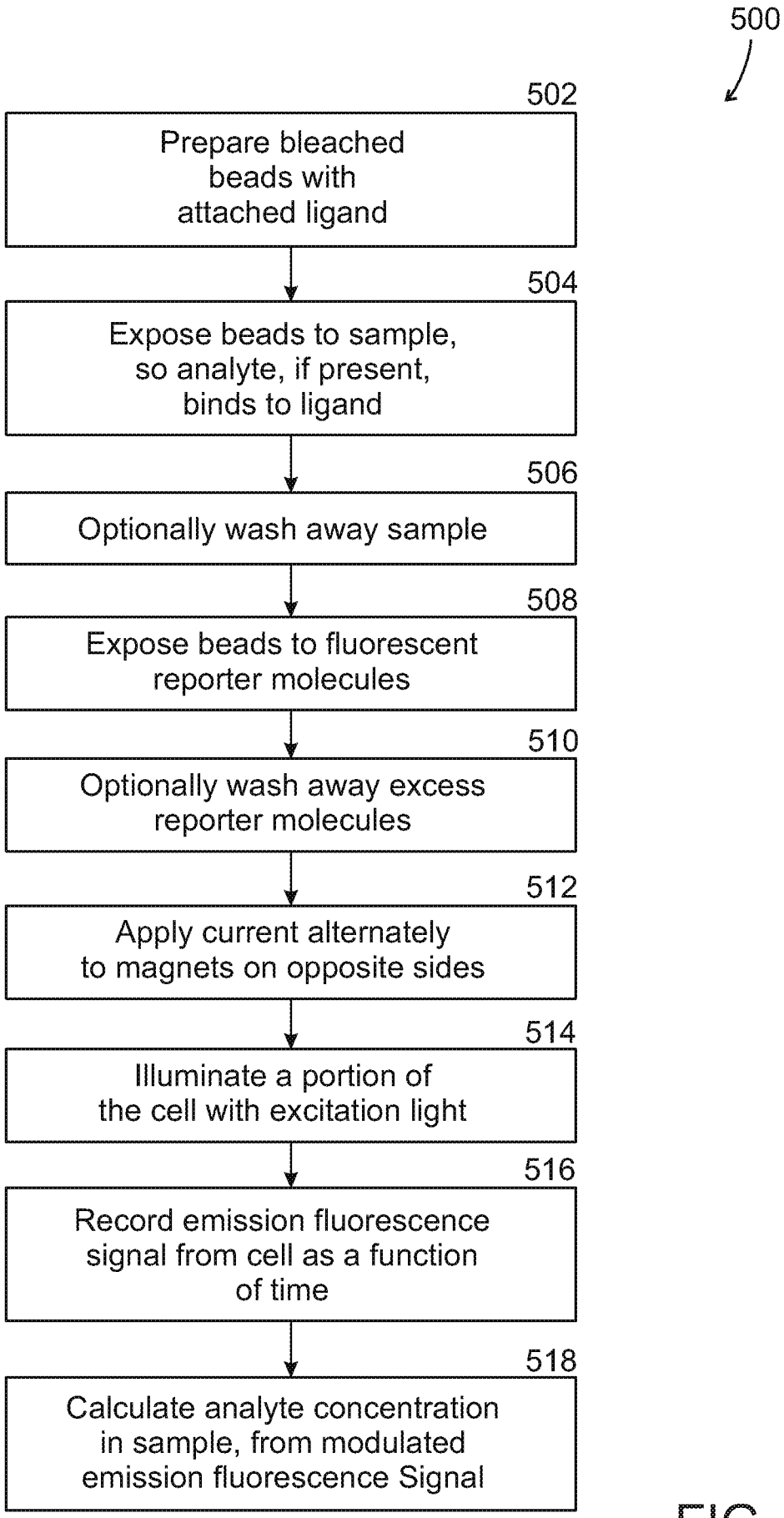

500
502
Prepare bleached beads with attached ligand
504
Expose beads to sample, so analyte, if present, binds to ligand
506
Optionally wash away sample
508
Expose beads to fluorescent reporter molecules
510
Optionally wash away excess reporter molecules
512
Apply current alternately to magnets on opposite sides
514
Illuminate a portion of the cell with excitation light
516
Record emission fluorescence signal from cell as a function of time
518
Calculate analyte concentration in sample, from modulated emission fluorescence Signal

FIG. 5

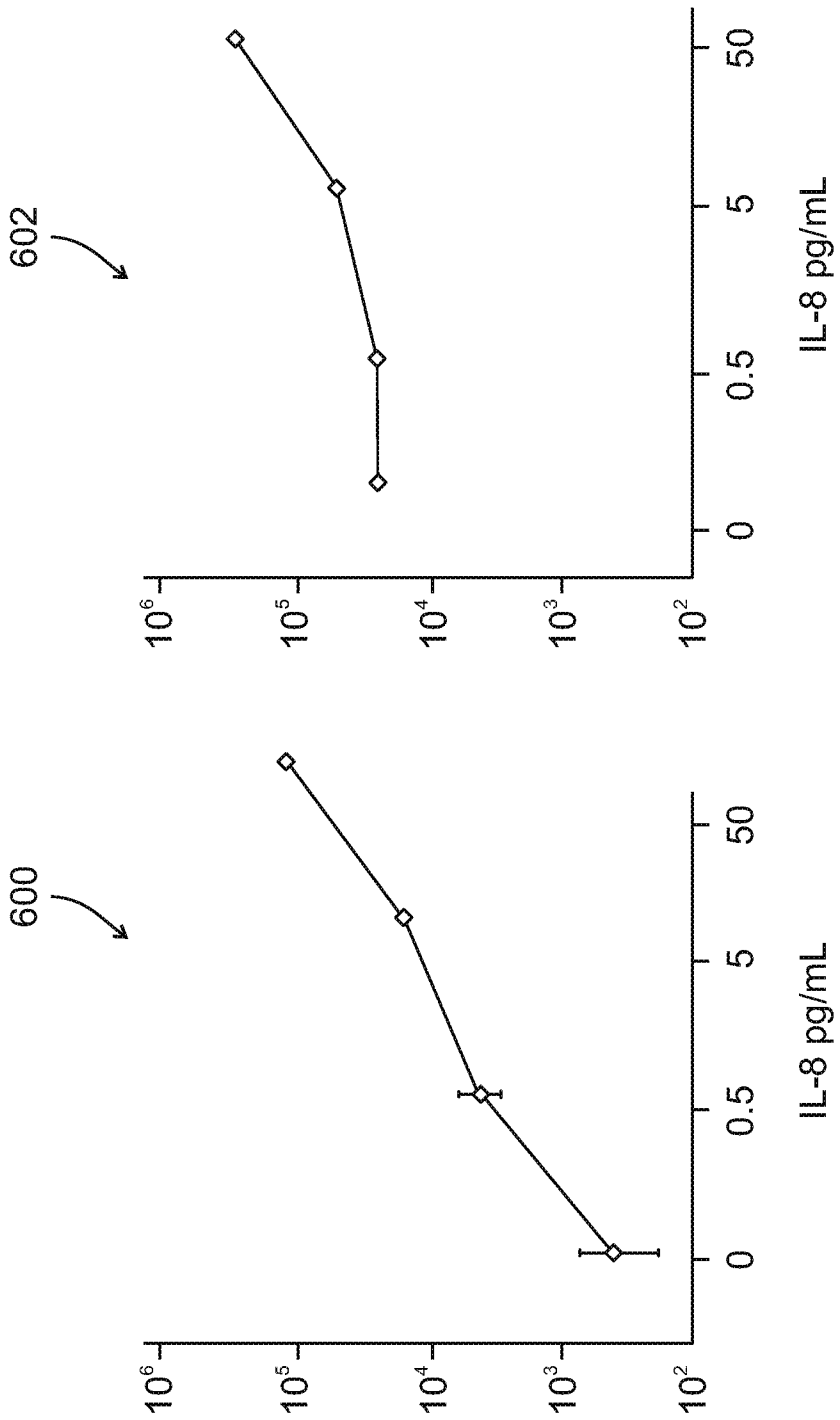
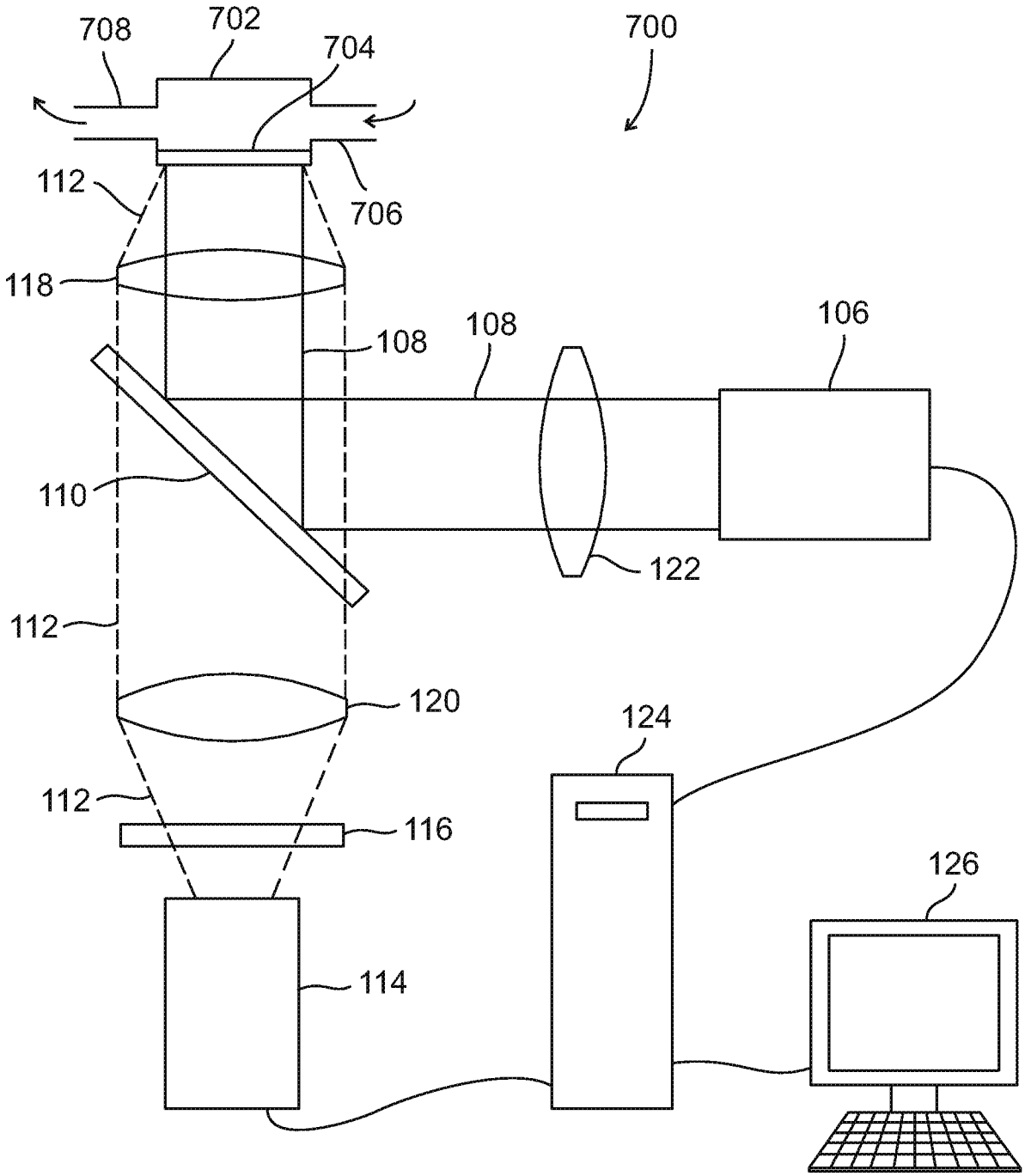


FIG. 6


708
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704
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112
706
118
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108
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110
122
112
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124
112
116
126
114

FIG. 7

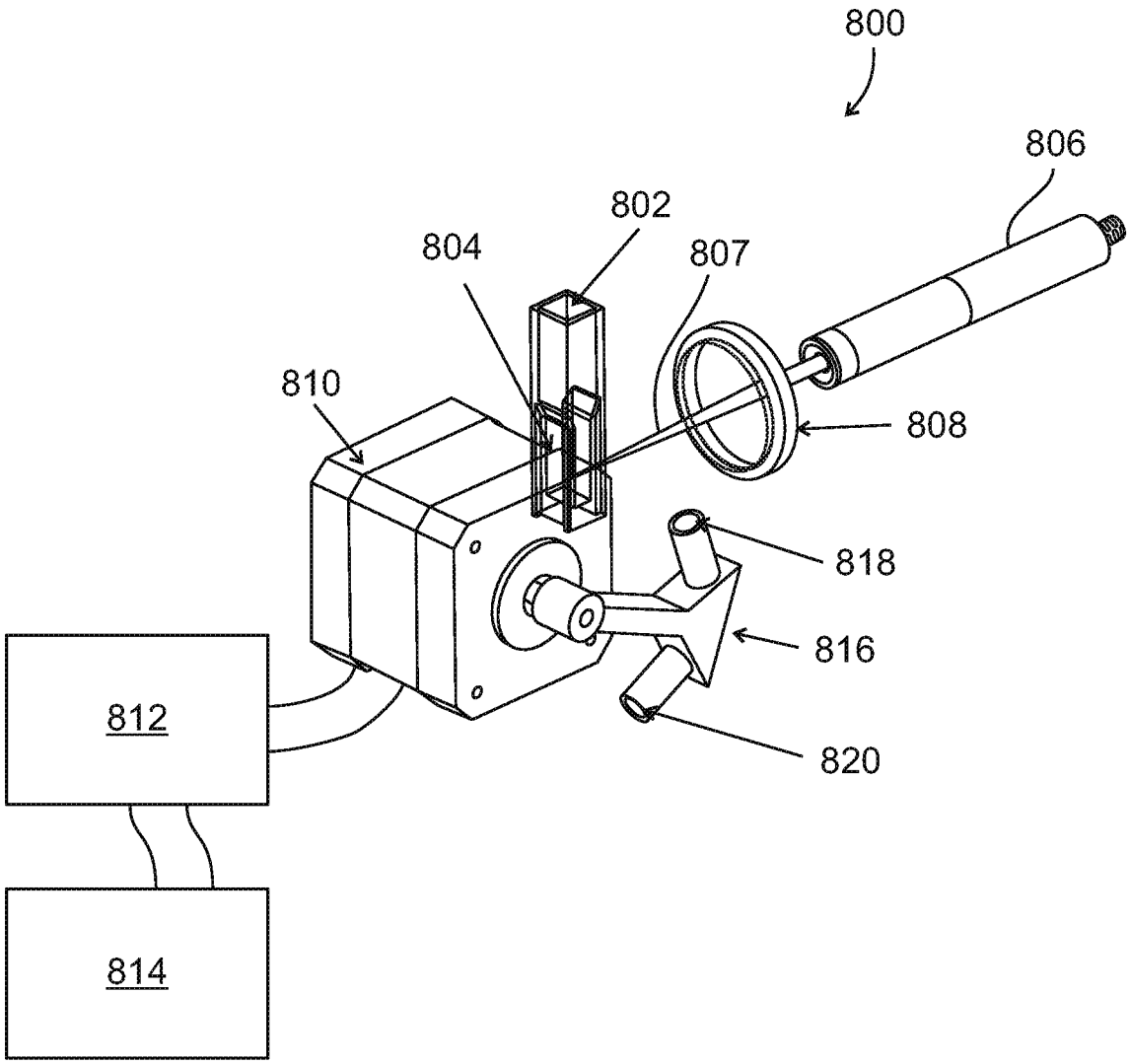

FIG. 8

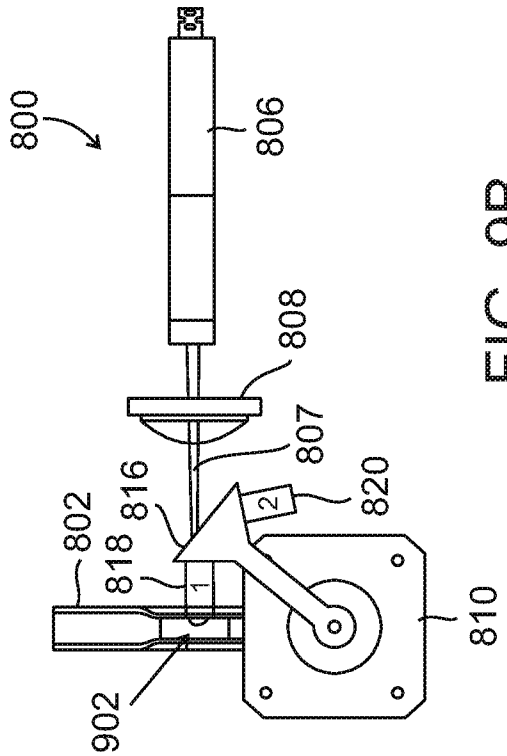

FIG. 9B

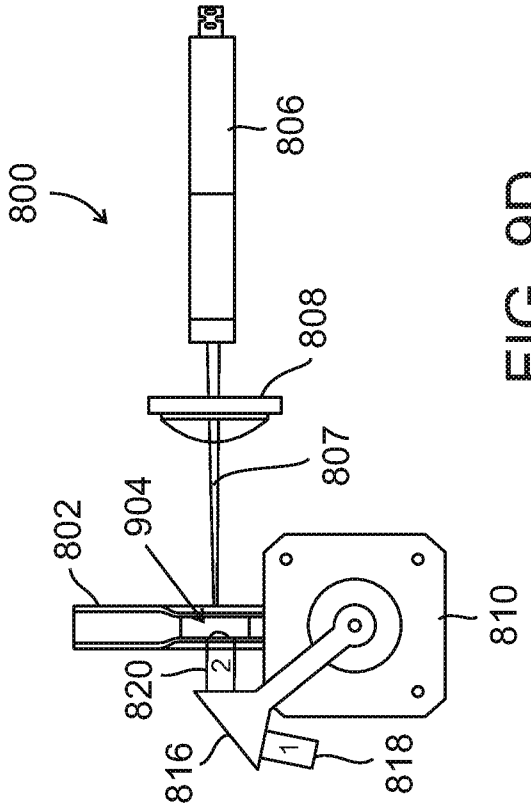

FIG. 9D

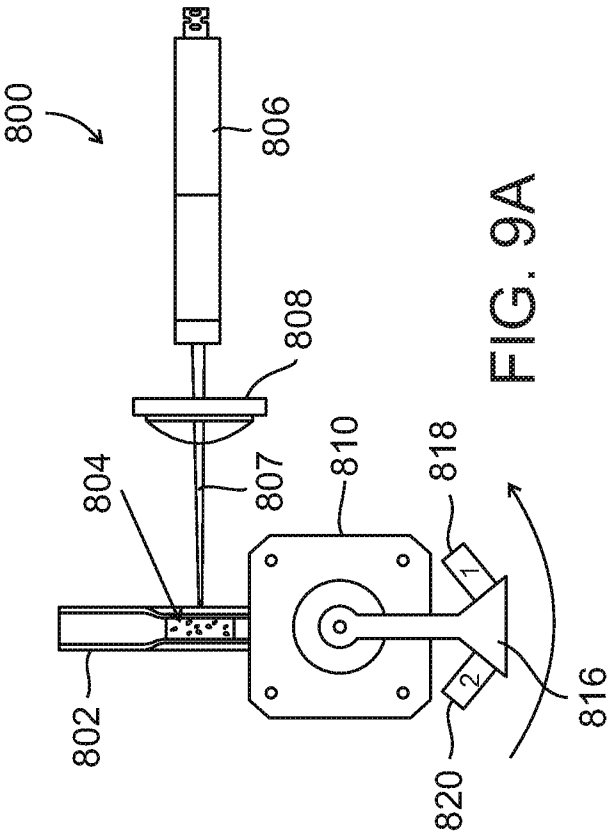

FIG. 9A

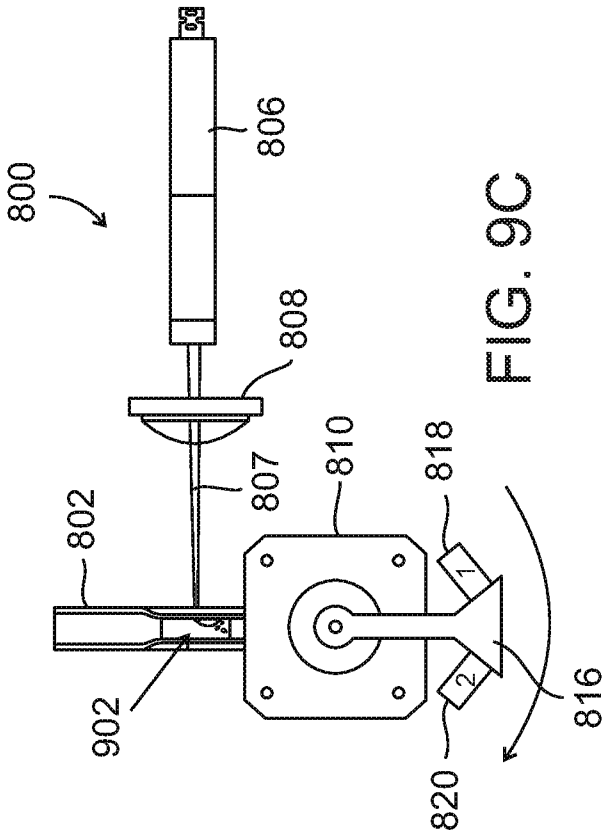

FIG. 9C

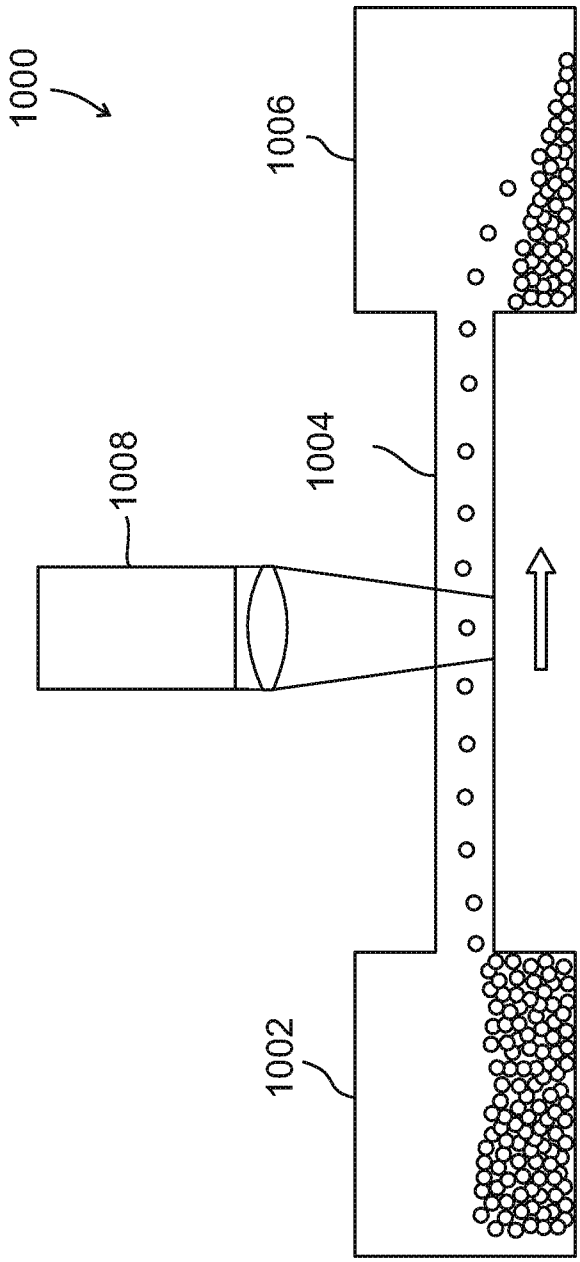

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2018/052318

A. CLASSIFICATION OF SUBJECT MATTER
C04B28/04 Version=2018.01

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)

C04B

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

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

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KR200417316 Y1 (RYU CHEOL JU[KR]) 26 May 2006 (26-05-2006) abstract, claims	1-8
Y	KR100734553 B1 (RYU CHEOL JU[KR]) 06 July 2007 (06-07-2007) abstract, claims	1-8
Y	DE3440575 A1 (ZIEGLER,WALTER [DE]) 07 May 1986 (07-05-1986) abstract	1-8
Y	KR100794925 B1 (LEE OK SIN, [KR]) 21 January 2008 (21-01-2008) abstract	1-8

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

* Special categories of cited documents:

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

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"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
27-07-2018	27-07-2018

Name and mailing address of the ISA/	Authorized officer
Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Parameswar Sau Telephone No. +91-1125300200