



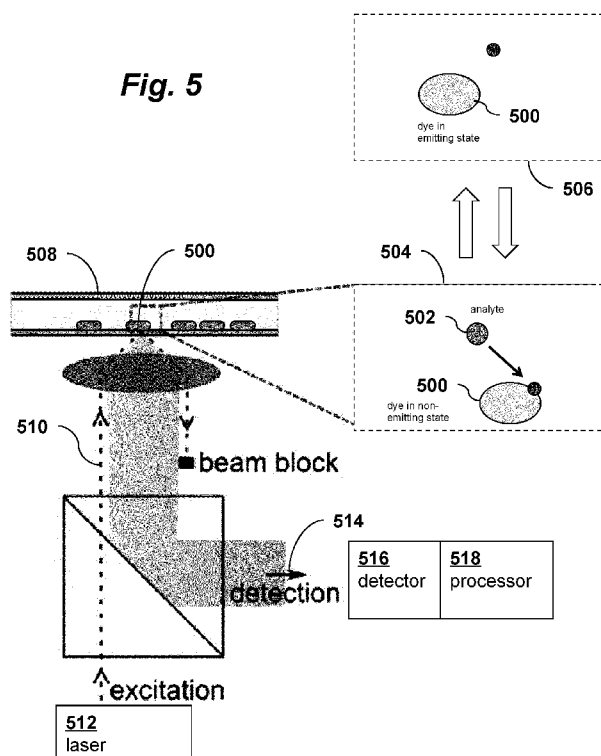
- (51) International Patent Classification:
C12Q 1/00 (2006.01) *G01N 33/542* (2006.01)
- (21) International Application Number:
PCT/EP2015/079872
- (22) International Filing Date:
15 December 2015 (15.12.2015)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
62/092,758 16 December 2014 (16.12.2014) US
62/092,763 16 December 2014 (16.12.2014) US
62/132,096 12 March 2015 (12.03.2015) US
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- (81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,

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- (54) Title: BIOSENSOR BASED ON SINGLE-MOLECULE FLUORESCENCE DETECTION



(57) Abstract: A method for sensing an analyte [502] includes bringing a matrix containing the analyte into contact with a sensor device having a sensing volume containing fluorescent emitters [500] that switch from a first state [504] to a second state [506] when activated by chemical activation by the analyte. The fluorescent emitters are excited with optical radiation [510] to generate fluorescence optical radiation [514]. The excitation induces photo-bleaching of the activated fluorescent emitters, which limits the duration of the generated fluorescence optical radiation. Each resulting pulse of the fluorescence optical radiation is associated with a single one of the fluorescent emitters. The pulses are detected [516] and processed [518] to determine the presence/concentration of the analyte from changes in the detected fluorescence optical radiation.

WO 2016/096908 A1



SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, **Published:**
GW, KM, ML, MR, NE, SN, TD, TG).

— *with international search report (Art. 21(3))*

BIOSENSOR BASED ON SINGLE-MOLECULE FLUORESCENCE DETECTION

FIELD OF THE INVENTION

- 5 The present invention relates generally to a biosensor technique based on single-molecule fluorescence detection.

BACKGROUND OF THE INVENTION

Most biosensing principles for biochemical markers have been developed for use in *in-vitro*
10 *diagnostics*, where a sample is taken (e.g., blood or saliva) and is transferred to an artificial device (e.g., a plastic disposable) outside a living organism. In such biosensing assays, a wide range of sample pre-treatment steps can be applied (e.g., separation or dilution steps) and multiple reagents can be introduced in the assay (e.g., for target amplification, signal amplification, or washing steps). Examples of *in-vitro* biosensing assays are: immunoassays,
15 nucleic acid tests, tests for electrolytes and metabolites, electrochemical assays, enzyme activity assays, cell-based assays, etc. (see Tietz, Textbook of Clinical Chemistry and Molecular Diagnostics, 2005).

In *in-vivo* biochemical sensing, at least a part of the sensor system remains connected to or is
20 inserted in the human body, e.g., on the skin, or in the skin, or below the skin, or on or in or below another part of the body. Due to the contact between the biosensor and the living organism, *in-vivo* biochemical sensing sets high requirements on biocompatibility (e.g., inflammation processes should be minimized) and the sensor system should operate reliably within the complex environment of the living organism. For monitoring applications, the system
25 should be able to perform more than one measurement over time and the system should be robust and easy to wear.

An important application of *in-vivo* biochemical sensing is continuous glucose monitoring (CGM). Continuous glucose monitoring is based on enzymatic electrochemical sensing. These
30 sensors show drift and need regular recalibration. Single-molecule sensitivity is not achieved.

Commercial systems for in-vivo glucose monitoring are available from Dexcom and Medtronic. A disadvantage of present-day CGM systems is that the sensor response shows drift, and therefore the systems require regular recalibration by an in-vitro blood glucose test (for a review, see e.g., Heo and Takeuchi, Adv.Healthcare.Mat. 2013, vol. 2, p. 43-56).

One alternative approach to real time glucose measurement are in vivo biosensing techniques that use fluorophores which change fluorescence emission in the presence of an analyte. These approaches, however, have problems with photobleaching (referring to irreversible destruction of the fluorophore, not reversible photobleaching).

SUMMARY OF THE INVENTION

In one aspect, the invention provides a single molecule fluorescence detection technique in which the presence of an analyte (directly or indirectly) chemically switches a fluorescent emitter such as a dye molecule (e.g., a fluorophore) from a non-emitting to an emitting state, resulting in a detectable fluorescence signal which quickly (less than 10s) subsides due to photobleaching of the fluorescent emitter. A key innovation is intentionally producing photobleaching as part of a fluorescence technique. This sequence (activation, detection and bleaching) can be repeated multiple times and the density of detection events per unit of time is related to the concentration of analyte.

The biosensing technique is useful for in-vivo biochemical monitoring. The sensing principle is sensitive, specific, stable, and biocompatible. It allows for the real-time probing of analyte concentrations in complex fluids (e.g., blood, saliva, interstitial skin fluid). Single-molecule resolution provides for high analytical sensitivity. Furthermore, high specificity may be reached by isolating specific from the non-specific interactions. This allows direct real-time measurements in complex fluids without repeated sample taking or intermediate filtering steps.

In some embodiments, a biomarker monitoring system is connected in a closed loop with a treatment system, e.g., a device that doses a drug (e.g., insulin in the case of diabetes) or a device that otherwise influences the body (e.g., a device that provides an organ with a physical stimulation, e.g., electrical).

Such a reliable and easy to use biochemical monitoring technology has many applications, including continuous glucose monitoring, without the need to regularly recalibrate the sensor system; electrolyte- and metabolite-monitoring, for patients that may become unstable e.g., in critical care; electrolyte-measurements are helpful to monitor kidney function, e.g., in cardiac patients; protein measurements can be helpful to monitor cardiac function, e.g., BNP is a key marker for heart failure; drug and/or drug metabolite measurements are helpful to monitor drug intake (compliance) and pharmacokinetics (aiming to keep the drug within the desired concentration window); and drug response measurements are helpful to monitor drug effectiveness. The technique is also relevant for in-vitro diagnostic testing, particularly for point-of-care testing, where it is advantageous if a specific molecular binding process leads to a signal that is detectable by optical means, preferably with little further chemical, biochemical, or fluidic processing. The current invention may also find application in biological, biomedical and pharmaceutical research, e.g. to monitor assays with live cells, tissue, organs, etc. Analytes can be electrolytes, small molecules, lipids, carbohydrates, peptides, hormones, proteins, oligonucleotides, DNA, RNA, etc.

In one aspect, the invention provides a method for sensing an analyte. The method includes bringing a matrix containing the analyte into contact with a sensor device having a sensing volume containing fluorescent emitters that switch from a first state to a second state when activated by chemical activation by the analyte. The chemical activation of the fluorescent emitters by the analyte may be direct activation by the analyte or indirect activation by an intermediate chemical activator molecule produced by enzymatic conversion of the analyte. The fluorescence emitters may be, for example, fluorescent dye molecules, fluorescent organic molecules, fluorescent emissive metal complexes, fluorescent organometallic complexes, or fluorescent quantum dots. In preferred embodiments, the second state has an extinction coefficient for light with a wavelength for fluorescence excitation of the emitters, and the extinction coefficient is at least 10 times larger than the extinction coefficient in the first state for light with the same wavelength.

The method also includes exciting the fluorescent emitters in the sensing volume with optical radiation at the excitation wavelength (preferably from a light source with a line width larger than 5 nm or from a superluminescent diode) to generate fluorescence optical radiation. The excitation induces photo-bleaching of the activated fluorescent emitters, which limits the duration of the generated fluorescence optical radiation. Pulses of the fluorescence optical radiation from the fluorescent emitters in the sensing volume are detected, where each of the pulses is associated with a single one of the fluorescent emitters. The presence/concentration of the analyte is determined from changes in the detected fluorescence optical radiation while the matrix containing the analyte is in contact with the sensor device. The determination of the presence/concentration of the analyte may include performing histogram and/or histogram processing to suppress background noise and enhance specificity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a schematic diagram illustrating the principle of a single molecule fluorescence detection technique involving direct interaction of a target analyte with a fluorescent emitter, according to an embodiment of the invention.

FIG. 1B is a graph of a single molecule fluorescence pulse whose duration is limited by photobleaching of the emitter, according to an embodiment of the invention.

FIG. 1C is a schematic diagram illustrating the principle of a single molecule fluorescence detection technique involving indirect interaction of a target analyte with a fluorescent emitter, according to an embodiment of the invention.

FIG. 1D is a schematic diagram of a portion of a biosensing device having a neutralization compartment positioned above a sensing compartment, according to an embodiment of the invention.

FIG. 2 illustrates an example of fluorescent emitter functionality for a fluorescent dye having a protective group that is cleaved by an analyte to switch from a non-emitting state to an emitting state which is capable of emitting a fluorescence pulse of limited duration due to photobleaching, according to an embodiment of the invention.

FIGS. 3A-B are schematic illustrations of an optical apparatus based on an optical fiber in which the sensing and neutralization compartment are incorporated, according to an embodiment of the invention.

FIGS. 4A-B are schematic illustrations of optical arrangements for excitation and detection through a hollow core fiber, according to embodiments of the invention.

FIG. 5 is a schematic diagram of a system for sensing the presence of a target analyte using optical excitation and detection of fluorescent emitters, according to an embodiment of the present invention.

FIGS. 6A-B illustrate two implementations of a combined microscopy and spectroscopy method, according to embodiments of the present invention.

DETAILED DESCRIPTION

Fig. 1A is a schematic diagram illustrating the principle of a single molecule fluorescence detection technique according to an embodiment of the invention. The presence of an analyte 100 (directly, or indirectly by means of intermediate chemical activator) chemically switches a fluorescent emitter such as a dye molecule (e.g., a fluorophore) from a non-emitting state 102 to a fluorescent emitter in an emitting state 104. In this example, the fluorescent emitter has a protective chemical group 106 that is cleaved by the analyte 100, changing non-emitting fluorescent emitter 102 to emitting fluorescent emitter 104. In the presence of suitable excitation, the switching from non-emitting state 102 to emitting state 104 results in a sudden rise in a fluorescence signal in the presence of the analyte 100. Due to photobleaching, however, the fluorescence signal has a finite duration, even in the continued presence of the photo-excitation signal. Thus, the switching of the emitter state results in a detectable fluorescence signal which quickly (i.e., in less than 10 s) subsides due to photobleaching of the emitter, i.e., a fluorescence pulse is generated as shown in Fig. 1B. A key feature of the technique is intentionally producing photobleaching as part of a fluorescence technique. The photobleaching results in the generation of a fluorescence pulse of finite duration. The single-molecule pulses are detected, identified and counted. The pulse signals are indicative for the presence and/or concentration of the analyte 100.

The biosensing principle in Fig. 1A consists of a single cleavage step. The analyte can for example be a reactive oxygen species (ROS) like H_2O_2 , NO, or O_2 . ROS are interesting because they are markers for e.g., inflammation.

Preferably, a biosensing device according to embodiments of the present invention contains a plurality of fluorescent emitters, so that signal generation is analyte-limited rather than emitter-limited. The emitter can be present at a high concentration (e.g., micromolar, millimolar or more) because most of the emitters are normally in a state where they do not generate fluorescence. Only in the presence of the analyte do the molecules switch to a fluorescence emitting state. Having the fluorescence emitter at high concentrations avoids depletion problems, ensuring a large dynamic range and long lifetime of the sensor.

There are several advantages of the sensing principle used in embodiments of the invention. Single-molecule fluorescence pulses have a very characteristic shape. Signal processing will lead to a high signal-to-noise ratio, giving high specificity, reliable quantitation, and high accuracy. The principle is based on single molecule detection, so it yields a high sensitivity and therefore low concentrations can in principle be measured. When the fundamental single-molecule limit is reached, maximum data is collected from the molecular binding and/or conversion process, giving optimal statistics and optimal precision.

In a variation of the principle shown in Fig. 1A, the biosensing principle can involve a cascade of molecular process steps. Fig. 1C sketches such a two-step process with an enzyme 112, where the analyte 110 is a substrate for the enzyme. The enzyme converts the analyte 110, which generates a reactive product (chemical activator) 114 that cleaves protective chemical group 120 from the fluorescent emitter (e.g., dye molecule) 116 in a non-emitting state to switch it to a fluorescent emitter in an emitting state 118. In one specific implementation, the analyte 110 can be a metabolite like glucose, the enzyme 112 can be glucose oxidase, and the reactive product 114 can be H_2O_2 .

Several cascade reactions are known in literature. For example, Heo and Crooks (Anal. Chem. 2005, 77, 6843-6851) reported enzymatic glucose sensing in a fluorescence assay, employing GOx, HRP and Amplex Red, resulting in production of fluorescent resorufin. The recorded data shows a dependence of the overall fluorescence intensity on glucose concentration. In this prior literature, however, strong illumination was avoided in order to suppress photobleaching. In contrast, in the present technique, it is desired to induce photobleaching in order to achieve

single-molecule detection, because the inventors have discovered that this can be used, surprisingly, to improve the detection sensitivity, specificity, and accuracy.

In alternate implementations, an enzyme can serve the role of the analyte. For example, substrates are available in the biosensing system and the reactive product is generated when the enzyme is present in the sample. For example, the enzyme analyte can be a liver enzyme, such a ALAT (alanine aminotransferase).

Alternatively, the sensing cascade may involve affinity binding. For example, an allosteric reporter such as an enzyme with analyte affinity binding, where the enzymatic activity is analyte-dependent, e.g., the allosteric reporter becomes enzymatically active or inactive upon analyte binding. For example, the reporter may be an enzyme-inhibitor complex to which the analyte can bind; when the analyte binds, the distance between inhibitor and enzyme is increased and the enzyme acquires an enzymatic activity. For example, the analyte could be a protein, e.g., an antibody. The enzyme may operate directly on a fluorogenic substrate, or it can be a multistep process, e.g., the enzyme generates a product that activates a fluorescent emitter.

Alternatively, the molecular sensing cascade may involve affinity-based displacement. For example, a substrate-conjugated analyte-analogue is displaced from an affinity binder; the displacement releases the substrate, which is subsequently converted by an enzyme, which then releases a reactive product that activates a fluorescent emitter, such as a dye molecule. A biomarker of interest could e.g., be BNP (brain natriuretic peptide). Alternatively, the analyte can be measured involving a competitive process.

The biological fluid that is being probed for the analyte may contain molecules that can interfere in the molecular sensing cascade. For example, a sensor with an enzymatic sensing cascade as sketched in Fig. 1C may use a fluorescent emitter (e.g., dye) that is sensitive to ROS. In that case, ROS present in the biological fluid may generate non-analyte related background signals. This problem can be solved by including a neutralization process in the device, e.g., a neutralization compartment 130 above a sensing compartment 136, as shown in Fig. 1D. In case

of interference by an interfering molecule 132 such as ROS, the neutralization compartment 130 may contain a scavenging agent, e.g., antioxidants 134 such as ascorbic acid. The scavenging agent 134 in the scavenging compartment 130 contains a substance that de-activates interfering molecules 132. Analyte molecules 138 are able to pass through the scavenging compartment 130 and penetrate into the sensing compartment 136, where an analyte-dependent signal is generated when they interact with fluorescent emitter 140. Black dots on some of the emitters 140 in the figure represent protective chemical group that prevents fluorescence of the emitter 140. When the group is cleaved from emitter 140, the emitter is switched into an emitting state from a non-emitting state.

Several fluorogenic substrates sensitive to ROS can be used in embodiments of the invention (see e.g., Chang et al, Nat. Chem. Biol. 2011, 7, 504-511). These molecules comprise a fluorescent dye directly conjugated with a protecting group that quenches fluorescence. The dye could e.g., be a fluorescein, rhodamine, cyanine or phenoxazine (e.g., resorufin). The protecting group can be for example a boronic-acid group, or an acetyl group, or a pentafluorobenzenesulfonyl group, that is removed after reaction with ROS turning on the fluorescence. The dye is subsequently bleached. The number of photons detected before dye bleaching can vary, e.g., between 250 and 6000.

Fig. 2 illustrates an example of fluorescent emitter functionality. The fluorescent emitter in this example is a dye. It includes a protective group (PG) that is cleaved by ROS to switch from a non-emitting state 200 to an emitting state 202. Thereafter fluorescence is excited, until the dye is bleached 204.

Alternatively, the fluorescent unit can be conjugated to the protective group through an immolative linker. In this strategy, e.g., the analyte reacts with the protective group and immediately the linker eliminates in a cascade reaction, activating the fluorescence of the emitter (Shabat et al, Nature Protocols, 2014, 9, 27-36.).

In some embodiments, the fluorescent emitter can be directly conjugated to the optical detection system using amidation coupling. For example, N-hydroxysuccinimide (NHS) activated esters

of a fluorescent dye can be conjugated with an amine-functionalized glass surface of the optical system.

Alternatively, the optical detection system can be coated with a biocompatible transparent hydrogel comprising the fluorescent emitter. For example, a gel can be made of e.g., polyacrylamide (PAA), dextrans, collagen, chitosan or polyethylene glycol (PEG). Fluorescent dyes can be conjugated to the gel using, e.g., the same NHS-amine reaction. Alternatively, a dye functionalized with a monomer for polymerization (e.g., acrylamide) can be co-polymerized with the gel components. Or the dye may be physically entrapped in the gel.

The gel can also incorporate enzymes to perform the cascade reaction based sensing previously described. The detection system can be coated with different gel layers with different functions. An external gel layer functionalized with a neutralization moiety can be used (as in Fig. 1D). Gel thicknesses and densities can be tuned to modulate the properties (e.g., neutralization effectiveness, number of sensing dyes).

An optical system to detect the fluorescence pulses is selected in various implementations to be compatible with the desired use conditions. For example, for in-vivo conditions, the sensing module is adapted so that it may be brought into contact with the human body, e.g., on the skin, or in the skin, or below the skin, or on or in or below another part of the body. The optical system in one embodiment, for example, is based on an optical fiber (single-mode or multi-mode) in which the sensing and neutralization compartment are incorporated, e.g., in a hollow core, as illustrated in Figs. 3A-B. The figure shows a cross-section of a portion of a single fiber having a core 302 surrounded by a cladding 300. The core 302 contains a sensing compartment 304 and, optionally, a neutralization compartment 306. Optical excitation signal 310 and optical detection (fluorescence) signal 312 are guided through the fiber core 302 in opposite directions. The fiber can be thin, e.g., less than a few millimeters, which will facilitate insertion into the body. During operation, the fluid that is probed may be allowed to enter into the core passively (by diffusion) or actively (by applying e.g., pressure difference across the fiber). The geometry allows for easy multiplexing using several fibers bundled together, each with its own unique sensing compartment, as shown in Fig. 3B. The multiplexed detection allows simultaneous

biosensing of several distinct analytes using a bundle of hollow core fibers each with their own distinct sensing compartment 306 customized for the distinct target analyte.

In operation, excitation light can be coupled into the fiber by a lens. The excitation light is then confined to the core of the fiber to ensure a high irradiance of the sensing area. This allows for the use of a low cost laser or even a light-emitting-diode for excitation. The excitation wavelength can be visible (400-600 nm) or in the near-infrared wavelength window (600-800 nm). The latter wavelength window is preferable in some embodiments to provide a lower background signal due to reduced autofluorescence of biological fluids. Fluorescence pulses originating from the sensing area (sensing compartment) and scattered excitation light travel back through the fiber and can be detected at the fiber exit. In the design of Figs. 3A-B, the fluorescing species is already present in the fiber core, which ensures that a large fraction of the fluorescence signal is coupled back into the fiber core to the detection light path.

Two implementations of an optical arrangement for excitation and detection through a hollow core fiber suitable for embodiments of the invention are illustrated schematically in Figs. 4A-B. In the embodiment of Fig. 4A, optical excitation signal 400 is coupled into the apparatus at an end of fiber 402 and propagates through fiber 406 to neutralization and sensing area 404. A fluorescence signal from neutralization and sensing area 404 propagates in the opposite direction back through fiber 406 and is diverted by fiber beam splitter 408 to fiber 410. The fluorescence signal exits fiber 410 and passes through a wavelength filter 412 to produce a detection fluorescence signal 414 for detection by a detector (not shown). In the embodiment of Fig. 4B, the optical excitation signal 420 passes through beam splitter 422 and enters fiber 424 which terminates in neutralization and sensing area 426. The fluorescence signal propagates back through fiber 424 in the opposite direction and is reflected by beam splitter 422 so that it is directed through wavelength filter 428 to produce a detection fluorescence signal 430 for detection by a detector (not shown).

The detector is selected to detect the typical saturation intensity of a single fluorophore, e.g., 10^6 photons in 1 ms, depending on the fluorophore. The Stokes-shift of the fluorescent species in the sensing area is preferably greater than 20 nm to allow for efficient suppression of excitation light using affordable wavelength filters. The detected signal will contain a steady background

signal due to autofluorescence of biological fluids, with superimposed the fluorescence pulses originating from the sensing area. The distinct time-dependence of the fluorescence pulses allows one to extract the sensor signal from the background using signal processing. The repeated detection of fluorescence pulses then indicates e.g., the concentration of the analyte in the biological fluid.

Embodiments of the invention provide biosensing devices and techniques for the detection of analyte in fluid. In preferred embodiments, a plurality of fluorescent emitters are present in a sensing volume, the plurality of fluorescent emitters are irradiated with light at an excitation wavelength, the fluorescent emitters are in a first state where the excitation wavelength does not cause the emission of fluorescence (e.g., extinction coefficient of the first state less than 10% of the extinction coefficient of the second state), chemical activation switches at least one fluorescent emitter from a first to a second state, (e.g., cleavage of a chemical group, which changes the electronic state of the fluorescent emitter and thereby changes its fluorescence properties), the second state is a state wherein excitation light is absorbed and fluorescence is emitted, the fluorescent emitter is bleached by the excitation light within a time of less than 10 seconds, (so as to gather sufficient statistics on the timescale of minutes), the fluorescent emitter generates at least one fluorescence pulse, during the time between chemical activation and bleaching, the fluorescence pulse is detected, where the detected fluorescence pulse is an indication of the presence and/or concentration of analyte in fluid.

In some embodiments, a plurality of fluorescent emitters generates a time series of fluorescence pulses due to chemical activation, a series of fluorescence pulses is detected, and the detected series of fluorescence pulses is an indication of the presence and/or concentration of analyte in fluid.

Additional and/or alternative embodiments may be further characterized by any one or more of the following features and/or enhancements. In some embodiments, a histogram is established of at least one pulse characteristic. The histogram may be filtered in order to separate signal from noise, e.g., by using a photon intensity threshold. An average reaction rate may be determined from the at least one histogram

In some embodiments, the analyte reacts with a fluorescent emitter and thereby activates the fluorescent emitter for generating a fluorescence pulse, which may take place directly or indirectly. In a direct process (e.g., as shown in Fig. 1A), the analyte chemically activates the fluorescent emitter directly. In an indirect process (e.g., as shown in Fig. 1C), the analyte triggers a reaction whose product chemically activates the fluorescent emitter, in which case the product plays the role of the analyte in the direct process. In this case, the analyte binds to and/or reacts with a first moiety (e.g., enzyme), that transforms into and/or generates a second moiety (e.g., reactive product), that reacts with the at least one fluorescent emitter and thereby activates the fluorescent emitter for generating a fluorescence pulse. For example, in this indirect process, the analyte generates a product, e.g., via a specific enzymatic process, and the product causes chemical activation of the fluorescent emitter.

The fluorescent emitter and/or the adjuvant moieties may be conjugated, embedded, anchored, or entrapped in a matrix, e.g., a hydrogel or a polymer film.

During operation, a plurality of fluorescent emitters may be irradiated through an optical waveguiding system, e.g., a fiber. A fluorescence signal emitted by a fluorescent emitter is detected through an optical collection system, e.g., a lens and/or a fiber.

Embodiments may also include various calibration methods, controls, multiplexing, etc. Embodiments may include measures to block and reduce unwanted processes (e.g., non-specific processes that generate background signals) and to increase efficiency, stability, and selectivity of signal generation.

Commercial applications include in-vivo biosensing, but also for in-vitro biosensing. The techniques can be used in a variety of biosensors having single-molecule resolution.

There are many applications that can benefit from a reliable and easy to use biochemical monitoring system. Such applications include the following: continuous glucose monitoring for diabetic patients, without the need to regularly recalibrate the sensor system; electrolyte and metabolite monitoring for patients that may become unstable e.g., in critical care (patient monitoring systems); electrolyte measurements are helpful to monitor kidney function, e.g., in

cardiac patients; protein measurements can be helpful to monitor for cardiac function, e.g., using BNP as a key marker for heart failure; drug and/or drug metabolite measurements, which are helpful to monitor drug intake (compliance) and pharmacokinetics (aiming to keep the drug within the desired concentration window); drug response measurements which are helpful to monitor drug effectiveness; and monitoring for disease management, therapy control, compliance monitoring.

The sensing system may be part of a disease management system, which may include (bio)chemical and/or physical sensing, a system for data collection and processing, and a system for physical and/or (bio)chemical actuation. The system may be connected in a closed-loop format with a treatment system, e.g., a device that doses a drug (e.g., insulin in the case of diabetes) or a device that otherwise influences the body (e.g., a device that provides an organ with a physical stimulation, e.g., electrical).

Alternatively, the sensing device may be part of a feedback system during a medical procedure, e.g., a sensor on an endoscope or on a catheter.

Embodiments of the invention are also relevant for in-vitro diagnostic testing, particularly for point-of-care testing, where it is advantageous if a specific molecular binding process leads to a signal that is detectable by optical means, preferably with little further chemical, biochemical, and/or fluidic processing.

Generally, the sensing technology may be used for in-vivo, ex-vivo, or in-vitro applications. For example, the sensing technology may be used for applications on human subjects, or on non-human subjects, e.g., in veterinary applications or for testing of other biological systems. The sensing technology may also be used as part of a disposable probe that is in contact with the subject or with the biological system.

In another aspect of the invention, the sensing technology may be implemented as part of a disposable cartridge, e.g., a lab-on-a-chip cartridge, or a disposable used in laboratory-based testing, or another disposable such as a tube, a needle, a fiber, a catheter, a patch. The disposable or disposable cartridge may be attached to an instrument or an analyzer in order to power and/or

actuate and/or read out the disposable or disposible cartridge. In another aspect of the invention, the instrument is suited for processing signals from the probe or cartridge, and/or for communicating data between the instrument and the probe or cartridge, and/or for communicating data between the instrument and e.g., an information system or communication
5 network.

FIG. 5 is a schematic diagram illustrating a technique for optical excitation and detection of optical signals from a fluorescent emitter 500 that switches between an emitting state 506 and non-emitting state 504 depending on the presence of a target analyte 502. The fluorescent
10 emitter 500 is contained in a sensing cell 508. An illumination beam 510 from a laser 512 undergoes total-internal-reflection in the wall of cell 508 and excites the fluorescent emitter 500 (if it is in an emitting state). An optical signal 514 from a fluorescent emitter (e.g., fluorescent dye molecule) 500 is directed to an optical detector 516, where it appears against a dark background, ensuring a high signal-to-noise ratio. Processor 518 analyzes signals from detector
15 516 to determine the presence of target analyte.

The detector 516 may be an electron multiplying charge-coupled-device (EM-CCD) which can image many individual fluorescent emitters simultaneously. Alternatively, to reach microsecond integration times, the signal from a fluorescent emitter may be projected onto an analog
20 photodiode. Alternatively, the detector may be a CMOS camera.

The analyte biosensing technique has significant sensitivity by allowing for single-molecule detection. The selectivity is significantly higher than ensemble-averaged methods because single-molecule resolution allows for the discrimination between specific (strong, long-lived)
25 and non-specific (weak, short-lived) interactions on a per-molecule basis.

Embodiments of the present invention may use techniques of dark field scattering spectroscopy. Specifically, it may use techniques using a bright low-temporal coherence light source, preferably with a line width larger than 5 nm (e.g., superluminescent diode) for dynamically
30 measuring changes in the plasmon resonance peak of plasmonic particles.

Imaging and/or spectroscopy of scattering objects is usually performed with a *non-coherent* white-light source (emission bandwidth >1000 nm). It allows for the imaging of scattering objects against a homogeneous and low background, and it can be used to extract a broadband scattering spectrum by determining the scattering signal at many different wavelengths using the same illuminator. Commonly employed sources are incandescent lamps (e.g., halogen) or arc-discharge sources (e.g., Xenon). The main disadvantage of these sources is their extended emitting area ($> 1 \text{ mm}^2$), which does not permit tight focusing of the beam to achieve a high irradiance of the sample.

One way to overcome this is by using a narrowband and *coherent* light source such as a laser. The high coherence and low bandwidth (typically less than 1 nm) allow for the tight focusing of the beam to achieve a high irradiance of the sample. However, coherent laser irradiation has limitations because (1) interference fringes cause an inhomogeneous illumination pattern and (2) small spurious reflections and leakage of light in the optical setup cause background artefacts in the image. Such artefacts significantly reduce the signal-to-noise ratio and may fluctuate in time due to vibrations and thermal drifts of the optical setup.

FIGS. 6A-B illustrate two implementations of a combined microscopy and spectroscopy method that use a bright and low-coherence light source, a superluminescent diode (SLD) 600, for the time-dependent imaging of scattering objects 602. In the biosensing techniques of embodiments of the present invention, the time-dependent signal represents fluorescence pulses indicative of the presence of an analyte. The shorter coherence length of the SLD light 604 compared to laser light significantly reduces the artefacts caused by interference, while the brightness of SLDs is similar to common diode lasers.

The *low coherence* and intermediate bandwidth (e.g., 15-30 nm at near-infrared wavelengths) of the beam result in homogeneous illumination and low background intensity. The high brightness and small emitting area (e.g., less than $30 \text{ }\mu\text{m}^2$ when coupled to a single-mode fiber) ensure a high irradiance. The signal from an object excited with a superluminescent diode is high compared to the background and stable on short as well as long timescales.

In **FIG. 6A** the SLD 600 illuminates the sample 602 under an angle exceeding the angle for total internal reflection at a glass-water interface of a liquid cell 606. In this implementation, the light 604 is coupled to the sample 602 via a glass prism 608. In an alternate implementation, shown in **FIG. 6B**, the light 604 is coupled to the sample 602 via the back aperture of an objective lens 610. Because the implementation in **FIG. 6A** separates the excitation and emission light-paths, it leads to a lower background and higher signal-to-noise ratio than the implementation in **FIG. 6B**. The implementation in **FIG. 6B** may be useful if the space above the sample is to be used for other purposes, e.g., a technical component for temperature regulation. In both implementations, because the angle of illumination is higher than the angle for total-internal-reflection at the glass-water interface of the cell 606, all the excitation light is reflected. The presence of the particle 602 perturbs the total-internal-reflection, leading to a certain intensity of fluorescence pulse 612 that is partly collected by the objective and sent to an imaging sensor 614. The reflected beam is blocked by a beam-block, and the remaining light is sent to an imaging detector 614, preferably a camera with sufficient dynamic range and wavelength sensitivity to achieve single-molecule resolution. In another embodiment, the sample 602 can be mounted on an optical probe (e.g., optical fibre) to allow for measurements to be conducted directly in complex biological environments. Signals from detector 614 are then analyzed by a processor 616 to determine the presence of the target analyte.

The assay may involve e.g., a binding assay, a competitive assay, a displacement assay, a sandwich assay, an enzymatic assay, an assay with target and/or signal amplification, a multi-step assay, an assay with molecular cascade, etc. The assay may involve recognition moieties of different natures, e.g., peptides, proteins, nucleic acids, carbohydrates, etc. Embodiments may include various calibration methods, controls, multiplexing, etc. Embodiments may include measures to block and reduce unwanted processes (e.g., non-specific processes that generate background signals) and to increase efficiency, stability, and selectivity of signal generation.

Embodiments of the present invention include a system and technique for biosensing an analyte in a matrix using a large collection of fluorescent emitters. In preferred embodiments, for example, a biosensor based on hundreds of fluorescent dye molecules with single-molecule sensitivity are simultaneously monitored in real-time within a dark-field microscopy setup.

Hundreds of individual fluorescent emitters may be simultaneously monitored with single-molecule sensitivity in real-time within a dark-field microscopy setup. The approach allows for the statistical analysis of single-molecule interactions without requiring any labeling of the analyte. The ability to probe hundreds of fluorescent emitter simultaneously will provide a
5 sensor with a very large dynamic range in concentration.

Single-molecule detection has distinct advantages over ensemble-averaged techniques because it yields statistical distributions of molecular properties instead of averages, and reveals rare and unsynchronized events. Preferred embodiments of the invention include techniques for
10 monitoring hundreds of single-molecule fluorescent emitter in real-time using total-internal-reflection excitation in a standard microscope.

In one implementation of the embodiments shown in **FIGS. 6A-B**, the superluminescent diode is a Superlum, with center wavelength 795 nm, bandwidth 14 nm, maximum power 35 mW. The
15 detector 614 is a charge coupled device (CCD), e.g., with an area of $50 \times 50 \mu\text{m}^2$ on the sample surface.

The use of a superluminescent diode (SLD) as the light-source is important to achieve sufficient signal-to-noise ratio (S/N). The poor spatial coherence of light from an incandescent lamp
20 provided insufficient intensity to image the small particles, whereas the high temporal coherence of laser illumination resulted in interference artifacts that induce signal fluctuations. SLD's are semiconductor high-gain devices that generate amplified spontaneous emission. In this application the low temporal coherence of the SLD significantly reduced interference artifacts whereas the high spatial coherence ensured a high illumination intensity. This resulted in shot-
25 noise limited signals for an integration time of 100 ms.

In a typical single-molecule experiment an analyte is passed into the flow cell using a syringe pump. The CCD camera is used to record the time-dependent fluorescence signal (determined by a two-dimensional Gaussian fit of each spot in each frame). Fluorescence pulses caused by
30 the presence of the analyte are then observed as intensity changes as a function of time.

The detected intensity may be obtained by fitting a particle's diffraction-limited spot in each frame with a two-dimensional Gaussian. The S/N (defined as the ratio between the mean and the standard-deviation of the signal over 150 seconds) increases for brighter particles. The S/N approaches the shot noise limit with some excess noise that is likely caused by fluctuations of the SLD intensity and slight mechanical drift of the sample. From these measurements we conclude that step-wise signal-changes of 1% can be detected with a S/N of 3-5 for fluorescent emitters with an integrated intensity over 10^5 counts/s.

The precision with which an analyte concentration can be determined may be limited by counting statistics. For example, at least 100 molecules need to be detected in a defined time window in order to have a precision of concentration-determination of about $1/\sqrt{100} = 10\%$. Small fluorescent emitters have a limited number of receptor molecules on their surface, so an individual particle can capture only a limited number of analyte molecules. Furthermore, in the limit of very low analyte concentration, there is a high probability that a single fluorescent emitter molecule will not have captured even a single analyte molecule, even for long incubation times. To address this issue, a biosensing system according to preferred embodiments of the invention have at least 100 fluorescent emitters, and time traces are recorded on the individual particles. The data of the particles is combined by the processor in order to determine an analyte concentration. For low analyte concentration, preferably data is combined from at least 1000 fluorescent emitters, more preferably at least 10,000 fluorescent emitters.

Probes for biosensing with single-molecule resolution typically exhibit a limited dynamic range due to the low number of binding sites per probe, prohibiting the accumulation of sufficient statistics at low analyte concentrations. This limitation is overcome in embodiments of the present invention by the parallelized probing of many sensors, giving an extraordinary projected dynamic range of 7 decades in concentration. The ability to extract distributions of molecular interaction parameters enables the investigation of heterogeneity in a population of unlabeled molecules. The simple and cheap optical layout allows the sensor to be implemented easily with a microscope.

It is also preferable to use an optical system with a high frame rate. Preferably the frame rate or reciprocal integration time is higher than 100 s^{-1} , more preferably higher than 1000 s^{-1} .

A high signal-to-noise ratio and high frame rate can be achieved using a light source with high power. However, a high optical power can give an unacceptable temperature rise in the sample fluid, thereby affecting the biochemical materials. To monitor this potential issue, an optical thermometer may be integrated with the system. For example, using a phase-sensitive camera, or monitoring the blue-shifted emission from the metallic particles, may be implemented. To maintain an acceptable temperature the incident power can be adjusted.

Analyte multiplexing, i.e., measurement of different analytes at the same time, is advantageous for increased biomedical sensitivity and specificity. To provide for such multiplexing, some embodiments use fluorescent emitters that have different receptors on their surface. For reasons of counting statistics and precision, the number of particles should be at least equal to 100 for every distinct type of analyte. Also, some embodiments use fluorescent emitters with different optical properties, so that the different fluorescent emitters can be mixed. The fluorescent emitters preferably have at least two sub-populations that can be optically distinguished and that have different receptors on their surface. Also in this case, for reasons of counting statistics and precision, the number of fluorescent emitters is preferably at least 100 for every analyte. The minimal frame rate of the optical system is determined by the analyte with the highest event rate.

The features described in various separate embodiments of the invention are not necessarily exclusive and, in general, may be used in combination with each other. Such features and embodiments also include material disclosed in US provisional patent applications 62/092758 filed 12/16/2014, 62/092763 filed 12/16/2014, and 62/132096 filed 3/12/2015, all of which are incorporated herein by reference.

CLAIMS

1. A method for sensing an analyte, the method comprising:

bringing a matrix containing the analyte into contact with a sensor device having a sensing

5 volume containing fluorescent emitters, where the fluorescent emitters switch from a first state to a second state when activated by chemical activation by the analyte, where the second state has an extinction coefficient for light with a wavelength for fluorescence excitation;

exciting the fluorescent emitters in the sensing volume with optical radiation at the excitation

10 wavelength to generate fluorescence optical radiation, where the excitation induces photo-bleaching of the activated fluorescent emitters, which limits the duration of the generated fluorescence optical radiation;

detecting pulses of the fluorescence optical radiation from the fluorescent emitters in the sensing volume, where each of the pulses is associated with a single one of the fluorescent

15 emitters;

determining the presence/concentration of the analyte from changes in the detected fluorescence optical radiation while the matrix containing the analyte is in contact with the sensor device.

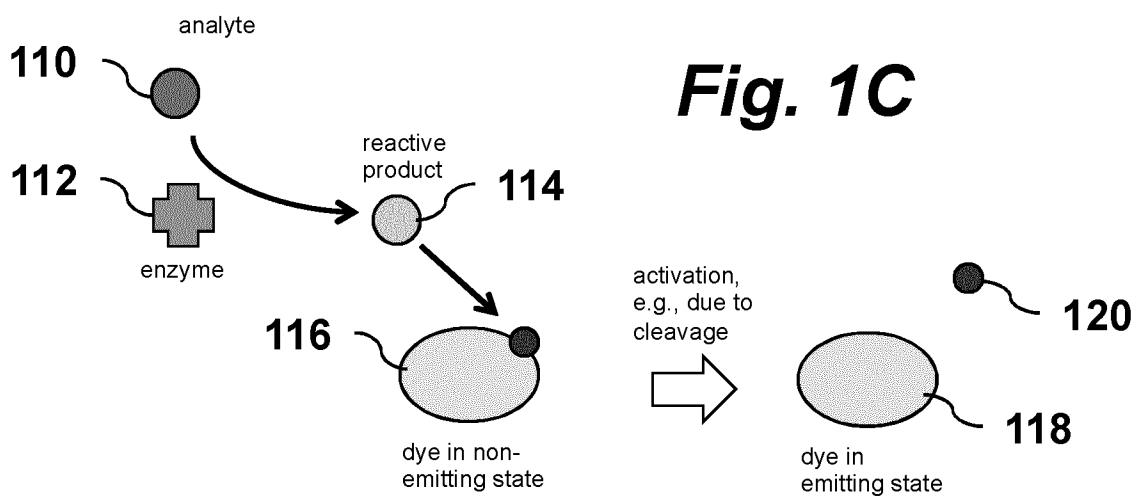
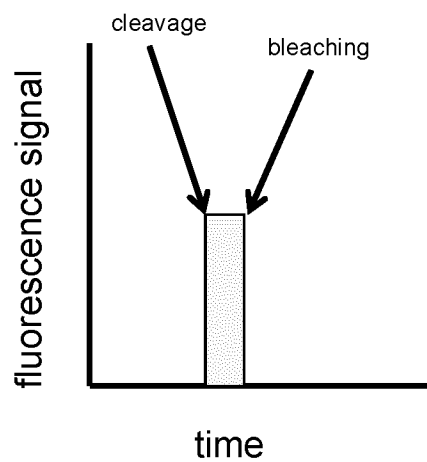
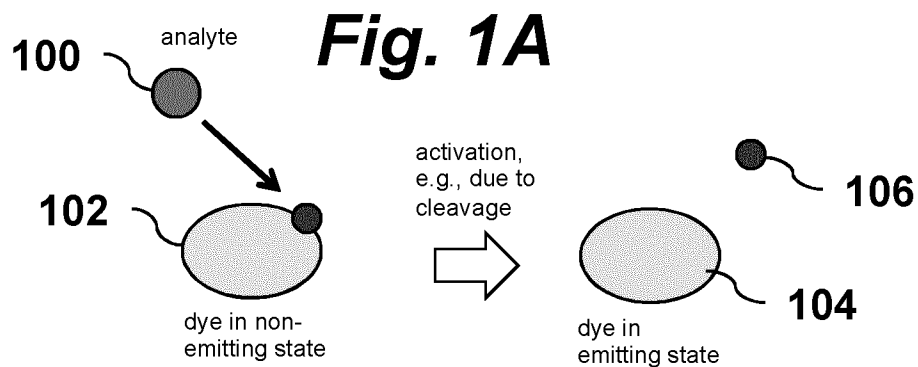
20 2. The method of claim 1 wherein the chemical activation of the fluorescent emitters by the analyte is direct activation by the analyte.

3. The method of claim 1 wherein the chemical activation of the fluorescent emitters by the analyte is indirect activation by an intermediate chemical activator molecule produced by enzymatic conversion of the analyte

4. The method of claim 1 wherein exciting the fluorescent emitters comprises exciting the fluorescent emitters by exposing the fluorescent emitters to optical light from a light source with a line width larger than 5 nm or a superluminescent diode.

5. The method of claim 1 wherein determining the presence/concentration of the analyte comprises performing histogram and/or histogram processing to suppress background noise and enhance specificity.
- 5 6. The method of claim 1 wherein the fluorescence emitters are fluorescent dye molecules, fluorescent organic molecules, fluorescent emissive metal complexes, fluorescent organometallic complexes, or fluorescent quantum dots.
7. The method of claim 1 wherein the extinction coefficient is at least 10 times larger than
10 the extinction coefficient for the same wavelength in the first state.

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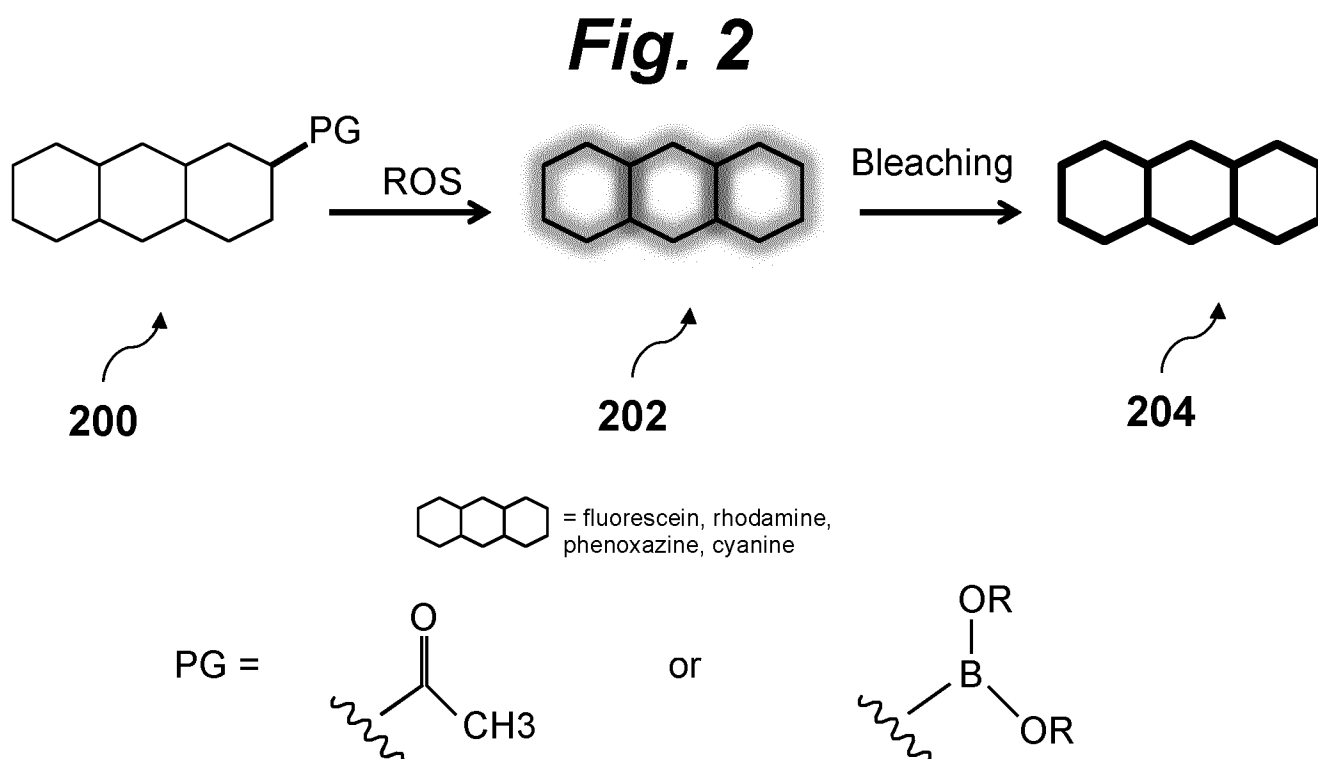
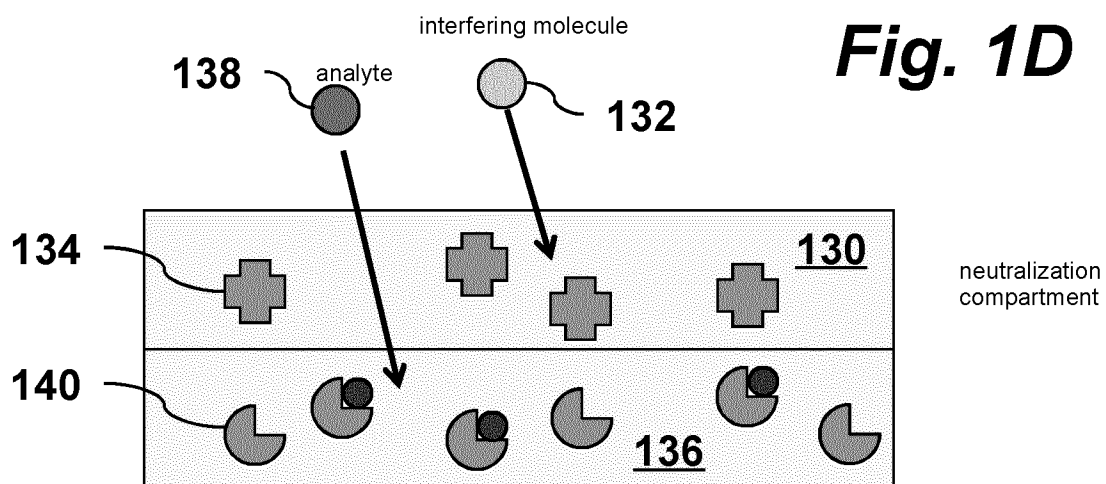


Fig. 3A

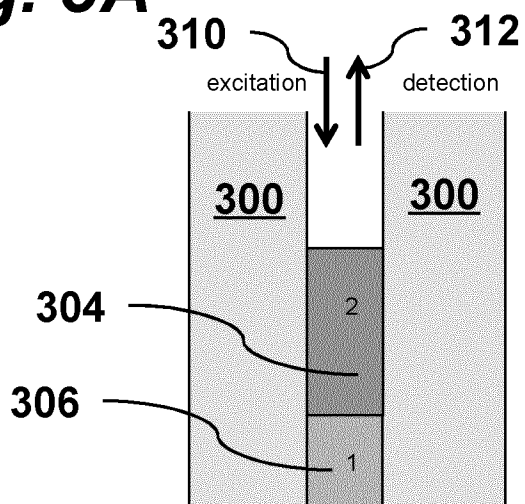


Fig. 3B

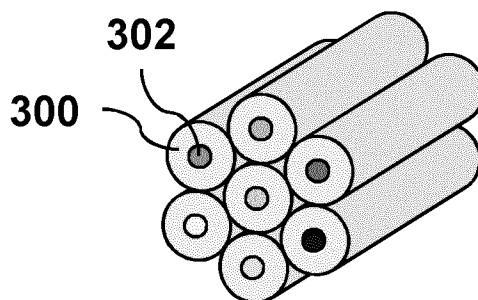


Fig. 4A

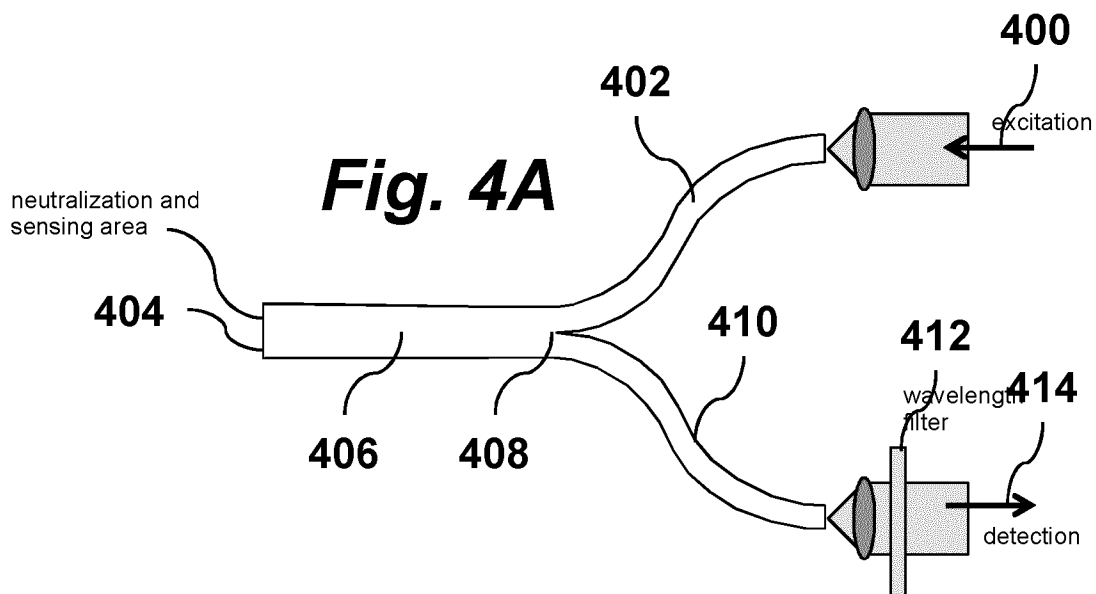


Fig. 4B

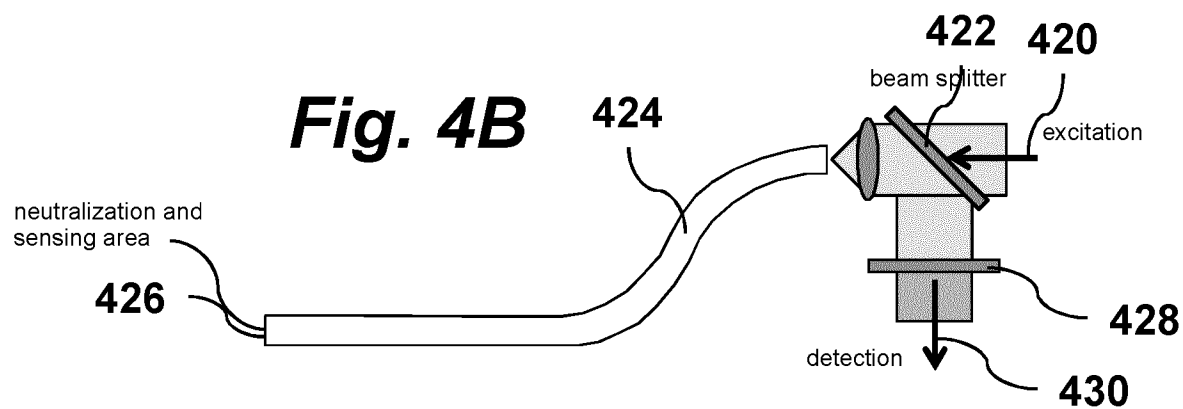


Fig. 5

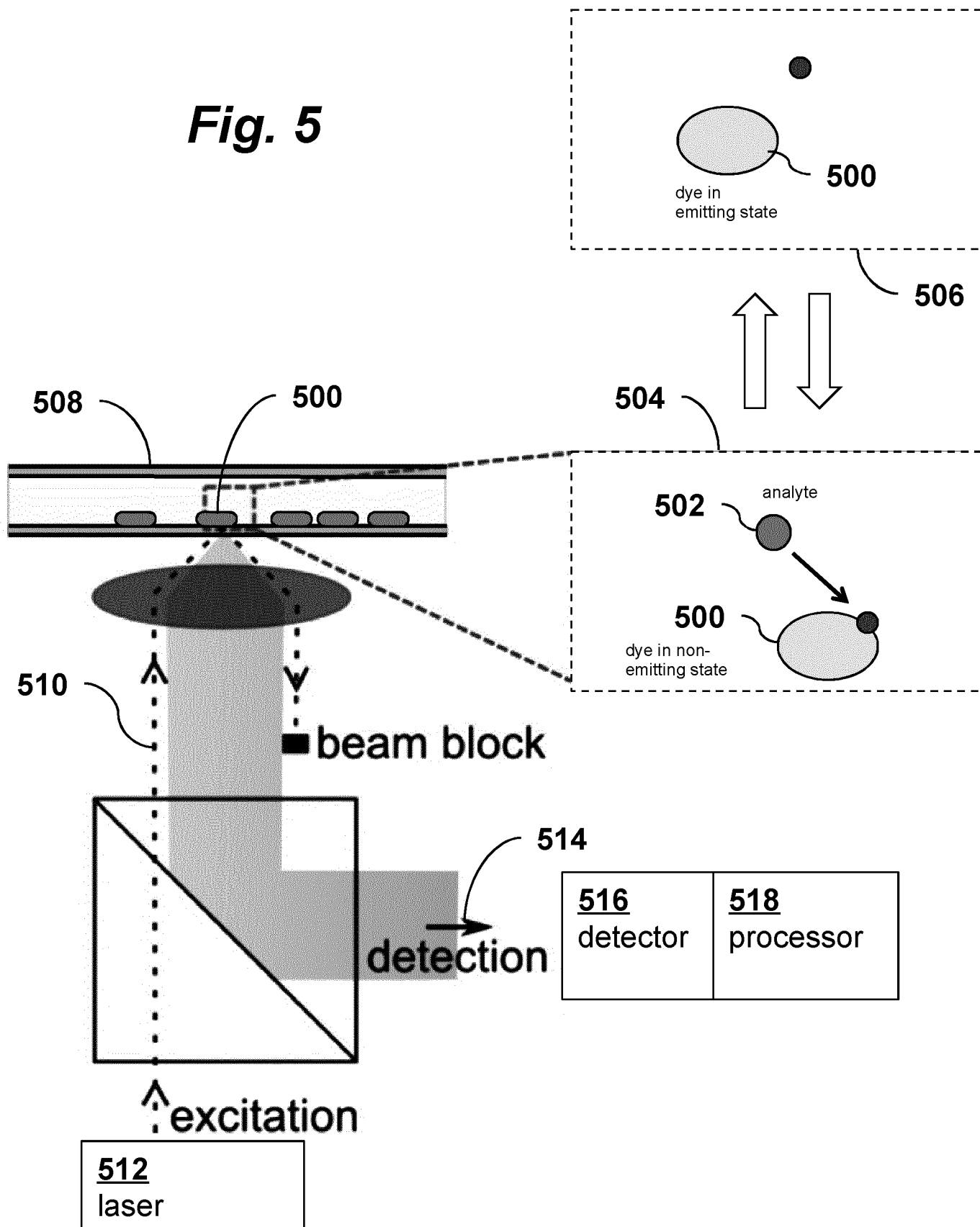
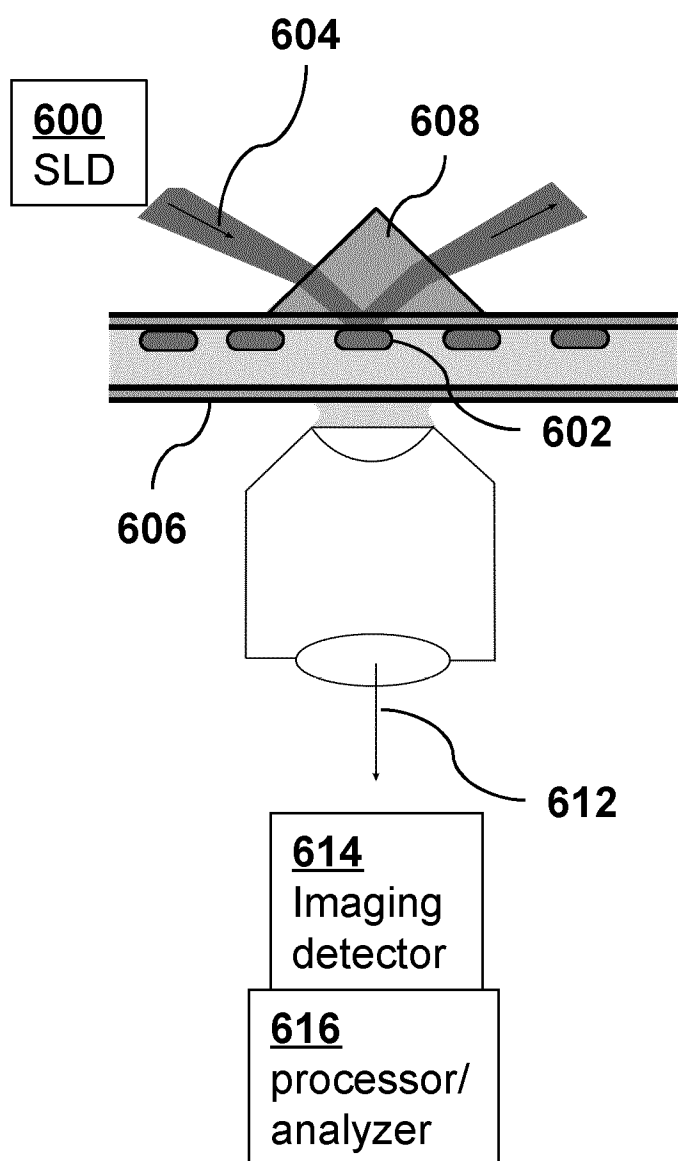
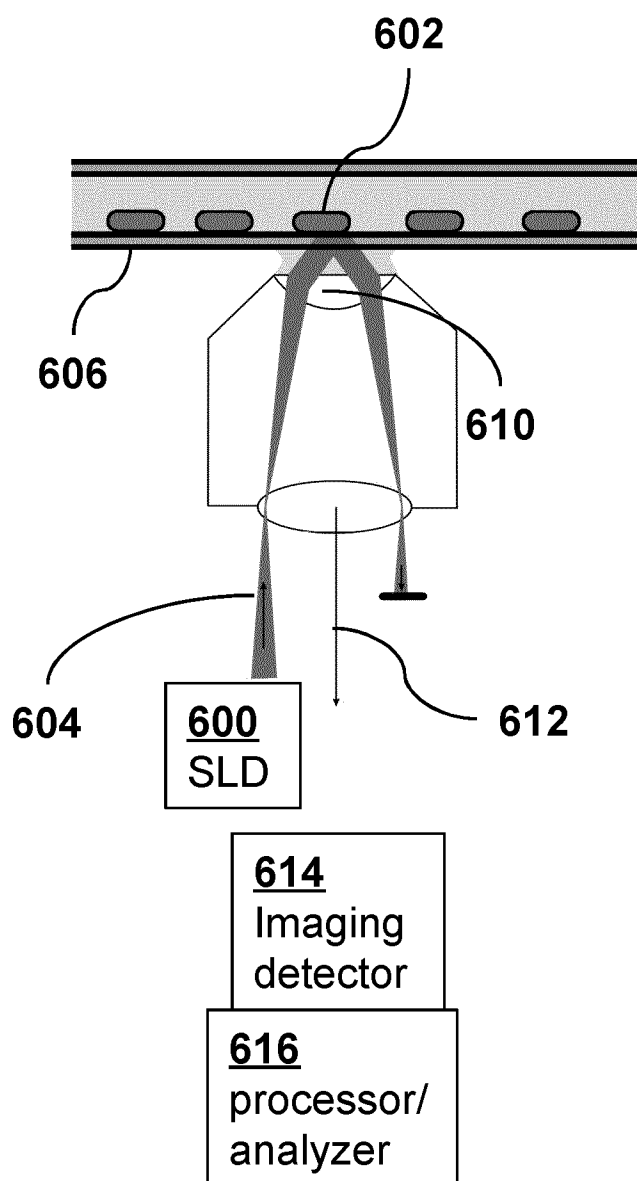


Fig. 6A**Fig. 6B**

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/079872

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/00 G01N33/542
ADD.

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)
C12Q G01N

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MATT PAIGE ET AL: "<title>Biomolecular applications of single-molecule measurements: kinetics and dynamics of a single-enzyme reaction</title>", PROCEEDINGS OF SPIE, vol. 4634, 29 March 2002 (2002-03-29), pages 92-103, XP055243740, US	1,3,6,7
Y	ISSN: 0277-786X, DOI: 10.1117/12.463828 ISBN: 978-1-62841-730-2 the whole document in particular: page 95, paragraph 2.2 - page 96 figures 3,4 ----- -/--	4,5



Further documents are listed in the continuation of Box C.



See patent family annex.

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

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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

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"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

22 January 2016

Date of mailing of the international search report

02/02/2016

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Authorized officer

Tuynman, Antonin

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/079872

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARISSA K. LEE ET AL: "Enzymatic activation of nitro-aryl fluorogens in live bacterial cells for enzymatic turnover-activated localization microscopy", CHEMICAL SCIENCE, vol. 4, no. 1, 1 January 2013 (2013-01-01) , pages 220-225, XP055243742, United Kingdom ISSN: 2041-6520, DOI: 10.1039/C2SC21074F	1,3,5-7
Y	the whole document in particular: abstract page 222; figures 1,2 figure 5 page 222, right-hand column, line 1 - page 223, left-hand column, line 8 -----	4
X	BENNO N. EHRL ET AL: "Single molecule kinetics of horseradish peroxidase exposed in large arrays of femtoliter-sized fused silica chambers", THE ANALYST, vol. 138, no. 15, 1 January 2013 (2013-01-01), page 4260, XP055243660, GB ISSN: 0003-2654, DOI: 10.1039/c3an00809f	1,3,6,7
Y	the whole document in particular: page 4263; figure 2 -----	4,5
X	QI YAN ET AL: "Localization Microscopy using Noncovalent Fluorogen Activation by Genetically Encoded Fluorogen-Activating Proteins.", CHEMPHYSICHEM - A EUROPEAN JOURNAL OF CHEMICAL PHYSICS & PHYSICALCHEMISTRY., vol. 15, no. 4, 5 November 2013 (2013-11-05), pages 687-695, XP055243731, DE ISSN: 1439-4235, DOI: 10.1002/cphc.201300757	1,2,6,7
Y	the whole document in particular: abstract page 689; figure 1 page 690; figure 7c par. "Localization Microscopy"; page 694 page 689, right-hand column, paragraph 2 bridging paragraph; page 688, left-hand column - right-hand column -----	4,5
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/079872

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TURNER E H ET AL: "Reaction of fluorogenic reagents with proteins", JOURNAL OF CHROMATOGRAPHY, ELSEVIER SCIENCE PUBLISHERS B.V, NL, vol. 1194, no. 2, 20 June 2008 (2008-06-20), pages 253-256, XP022696663, ISSN: 0021-9673, DOI: 10.1016/J.CHROMA.2008.04.046 [retrieved on 2008-04-24]	1,2,6,7
Y	the whole document in particular: abstract page 255, paragraph 3.2 - page 256, paragraph 3.3 page 255; figure 3	4,5
Y	----- SLAVIK ET AL: "Ultrahigh resolution long range surface plasmon-based sensor", SENSORS AND ACTUATORS B: CHEMICAL: INTERNATIONAL JOURNAL DEVOTED TO RESEARCH AND DEVELOPMENT OF PHYSICAL AND CHEMICAL TRANSDUCERS, ELSEVIER S.A, CH, vol. 123, no. 1, 30 March 2007 (2007-03-30), pages 10-12, XP022011170, ISSN: 0925-4005, DOI: 10.1016/J.SNB.2006.08.020 the whole document abstract	4
Y	----- EUGENIO GALLO ET AL: "Fluorogen-activating-proteins as universal affinity biosensors for immunodetection", BIOTECHNOLOGY AND BIOENGINEERING., vol. 111, no. 3, 1 March 2014 (2014-03-01), pages 475-484, XP055243677, US ISSN: 0006-3592, DOI: 10.1002/bit.25127 the whole document in particular: abstract figures 3,4 -----	5