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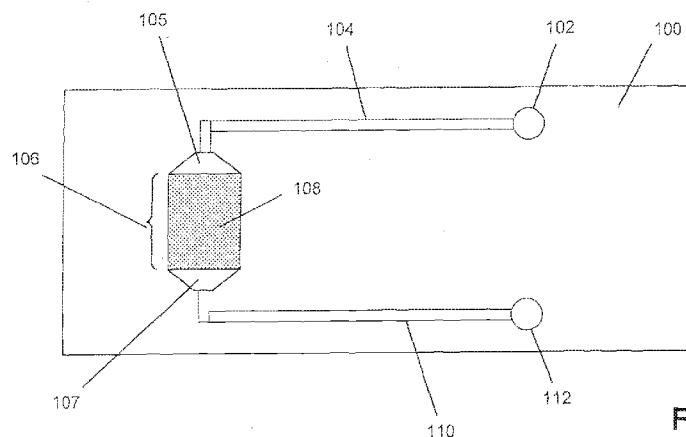


Fig. 1A

(57) Abstract: In one aspect, the invention provides a disposable cassette for performing immunoassays, particularly in conjunction with an imaging apparatus. An important feature of the cassette is a sample chamber containing a polymer coating for holding and releasing assay reagents when exposed to a biological fluid, such as blood or saliva. Preferably, the polymer coating has the following characteristics: (i) it immobilizes and stabilizes assay reagents for prolonged shelf live, (ii) it retards sample release during sample loading, and (iii) it rapidly releases assay reagents after sample has filled the chamber to form a uniform reagent concentration throughout the sample chamber.



ASSAY CASSETTE AND SYSTEM

Background

Point-of-care testing and the search for effective biomarkers are important themes in biomedical research, e.g. Holland et al, *Curr. Opin. Microbiol.*, 8: 504-509 (2005); Yager et al, *Nature*, 442: 412-418 (2006); Frank et al, *Nature Reviews Drug Discovery*, 2: 566-580 (2003); Sidransky, *Nature Reviews Drug Discovery*, 2: 210-218 (2002). Both endeavors are meant to improve the access and effectiveness of healthcare while reducing its costs. Point-of-care testing is analytical testing performed outside a central laboratory using a device that can be easily transported to the vicinity of the patient and that can be operated under field conditions without highly specialized personnel. In many acute care medical and bio-defense monitoring applications, rapid sample processing and test readouts are also required, e.g. Raja et al, *Clinical Chemistry*, 48: 1329-1337 (2002).

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention, Atkinson et al, Clin. Pharmacol. Ther., 69: 89-95 (2001).

Biomarkers vary widely in nature, ease of measurement, and correlation with physiological states of interest, e.g. Frank et al (cited above). Most point-of-care devices are designed to measure molecular biomarkers that have been extracted from a sample or specimen or that are found directly in a biological fluid, such as blood, Holland et al (cited above). There is significant interest in measuring cellular markers in point-of-care devices, but cellular markers typically require some form of imaging or a fluidics system in order to make cell-specific measurements, thereby adding a significant technical challenge over that posed by the measurement of molecular markers, e.g. Shapiro, *Cytometry A*, 60A: 115-124 (2004); Shapiro et al, *Cytometry A*, 69A: 620-630 (2006); Rodriguez et al, *PLOS Medicine*, 2(7): e182 (2005); Janossy et al, *Clinical Cytometry*, 50: 78-85 (2002); Toner et al, *Annu. Rev. Biomed. Eng.*, 7: 77-103 (2005); and the like.

Point-of-care tests could be carried out on a wide range of sample types, including not only samples from individual organisms, such as medical, veterinary, or plant samples, but also samples from various environments, such as soils, water systems, air conditioner systems, surfaces in public places, such as transportation systems, and the like. Among

medical samples, biological fluids, such as blood, saliva, tear duct fluid, urine, and the like, are especially amenable for use with point-of-care assays, as they are usually much more accessible than solid tissues. Among such biological fluids from which cellular or molecular markers can be obtained, blood is the sample of choice, whenever biologically relevant, because it systemic, it is easily accessible, and it contains a rich and dynamic suspension of cells and molecules whose composition reflects states of health and disease. In particular, there is great interest in being able to count certain subsets of non-red blood cells that are correlated with disease susceptibilities, disease progression, drug responsiveness, and the like, e.g. Guisset et al, Intensive Care Med., Epub (November 8, 2006); Shaked et al, Curr. Cancer Drug Targets, 5: 551-559 (2005); Madjid et al, J. Am. Coll. Cardiol., 44: 1945-1956 (2004); Janossy et al (cited above); Rodriquez et al (cited above). Unfortunately, currently available analyzers for such markers suffer from one or more drawbacks that limit their widespread use, including complex preparation steps involving separation and/or cell lysis, involvement of specialized personnel, lack of portability, high cost, lack of sensitivity, and the like.

In view of the above, several medical and biotechnology fields would be significantly advanced with the availability of techniques, capable of point-of-care operation, which permitted facile and flexible measurements of cellular markers, particularly in biological fluids, such as blood.

SUMMARY OF THE INVENTION

The present invention provides a system, associated assay cassettes and methods for detecting, measuring and/or counting cells in a biological sample, particularly non-red blood cells in a sample of whole blood. In one aspect, the invention includes a cassette for optical analysis of cells in a sample, which comprises (a) a sample chamber capable of receiving a sample, the sample chamber being disposed in a body and having an optically transmissive wall and a dimension perpendicular to such wall substantially equivalent to the depth of field of a detector for collecting optical signals generated by probes attached to cells in the sample; and (b) a polymer coating on a surface of the sample chamber substantially parallel to, and co-extensive with, the optically transmissive wall, the polymer coating releasing a composition of probes in a uniform concentration throughout the sample chamber whenever sample is loaded therein, the composition of probes comprising a plurality of analyte-specific

probes, each analyte-specific probe comprising an optical label and a binding compound capable of binding specifically to a different cellular analyte of cells in the sample.

Preferably, the cassette is provided in a single-use, or disposable, form, usually made of plastic. In one embodiment, the sample is whole blood and the perpendicular dimension of the sample chamber is substantially equivalent to the size of a non-red blood cell being
5 detected so that optical signals generated by probes attached to cellular analytes thereof are not obstructed by red blood cells of the sample.

In another aspect, the invention includes a system for imaging multiple features of cells in a sample, the system comprising: (a) one or more light sources capable of
10 successively generating illumination beams each having a distinct wavelength band; (b) a composition of probes each having a differentially excitable label capable of labeling cells comprising multiple features, such that each different feature is labeled with a different differentially excitable label; (c) a controller operationally associated with the one or more light sources for successively directing illumination beams onto the sample so that each of the
15 different differentially excitable labels is successively caused to emit an optical signal within the same wavelength band; (d) an optical system capable of collecting such emitted optical signals and forming successive images corresponding to the labeled features of cells in the sample on a light-responsive surface to form successive sets of image data thereof; and (e) a cassette operationally associated with the optical system, the cassette comprising (i) a sample
20 chamber capable of receiving the sample, the sample chamber being disposed in a body and having an optically transmissive wall and a dimension perpendicular to such wall substantially equivalent to the depth of field of the optical system for collecting optical signals generated by probes attached to cells in the sample, and (ii) a polymer coating on a surface of the sample chamber substantially parallel to, and co-extensive with, the optically
25 transmissive wall, the polymer coating releasing the composition of probes in a uniform concentration throughout the sample chamber whenever sample is loaded therein.

In another aspect, the invention includes a method for analyzing predetermined non-red blood cells in a sample of whole blood, which comprises the steps of (a) providing a composition of probes each having a differentially excitable label capable of labeling cells
30 comprising multiple features, such that each different feature is labeled with a different differentially excitable label; (b) loading the sample into a cassette comprising (i) a sample chamber capable of receiving the sample, the sample chamber being disposed in a body and

having an optically transmissive wall and a dimension perpendicular to such wall substantially equivalent to the depth of field of an optical system for collecting optical signals generated by probes attached to non-red blood cells in the sample, and (ii) a polymer coating on a surface of the sample chamber substantially parallel to, and co-extensive with, the optically transmissive wall, the polymer coating releasing the composition of probes in a uniform concentration throughout the sample chamber; (c) successively directing illumination beams onto the sample so that different differentially excitable labels are successively caused to emit an optical signal; (d) collecting such successively emitted optical signals with the optical system and forming successive images corresponding to the labeled features of non-red blood cells in the sample on a light-responsive surface to form successive sets of image data thereof; and (e) enumerating the non-red blood cells in the sample by analyzing the successive sets of image data. In one embodiment, the non-red blood cells are CD4+ lymphocytes. In another embodiment, the non-red blood cells are microorganisms, such as malaria.

The invention overcomes many cost and efficiency drawbacks of prior art approaches to point-of-care systems for rapid analysis of medical and environmental samples, including blood, saliva, urine, and the like. Particular embodiments of the invention are well suited for low cost and efficient detection and counting of a variety of cellular components and/or pathogens that may be present in whole blood, including, but not limited to, non-red blood cells, lymphocytes, such as CD3+ cells, CD4+ cells, CD8+ cells, blood parasites, such as malaria, and the like.

Brief Description of the Drawings

Figs. 1A-1E illustrate one embodiment of the cassette of the invention and the operation of the polymer coating upon exposure to sample.

Figs. 1F-1H illustrate another embodiment of the cassette of the invention wherein the polymer coating is in the form of droplets.

Figs. 2A-2B illustrate diagrammatically an embodiment of cassette for detecting and analyzing non-red blood cells and/or other cells or microorganisms in whole blood.

Fig. 3 illustrates diagrammatically an optical system for use with the invention.

DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention may employ, unless otherwise indicated, conventional techniques from molecular biology (including recombinant techniques), cell biology, immunoassay technology, microscopy, image analysis, and analytical chemistry, which are within the skill of the art. Such conventional techniques include, but are not limited to, detection of fluorescent signals, image analysis, selection of illumination sources and optical signal detection components, labeling of biological cells, and the like. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual*, *Cells: A Laboratory Manual*, *PCR Primer: A Laboratory Manual*, and *Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press); Murphy, *Fundamentals of Light Microscopy and Electronic Imaging* (Wiley-Liss, 2001); Shapiro, *Practical Flow Cytometry*, Fourth Edition (Wiley-Liss, 2003); Herman et al, *Fluorescence Microscopy*, 2nd Edition (Springer, 1998); all of which are herein incorporated in their entirety by reference for all purposes.

In one aspect, the invention provides a disposable cassette for performing immunoassays, particularly in conjunction with an imaging apparatus, such as described in Goldberg et al, U.S. patent publication 2008/0212069. An important feature of the cassette is a sample chamber containing a polymer coating for holding and releasing assay reagents when exposed to a biological fluid, such as blood or saliva. Preferably, the polymer coating has the following characteristics: (i) it immobilizes and stabilizes assay reagents for prolonged shelf live, (ii) it retards sample release during sample loading, and (iii) it rapidly releases assay reagents after sample has filled the chamber to form a uniform reagent concentration throughout the sample chamber.

The cassette of the invention achieves the above (1) in part by providing a sample chamber having a flat geometry with large width and length dimensions and a height either (a) substantially equivalent to the depth of field of an objective lens of a detector, or (b) just slightly larger than the cells to be analyzed in a sample, and (2) in part by providing a polymer coating on a large-area wall of the chamber for releasing assay reagents uniformly into the sample. The polymer coating is selected so that reagents are rapidly released into the chamber over a large area where they are required to diffuse only a short distance to establish

a uniform concentration throughout the chamber, after a sample is loaded. Preferably, the polymer coating does not interfere with the loading of the sample into the sample chamber, e.g. by blockage or other inhibition of flow when the sample enters the sample chamber, or with the assay reaction or the detection of probes or other assay components. In one aspect, the polymer coating comprises a cross-linked hydrophilic polymer that is capable of adhering to a wall of the sample chamber, so that it is not displaced or disrupted when sample is loaded. Such polymer coatings include hydrogels that swell upon contact with sample so that the gel pore size increases to release assay reagents. The polymer coatings may be in the form of a continuous film or a discontinuous array of droplets. In a particular embodiment, the polymer coating comprises a polyvinyl alcohol cross-linked with borate. In further embodiments, the polymer coating may comprise more than one layer of polymer materials. For example, in such a multi-layer format, a first layer may hold assay reagents and a second layer may regulate the release of assay reagent into the chamber upon loading of a sample. Such multi-layer structures are disclosed in Walter, *Methods in Enzymology*, Vol. 137, pgs. 394-420 (1988), and like references.

Embodiments and features of the cassette of the invention are illustrated in Figs. 1A through 2B. Fig. 1A is a top view of one embodiment of the cassette. Preferably, cassettes of the invention comprise a molded plastic body (100) and an optically transmissive cover that is sealingly attached to body (100) so that fluid passages and chambers are formed from etched or molded cavities in body (100), for example, using conventional techniques for making microfluidics devices. In one form, plastic body (100) has the dimensions roughly equivalent or similar to those of a standard microscope slide. Port (102) is in fluid communication with sample chamber (106) through passage (104). Passage (104) connects to chamber (106) at one end by way of inlet (105). Chamber (106) also comprises outlet (107), shown in this embodiment at the opposite end of the chamber, which allows air or gas to vent from chamber (106) as a sample is loaded and exhaust through passage (110) and port (112). Obviously, many alternative geometries of such a fillable chamber are available for use in conjunction with the invention. As noted above, an important element of chamber (106) is polymer coating (108) that stores and then releases assay reagents upon sample being introduced into chamber (106). As illustrated in Fig. 1B, sample, such as whole blood, may be introduced into port (102) by a sample collecting device, such as pipette (114), after which

it travels (116) through passage (104), e.g. by capillary action, to chamber (106).

Alternatively, sample could be pumped or injected into chamber (106). Preferably, surfaces of chamber (106) and the composition of polymer coating (108) are selected so that loaded sample fills chamber (106) uniformly and without the formation of bubbles that might disrupt optical measurements. During and after the time sample is loaded into chamber (106), as shown in Fig. 1C, polymer coating (108) changes in response to the sample so that assay reagents embedded in the polymer are released into chamber (106). In one aspect, polymer coating (108) is a hydrogel that upon exposure to sample expands so that pore size increases and assay reagents are released. In another aspect, polymer coating (108) is a degradable gel that upon exposure to sample degrades to release assay reagents.

Fig. 1D shows cross section of chamber (106) along vertical plane (118) (shown in Fig. 1C). Cover (120) is attached to body (100) to form chamber (106), which comprises polymer coating (108). Prior to loading sample, when polymer coating (108) is a hydrogel it is in a dried or dehydrated form of reduced volume (122) and with assay reagents trapped in the matrix of the polymer. Upon loading (125) of sample, volume (124) of polymer coating (108) increases by hydration from the sample, so that pore size in the polymer matrix increase and assay reagent are released (126) into chamber (106). The composition and dehydrated volume of polymer coating (108) are selected so that upon exposure to sample, the volume of polymer coating (108) increases so that its top surface rises in chamber (106) to a predetermined level (121). Predetermined level (121) corresponds to a height of chamber (106) that is approximately equal to the size of cells of interest in the sample or the depth of field of the detection system for collecting optical signals from the assay reagents, as explained more fully below.

Polymer coating (108) may be applied to a wall of chamber (106) in a variety of ways. For example, in Figs. 1A-1E, polymer coating (106) is shown as a continuous film. Alternatively, polymer coating (108) may be applied as an array (130) of droplets (132) of the polymer that adhere to a wall of chamber (106), as illustrated in Fig. 1F. Fig. 1G shows chamber (106) of Fig. 1F in cross section (134), where polymer coating (108) is in the form of an array of droplets (132). Upon sample loading (136), as illustrated in Fig. 1H, droplets (138) of the polymer coating (108) swell and release assay reagents. The size and density of droplets in array (130) are design choices within the scope of the invention. Likewise, array

(138) may be a regular array, such as rectilinear, hexagonal, or the like, or array (130) may be random, such as a Poisson distribution of droplets at a predetermined density.

An important feature of cassette (100) is the collection of optical signals from a defined or determinable volume within sample chamber (106)(referred to herein as the
5 “Optical Sample Volume”) so that concentration determinations can be made from image data, e.g. of selected cell types. An Optical Sample Volume is defined by the distance (e.g. 228 in Fig. 2B) between top wall (214) and bottom wall (216) of cassette (100) and the area, or field of view, of the imaging optics. An important feature of the optical system of the invention in this embodiment is that the depth of field of the objective be greater than or
10 equal to the distance (228 or 218) between top wall (214) and bottom wall (216), so that optical signals from all the objects in Optical Sample Volume are collected. Preferably, top wall (214) is suitable for passing optical signals for collection and is substantially parallel with bottom wall (216).

As mentioned above, the distance between the top wall (214) and bottom wall (216)
15 of sample chamber (202) is important for analysis of whole blood samples. If the distance is too great, e.g. (218) of Fig. 2A, then enucleate red blood cells (220) may obstruct (226) the passage optical signals generated from cell types of interest (222), in which case such cells may not be counted, leading to an under estimate of cell numbers or concentration. In accordance with the invention, and as illustrated in Fig. 2B, distance (218) between top wall
20 (214) and bottom wall (216) of sample chamber (202) is substantially equivalent to the diameter, or effective diameter, of cell types of interest (222), so that obstructing layers of enucleate red blood cells cannot form between a cell of interest (222) and top wall (214), and optical signals therefrom (224) all from sample chamber (202) to the imaging optics. In one aspect, sample chamber (202) has a distance (218) substantially equivalent to the depth of
25 field of the imaging optics. In another aspect, sample chamber (202) has a distance (218) in the range of from 10 to 100 μm , or from 10 to 50 μm , or from 20 to 50 μm .

The invention includes kits comprising a cassette, as described above, packaged so that its polymer coating is protected against drying or assay reagent degradation until used. Such packaging includes controlled humidity packaging.

Polymer Coatings and Assay Reagents

Preferably, polymer coatings in the cassette, and any reagents or excipients included therein, (i) do not cause aberrant flow of the sample in the chamber and (ii) allow even distribution and release of assay reagents. In one aspect, a formulation of polymer coating material preferably minimizes the amount of polymer added per unit volume of sample, while at the same time providing a matrix capable of immobilizing the necessary amount of assay reagents, e.g. dye-antibody conjugates. That is, in one aspect, polymer coatings preferably satisfy the following performance criteria: (1) polymer coatings permit reagent release when used at very low reagent concentrations (and do not permanently sequester or bind reagent), (2) polymer coatings adhere well to the chamber wall (thereby avoiding detachment and obstruction of sample loading), (3) polymer coatings wet well and allow even chamber fill with sample, such as whole blood, (4) polymer coatings do not dissolve significantly during initial wetting or after prolonged contact with the sample, and (5) polymer coatings can be dried (i.e. have water removed) to stabilize reagents, especially antibody-based reagents. In other aspects, polymer coatings may include polymer materials capable of degrading in the presence of sample (for example, by eroding, dissolving, or the like), provided that such process does not interfere with loading sample and the uniform dispersal of assay reagents.

In one aspect, the main component of a polymer coating is preferably a polymer that could be either natural or synthetic (man made). One of the preferred matrices is a system based on polyvinyl alcohol (PVA, for example, MW 85-146kDa), cross-linked using borate (at different concentrations), preferably containing a carrier protein, preferably gelatine (others include BSA, collagen, casein) and containing one or more antibody-dye conjugate (dye is preferably fluorescent). A preferred matrix formulation was identified that is based on following composition: 1% PVA, 5-10 mM Borate, 0.2% Gelatin and the Ab conjugates at 0.4 ug/mL CD4-PECy5 + 1.0 ug/mL CD3-APC + 0.5 ug/mL anti-CD45RA-APC.

Whole blood compatibility and slow release of dye conjugates were achieved with hydrophilic long-chain vinyl polymers, such as polyvinyl acetate glue (PVAc), Eudragits (including Plastoids, which are formulated from Eudragit polymers. Eudragits are a family of methacrylate polymers, which are typically marketed for controlled drug release. The following types of Eudragit polymers were evaluated:

- Eudragit RL 30 D – This is a 30% aqueous dispersion of Eudragit RL 100. Eudragit RL 100 is a copolymer of acrylic and methacrylic acid esters with a low content of quaternary ammonium groups. A variety of such polymers, including neutral forms designated as NE and NM, can be obtained through Evonik (www.evonik.com).

- Plastoid L50 – This is a solution of Eudragit L-100 in water, along with some glycerol (glycerol % not given). Eudragit L-100 is a copolymer of methacrylate ester and methacrylic acid.

- Plastoids E35M and E35L – These are solutions of Eudragit E-100 in water, along with some glycerol (glycerol % not given). Eudragit E-100 is a copolymer of methacrylic acid and an aminoalkyl methacrylate.

Linear amorphous polymers may dissolve after swelling, but cross-linked polymers, polymers with significant chain entanglements or partial crystallinity, that are partially insoluble are of general interest as well for use in polymer coatings of the invention. The use of cross-linked polymers are preferred and offer an opportunity for creating a surface that releases reagent without adding significantly to the solids content of the blood sample as it fills the chamber. Polymers may also be cross-linked in situ via addition of appropriate cross-linkers. Crosslinkers could be of ionic nature (Borate or other multivalent ions such as calcium) or could be reactive moieties that generate covalent bonds between individual polymer strands. Gantrez AN119 (a copolymer of methyl vinyl ether and maleic anhydride, nominal molecular weight 200,000 kDa) may be used as a polymer coating or as a component of a polymer coating. This polymer, which is highly soluble when neutralized, can be cross-linked with PVA or with a polyamine. For example, Gantrez, an anhydride containing polymer, can be crosslinked with diols (polyethylene glycols) or diamines, polyols, or polyamines, amino-alcohols or proteins. Cross-linking levels can be controlled and adjusted by tuning the concentration of cross-linker. Combination of different types of polymers might be used also.

Additional formulations for polymer coatings investigated include cellulose polymers such as hydroxypropyl cellulose (HPC) and hydroxypropyl methyl cellulose (HPMC), at a variety of molecular weights as shown in Table 1 below, added to the basic formulation of 10 mM Na phosphate, pH 7.4, 10 mM EDTA, 3% (or more) trehalose, and 1 mg/mL of either BSA or gelatin.

Table 1.
Cellulose Polymers Tested

Cat. No.	Description	Lot No.
Sigma 435007-5G	Hydroxypropyl cellulose, Mw 80,000	07413KH
Sigma 191884-5G	Hydroxypropyl cellulose, Mw 100,000	17009PD
Sigma 191892-5G	Hydroxypropyl cellulose, Mw 370,000	10206ED
Sigma 191906-5G	Hydroxypropyl cellulose, Mw 1,000,000	08031ED
Sigma 442755-25G-A	(Hydroxypropyl)methyl cellulose, Mn 10,000	12816TD
Sigma 423181-25G	(Hydroxypropyl)methyl cellulose, Mn 90,000	09226KU
Sigma 423173-25G	(Hydroxypropyl)methyl cellulose, Mn 120,000	05028JE

Formulations using HPC and HPMC polymers include 10 mM Na Phosphate, pH 7.4, 10 mM EDTA, 10% Trehalose, 1 mg/mL gelatin, 1.5% 10kDa hydroxypropyl methyl cellulose (HPMC), 0.01% Tween 80.

Other natural polymers (positively or negatively charged) and of different sizes for use in the invention include: Dextrans, xanthan and guar gums. Agarose, alginates and carrageenans, Ficol and heparin, dried egg and whole milk components.

Detergents, excipients and stabilizers that are used in general lyophilization processes are also useful her to stabilize the reagents and facilitate wetting and rehydration may be used in polymer coatings. Detergents to improve and adjust wetting and rehydration properties include the following:

Tween 20, Tween 80 and Tritons are effective in wetting the polymers listed above when they are dried down. The Pluronics (25R2 and Pluronic L92) facilitate dissolution of a dried matrix.

Zonyl FSH, from DuPont, may be used as a wetting agent. Formulations prepared with fluorosurfactant were capable of wetting out very well on a surface of a cassette made of PMMA. Combination of detergents are also considered useful.

Excipients and other small molecules such as sucrose and trehalose well known to those skilled in the art of lyophilization may also be used in polymer coatings of the invention. Furthermore addition of glycerol and similar small molecules help in stabilizing the reagents as well as facilitate rehydration of the matrix. Additional assay reagent stabilizers include carriers proteins, for example, BSA, Gelatin, Collagen, Fish Skin gelatin, Casein and the like.

Methods for applying a polymer coating to a surface of a cassette include streaking, spotting, spraying using automated or robotic systems. Combination of spotting and spraying

are also considered. A preferred reagent deposition method is based on automated spotting of nanoliters size drops that are evenly spaced across the assay chamber. These drops are allowed to merge by judicious choice of excipients and condition such as relative humidity and temperature. Thus a continuous film of reagent is generated across the entire chamber.

5 In another aspect, polymer coatings may be applied to a cassette surface by sequential deposition of the individual components. For example, the polymer could be deposited first, such that it adheres to the surface. This could be followed with a crosslinker that further stabilizes the polymer. The reagents including stabilizers may be deposited next either as a cocktail or individually, followed by a protective layer that allows proper wetting by the
10 sample and subsequent facile release of the assay reagents.

 In one aspect, polymer coatings used in the present invention comprise a controlled release polymer and a releasable reagent formulation able to react irreversibly or reversibly with a pre-chosen analyte of interest, such as a molecule, molecular complex, receptor,
15 biological cell, or the like. The purpose of an assay reagent formulation is to react with the analyte of interest and form a reaction product which is optically detectable. In contrast, the role of the polymer coating is to provide a controlled release over time and space of the chosen assay reagent formulation.

 Controlled release polymers for use with the invention, their properties and
20 characteristics, their structures and formulations, and their modes of action have been an area of intensive investigations in unrelated technical fields, particularly as a form of drug administration and in delivery systems of drugs in-vivo.. Controlled release polymers, as a class of compositions, are able to release a ligand of choice over a large range of disparate time periods and at greatly varying rates of release; and act almost independently of markedly
25 different environmental conditions in-vitro and in-vivo. Merely representative of the information, knowledge, and applications conventionally known for controlled release polymers are the following publications, each of which is expressly incorporated by reference herein: Langer, R., Science 249:1527-1533 (1990) and the references cited therein; Benita et al., J. Pharm. 73:1721-1724 (1984) and the references cited therein; Watts et al., J. Controlled
30 Release 16:311-318 (1991) and the references cited therein; and U.S. Pat. Nos. 4,897,268; 4,897,267; 4,897,268; and the references cited therein. The present invention intends that the entire membership comprising the class of controlled release polymers, individually and

collectively, be available for use in the present invention regardless of the particular formulation and structure, format requirements, or mechanism of action by which a chosen assay reagent becomes controllably released.

In one aspect, the invention will employ only one particular controlled release polymer in combination with one or more specific assay reagents when constructing a polymer coating. In other aspects of the present invention, polymer coatings may be constructed utilizing multiple and different controlled release polymers in combination with multiple and different reagent formulations to form a range of divergent and heterogeneous discrete polymer carriers, each of which has its own singular controlled release polymer and its own individual reagent formulation. In this complex mode of construction, there exists a range of differently formulated polymer carriers which may be varied individually in the kinds and assortment of reagent formulations released, in the rates at which each of the different reagent formulations are released, and the different time intervals over each of which the various individual reagent formulations are released. Thus, within a single polymer coating, the use of a mixture of different discrete polymer carriers can provide a range of slow, intermediate, or fast rates of controlled release for a single reagent formulation or a pre-chosen set of different assay reagent formulations. Similarly, using a mixture of different discrete polymer carriers can provide a divergent range of differing time durations for controlled release of one or more reagent formulations - such timing being continuous, intermittent at preset intervals, or irregular as needed or desired by the user or the specific application.

A representative listing of fluid-erodible, non-erodible, and other controlled release polymers is provided by Table 2.

Table II

Representative Polymer Carriers for Use
In a Polymer Coating

I. Erodible Controlled Release Polymer
Polyanhydrides (and copolymers and homopolymers)
polysebacic acid
poly(p-carboxyphenoxy)propane
poly(p-carboxyphenoxy)hexane
poly-isophthalic acid

- Vinyl Polymers (and copolymers and homopolymers)
 ethylene vinyl acetate copolymer
 polyvinylpyrrolidone
 polyvinyl alcohol
 5 Polyacrylamides
 poly-2-hydroxyethyl methacrylate
 Polyglycolic Acids
 polyglycolide
 polylactide
 10 pollyglycolide/lactide copolymer
 polyhydroxybutyrate
 polyhydroxy valerate
 polycaprolactones
 II. Non-erodible Controlled Release Polymers
 15 Hydrogels
 polyacrylamide
 polyvinyl alcohol
 poly(2-hydroxyethyl methacrylate)
 poly-N-(2-hydroxypropyl methacrylamide)
 20 poly vinylpyrrolidone
 polymethyl methacrylate (as adjuvants)
 Hydrophobic polymers
 ethylene vinyl acetate copolymer
 silicone elastomers
 25 microporous polypropylene
 cross-linked (meth)acrylates
 III. Biodegradable Controlled Release Polymers
 polyactic acid (polylactide)
 polyglycolic acid (polyglycolide)
 30 poly (lactic acid-co-glycolic acid)
 poly (ϵ -caprolactone): polyvalerolactone
 poly (hydroxybutyric acid-co-hydroxyvaleric acid)
 poly orthoesters
 poly alkylcyanoacrylates
 35 synthetic polypeptides
 cross-linked polypeptides and proteins
 natural polymers: albumin, gleatin, starch
 polyanydrides:
 monomers for: sebacic acid
 40 bis(p-carboxy-phenoxy)-propane
 dodecandedioic acid
 IV. pH Sensitive Controlled Release Polymers
 cellulose acetate trimellitate
 hydroxypropyl methyl cellulose phthalate
 45 cellulose acetate
 cellulose acetate propioinate
 cellulose triacetate

copolymers of methacrylic acid and methacrylic acid methyl
ester
(Eudragit L100^R)

5

Assay reagent formulations for use in a polymer coating may be selected from a wide and diverse range of compositions and properties. The releasable assay reagents may include
10 known indicator or dye compounds useful within ultraviolet, visible, fluorescent, phosphorescent, and other well defined optical systems and methods. Accordingly, these include the known colorimetric compositions known as absorbers, protoabsorbers, absorption complexes, chromophoric and chromogenic compositions, and fluorophoric and fluorogenic compounds--all of which are known and described in the relevant scientific and industrial
15 literature. In addition, the releasable assay reagent formulations may also have other attributes and capabilities such as specific binding properties. Accordingly, such reagent formulations include all the conventionally known labeled immunological compositions including labeled antigens, haptens, and other antibody or cellular immunological and/or immunochemical components. Moreover, there are many applications where the components
20 of known enzyme systems are valuable for use as the releasable assay reagent formulations. Preferably, assay reagent formulations comprise one or more labeled antibodies, as a probe, that specifically bind to pre-chosen cell surface molecules of cells in a sample. Preferably, such antibodies are directly or indirectly labeled with fluorescent dyes, using conventional techniques.

25 Preferably, assay reagents of the invention comprise fluorescently labeled antibodies for identifying CD4+ cells in human whole blood. Such assay reagents include the antibody-based probes disclosed in Goldberg, U.S. patent publication 2009/0047690, which is incorporated herein by reference.

30

System and method of the Invention

The invention includes a system for use with cassettes described above, which generally holds a cassette in proper orientation with respect to an optical system which, in turn, detects optical signals from probes bound to cells. Typically, an optical system includes an objective lens, having a depth of field, and other optical components for collecting optical

signals, e.g. fluorescent signals, and imaging them on a photodetector, such as a CCD.

Where fluorescent labels are employed, an optical system also includes one or more light sources for generating illumination beams for exciting the fluorescent labels.

Preferably, the system of the invention comprises a low-cost apparatus suitable for
5 point-of-care operation. One such embodiment is disclosed in Goldberg et al, U.S. patent
publication 2008/0212069, which is incorporated herein by reference. Briefly, labeled cells
in a cassette are measured or counted by sequentially illuminating a sample with illumination
beams having different wavelength ranges that correspond to the excitation bands of labels
directly or indirectly bound or attached to the cells, or particles in the sample. After each
10 illumination in such a sequence, optical signals are collected to form an image, so that a set of
images are formed each containing image data that is analyzed to provide counts and/or
measurements of the population of cells, particles, and/or analytes. In one aspect, a plurality
of illumination beams is employed that have substantially non-overlapping wavelength
ranges. Such plurality of illumination beams may be in the range of from 2 to 6, or in the
15 range of from 2 to 4, or in the range of from 2 to 3. A plurality of illumination beams may be
generated by a variety of methods and apparatus available to those of ordinary skill, including
by lasers, filament and arc lamps, and the like. In one embodiment, illumination beams are
generated using light emitting diodes (LEDs), or like solid state devices. Exemplary LED
light sources include Luxeon™ LEDs that have wavelength peaks in green (530 nm), cyan
20 (505 nm), blue (470 nm), and royal blue (455 nm), commercially available from Lumileds
Lighting LLC (San Jose, CA). Guidance in selecting particular LEDs for use with the
invention is widely available in the technical literature, such as Luxeon Star Technical Data
Sheet DS23 (Philips Lumileds Lighting Company, San Jose, 2006); Luxeon Star V Technical
Data Sheet DS30 (Lumileds Lighting, U.S., LLC, San Jose, CA, September 20, 2004); and
25 the like. Usually, light sources are used with conventional filters and other optical
components for generating illumination beams of desired wavelength ranges and intensity
distributions.

A wide variety of optical systems can be employed with the apparatus. Generally,
such systems provide one or more illumination beams for sequentially illuminating a sample
30 in distinct wavelength ranges, an image collection device for recording image data from the
illuminated sample, and a controller that controls the operation of the illumination beams and
image collection device so that image data sets are sequentially collected.

In one aspect, the system comprises an image collection device used in concert with sets of differentially excitable dyes attached to probes specific for cell, particles, or analytes of interest in a sample. In other words, such a system comprises an apparatus of the following components for imaging samples or specimens labeled with a plurality

5 differentially excitable labels: (a) multiple light sources each capable of illuminating the specimen with an illumination beam having a distinct wavelength band; (b) a controller coupled to the multiple light sources for successively directing the illumination beam of each light source onto the specimen so that each of the plurality of differentially excitable labels is successively caused to emit an optical signal within the same wavelength band; and (c) an

10 optical system capable of collecting such emitted optical signals and forming successive images corresponding thereto on a light-responsive surface to form successive sets of image data. One embodiment of the above apparatus is illustrated in Fig. 3. System (300) comprises several components, including a plurality of light sources, shown as LED 1 (302) and LED 2 (304), for sequentially illuminating observation area (307) of sample (314)

15 disposed on or in sample platform (316), imaging optics (306) for collecting optical signals (309) generated from probes in and/or on the sample in response to illumination beams (303) and (305) and for directing (311) the collected signals to detector (308), which comprises a light-responsive surface, such as a CCD or CMOS element, on which optical signals (309) form an image and from which successive sets of image data are recorded. Preferably,

20 operation of system (300) is under the control of computer (310) that (a) controls the timing and duration of illumination beams (303) and (305), (b) controls detector (308) for collecting and transferring image data to one or more databases, (c) analyzes image data to produce a readout for readout component (312), and like operations. Sample platform (316) may vary widely in design and functional capabilities, but generally requires that a sample be disposed

25 in a substantially planar geometry that is consistent with collecting a plurality of optical signals in parallel and forming an image on a detector. Preferably, a sample disposed on sample platform (316) is static and not flowing or moving; or if motion is present, it is sufficiently slow that successive images may be collected that are capable of alignment during image analysis. Sample platform (316) may comprise conventional microscope slides,

30 sample chambers or cuvettes used in microscopy, culture plates, microfluidic devices, or the like. In one aspect, described more fully below, sample platform (316) comprises a disposable cuvette that is designed for detection of non-red cell components in whole blood.

In another aspect, sample platform (316) comprises a cuvette having a sample chamber with a geometry that permits a known volume to be surveyed whenever such cuvette is used with system (300). In one embodiment, such a sample chamber has a substantially planar geometry wherein (a) a floor (or bottom wall) and a ceiling (or top wall) are parallel to one another and (preferably) perpendicular to the minimal light path to imaging optics (306) and (b) the perpendicular distance between the top and bottom walls is substantially equivalent to the diameter of the cells or particles being detected. Whenever such sample chamber is disposed in observation area (307), which is known or determinable, the cells or particles will be in a known (or determinable) volume, thereby permitting concentrations of the particles or cells to be measured. "Substantially equivalent" in reference to the perpendicular distance, or dimension, between the top and bottom walls of a sample chamber means that, in a whole blood sample, optical signals from non-red cells or particles in observation area (307) are detectable. In other words, a layer of red blood cells (or other debris) that may be between a labeled cell or particle and the top wall of the chamber does not completely obstruct transmission of optical signals. In one aspect, where white blood cells are labeled and detected, such as CD4+ cells, the perpendicular distance between a top wall and a bottom wall is in the range of from 40 to 120 μm , or in the range of from 50 to 100 μm . The nature of readout component (312) may vary widely from a simple numerical display to an information-rich graphic user interface. In one embodiment, a simple numerical readout is provided by readout component (312) that gives counts of one or more predetermined cell or particle types. In another embodiment, readouts comprise concentrations of one or more predetermined cell or particle types. And in still another embodiment, readouts comprise simple "yes or no" indicators as to whether threshold levels (e.g. counts or concentrations) of cells, particles, or other analytes have or have not been passed.

In another aspect, the invention employs compositions of differentially excitable probes for use in labeling one or more of a plurality of different analytes in a sample, e.g. different cell surface receptors on cells in a sample. Generally, probe compositions of the invention comprise a mixture of analyte-specific probes, each capable of binding specifically to a different analyte, wherein each probe is characterized by (a) a binding compound specific for an analyte, such as a cellular analyte, under binding conditions, and (b) attached to the binding compound an optical label, wherein the optical label of each different probe has a

different excitation band and the optical labels of all probes emits optical signals within the same wavelength range. Usually, the latter wavelength range does not overlap with any of the excitation bands. Preferably, optical labels are fluorescent labels, such as fluorescent dyes, capable of generating fluorescent signals. However, other optical labels may be used with the invention, such as plasmon resonance particle when used under dark field illumination conditions. In one aspect, probe compositions of the invention include at least one probe specific for each of a plurality of different analytes. In another aspect, such plurality is in the range of from 2 to 8; or in another aspect, in the range of from 2 to 4; or in another aspect, in the range of from 2 to 3; and in another aspect, such plurality is at least 3; or is in the range of from 3 to 4. An important feature of a probe composition of the invention is that analytes in a sample labeled with different probes of the composition may be detected sequentially by the successive excitation of the optical labels of each probe using the illumination beam specific for such optical label. Usually, such successive excitation is temporally non-overlapping in that when each illumination beam is directed to the sample in a separate time interval. In other words, the illumination beams are successively directed to a sample one at a time. Preferably, in operation, optical signals from each excitation are imaged on a light-responsive surface of a detector from which image data is generated and stored for analysis. When optical signals of the probes are restricted to a narrow wavelength range, image degradation due to chromatic aberrations of lens in the optical path is reduced or eliminated. A wide variety of fluorescent labels are available that fulfill the above requirements. An exemplary optical label of a first probe is cyanine 3-allophycocyanin (Cy3-APC), and an exemplary optical label of the second probe is cyanine 5 (Cy5). Exemplary optical labels for a three-probe composition includes cyanine 7 (Cy7) (as donor and acceptor for a first probe), APC-Cy7 (APC as donor and Cy7 as acceptor for a second probe), and PE-Cy7 (PE as a donor and Cy7 as acceptor for a third probe).

DEFINITIONS

Generally, terms used herein not otherwise specifically defined have meanings corresponding to their conventional usage in the fields related to the invention, including analytical chemistry, biochemistry, molecular biology, cell biology, microscopy, image analysis, and the like, such as represented in the following treatises: Alberts et al, Molecular Biology of the Cell, Fourth Edition (Garland, 2002); Nelson and Cox, Lehninger Principles of

Biochemistry, Fourth Edition (W.H. Freeman, 2004); Murphy, Fundamentals of Light Microscopy and Electronic Imaging (Wiley-Liss, 2001); Shapiro, Practical Flow Cytometry, Fourth Edition (Wiley-Liss, 2003); Owens et al (Editors), Flow Cytometry Principles for Clinical Laboratory Practice: Quality Assurance for Quantitative Immunophenotyping (Wiley-Liss, 1994); Ormerod (Editor) Flow Cytometry: A Practical Approach (Oxford University Press, 2000); and the like.

“Antibody” or “immunoglobulin” means a protein, either natural or synthetically produced by recombinant or chemical means, that is capable of specifically binding to a particular antigen or antigenic determinant. Antibodies are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. “Antibody fragment”, and all grammatical variants thereof, as used herein are defined as a portion of an intact antibody comprising the antigen binding site or variable region of the intact antibody, wherein the portion is free of the constant heavy chain domains (i.e. CH2, CH3, and CH4, depending on antibody isotype) of the Fc region of the intact antibody. Examples of antibody fragments include Fab, Fab', Fab'-SH, F(ab')₂, and Fv fragment. The term “monoclonal antibody” (mAb) as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each mAb is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they can be synthesized by hybridoma culture, uncontaminated by other immunoglobulins. Guidance in the production and selection of antibodies for use in immunoassays can be found in readily available texts and manuals, e.g. Harlow and Lane, Antibodies: A Laboratory Manual (Cold Spring Harbor Laboratory Press, New York, 1988); Howard and Bethell, Basic Methods in Antibody Production and Characterization (CRC Press, 2001); Wild, editor, The Immunoassay Handbook (Stockton Press, New York, 1994), and the like.

“Antigenic determinant,” or “epitope” means a site on the surface of a molecule, usually a protein, to which a single antibody molecule binds; generally a protein has several

or many different antigenic determinants and reacts with antibodies of many different specificities. A preferred antigenic determinant is a phosphorylation site of a protein.

“Binding compound” means a compound that is capable of specifically binding to a particular target molecule. Examples of binding compounds include antibodies, lectins,
5 nucleic acids, aptamers, and the like, e.g. Sharon and Lis, *Lectins*, 2nd Edition (Springer, 2006); Klussmann, *The Aptamer Handbook: Functional Oligonucleotides and Their Applications* (John Wiley & Sons, New York, 2006). Preferably, binding compounds are antibodies, and more preferably, binding compounds are monoclonal antibodies.

“CD2” means a cell surface molecule of the cluster designation (CD) system, also
10 known as T11 or LFA-2, that is expressed on T cells, thymocytes, and NK cells, and that functions as an adhesion molecule, binding CD58 (LFA-3). Preferably, CD2 refers to the human CD2 molecule.

“CD3” means a cell surface molecule of the cluster designation (CD) system, also known as T3, that is expressed on T cells and thymocytes, and that is associated with the T
15 cell antigen receptor (TCR), is required for cell surface expression of and signal transduction by TCR. Its cytoplasmic domains contain ITAM motifs and bind cytoplasmic tyrosine kinases. Preferably, CD3 refers to the human CD3 molecule.

“CD4” means a cell surface molecule of the cluster designation (CD) system, also known as T4 or L3T4, that is expressed on thymocyte subsets, helper and inflammatory T
20 cells (about two thirds of peripheral T cells), monocytes, macrophages, and that functions as coreceptor for MHC class II molecules, binds Lck on cytoplasmic face of membrane. CD4 is a receptor for HIV-I and HIV-2 gp120. Preferably, CD4 refers to the human CD4 molecule.

“CD45RA” means a cell surface molecule of the cluster designation (CD) system
25 that is expressed on B cells, T cell subsets (naive T cells), and monocytes, and that is a splice variant of CD45 containing the A exon. Preferably, CD45RA refers to the human CD45RA molecule.

“CD56” means a cell surface molecule of the cluster designation (CD) system, also known as NKH-1, that is expressed on NK cells, and that is a splice variant of the Neural
30 Cell Adhesion Molecule (NCAM). Preferably, CD56 refers to the human CD56 molecule.

“Complex” as used herein means an assemblage or aggregate of molecules in direct or indirect contact with one another. In one aspect, “contact,” or more particularly, “direct

contact” in reference to a complex of molecules, or in reference to specificity or specific binding, means two or more molecules are close enough so that attractive noncovalent interactions, such as Van der Waal forces, hydrogen bonding, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules. In such an aspect, a complex of molecules is stable in that under assay conditions the complex is thermodynamically more favorable than a non-aggregated, or non-complexed, state of its component molecules. As used herein, “complex” usually refers to a stable aggregate of two or more proteins. In one aspect, a “complex” refers to a stable aggregate of two proteins, such as an antibody specifically bound to an antigenic determinant of a target protein.

“Dried reagents” mean assay reagents, such as buffers, salts, active compounds, such as enzymes, co-factors, and the like, or binding compounds, such as antibodies, aptamers, or the like, that are provided in a dehydrated formulation for the purpose of improved shelf-life, ease of transport and handling, improved storage, and the like. The nature, composition, and method of producing dried reagents vary widely and the formulation and production of such materials is well-known to those of ordinary skill in the art as evidenced by the following references that are incorporated by reference: Franks et al, U.S. patent 5,098,893; Cole, U.S. patent 5,102,788; Shen et al, U.S. patent 5,556,771; Trembl et al, U.S. patent 5,763,157; De Rosier et al, U.S. patent 6,294,365; Buhl et al, U.S. patent 5,413,732; McMillan, U.S. patent publication 2006/0068398; McMillan et al, U.S. patent publication 2006/0068399; Schwegman et al (2005), *Pharm. Dev. Technol.*, 10: 151-173; Nail et al (2002), *Pharm. Biotechnol.*, 14: 281-360; and the like. Dried reagents include, but are not limited to, solid and/or semi-solid particulates, powders, tablets, crystals, films, coatings, and the like, that are manufactured in a variety of ways. In one aspect, dried reagents are lyophilized coatings or films on the inner walls of vessels or inside a chamber of a disposable cartridge for carrying out an assay in accordance with the invention. Dried reagents may include excipients, which are usually inert substances added to a material in order to confer a suitable consistency or form to the material. A large number of excipients are known to those of skill in the art and can comprise a number of different chemical structures. Examples of excipients, which may be used in the present invention, include carbohydrates, such as sucrose, glucose, trehalose, melezitose, dextran, and mannitol; proteins such as BSA, gelatin, and collagen; and polymers such as PEG and polyvinyl pyrrolidone (PVP). The total amount of excipient in the lyophilized particulate may comprise either single or multiple compounds. In some

embodiments, the type of excipient is a factor in controlling the amount of hygroscopy of a dried reagent. Lowering hygroscopy can enhance the a dried reagent's integrity and cryoprotectant abilities. However, removing all water from such a composition would have deleterious effects on those reaction components, proteins for example, that require certain amounts of bound water in order to maintain proper conformations.

"Granulocyte-specific marker" means any molecule that is present on or in substantially all granulocytes, but is substantially absent from other white blood cell types. Exemplary granulocytes-specific markers include, but are not limited to, the following molecules: 1C3, 3C4, 6D10, 2B2, 8C5, alkaline phosphatase, calprotectin, CD18, CD15, CD16, CD24, CD32, CD34, CD45, CD66b, CEACAM8, DH59B, EMR3, eosinophil cationic protein, granulocyte factor, GMP, Gr-1 (Ly-G6), granulocyte elastase, HIS48, IL-8, leukocyte alkaline phosphatase, LRG, myeloperoxidase, NKH1, and the like. Preferably, granulocytes-specific markers are cell surface molecules. More preferably, granulocytes-specific markers are the human equivalent of the markers listed above. Still more preferably, a granulocytes-specific marker is CD15.

"Kit" means to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of assays for analyzing white blood cells, such delivery systems include systems that allow for the storage, transport, or delivery of assay reagents (e.g., probes, ancillary labeling antibodies, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain buffers and a reaction cuvette, vessel, or the like, for use in an assay, while a second container may contain probes. Optional additional components may include, sample-extraction apparatus, e.g. skin puncturing devices for exposing small amounts of blood, germicidal swabs, or the like.

"Microfluidics device" means an integrated system of one or more chambers, ports, and channels that are interconnected and in fluid communication and designed for carrying out an analytical reaction or process, either alone or in cooperation with an appliance or instrument that provides support functions, such as sample introduction, fluid and/or reagent driving means, temperature control, detection systems, data collection and/or integration

systems, and the like. Microfluidics devices may further include valves, pumps, and specialized functional coatings on interior walls, e.g. to prevent adsorption of sample components or reactants, facilitate reagent movement by electroosmosis, or the like. Such devices are usually fabricated in or as a solid substrate, which may be glass, plastic, or other solid polymeric materials, and typically have a planar format for ease of detecting and monitoring sample and reagent movement, especially via optical or electrochemical methods. Features of a microfluidic device usually have cross-sectional dimensions of less than a few hundred square micrometers and passages typically have capillary dimensions, e.g. having maximal cross-sectional dimensions of from about 500 μm to about 0.1 μm . Microfluidics devices typically have volume capacities in the range of from 1 μL to a few nL, e.g. 10-100 nL. The fabrication and operation of microfluidics devices are well-known in the art as exemplified by the following references that are incorporated by reference: Ramsey, U.S. patents 6,001,229; 5,858,195; 6,010,607; and 6,033,546; Soane et al, U.S. patents 5,126,022 and 6,054,034; Nelson et al, U.S. patent 6,613,525; Maher et al, U.S. patent 6,399,952; Ricco et al, International patent publication WO 02/24322; Bjornson et al, International patent publication WO 99/19717; Wilding et al, U.S. patents 5,587,128; 5,498,392; Sia et al, Electrophoresis, 24: 3563-3576 (2003); Unger et al, Science, 288: 113-116 (2000); Enzelberger et al, U.S. patent 6,960,437.

“Monocyte-specific marker” means any molecule that is present on or in substantially all monocytes, but is substantially absent from other white blood cell types. Exemplary monocyte-specific markers include, but are not limited to, the following molecules: 125I-WVH-1, 63D3, CB12, CD11a, CD14, CD15, CD54, CD62L, CD163, cytidine deaminase, DH59B, Fc-receptors, Flt-1, hMGL, Ki-M1p, Leu-7, lysozyme, leucocyte tartrate-resistant acid phosphatase, mannosyl receptors, peanut agglutinin, thromboplastin, thymidine phosphorylase, TNF, urokinase, and the like. Preferably, monocytes-specific markers are cell surface molecules. More preferably, monocytes-specific markers are the human equivalent of the markers listed above. Still more preferably, a monocytes-specific marker is CD14.

“Sample” means a quantity of material from a biological, environmental, medical, or patient source in which detection or measurement of predetermined cells, particles, beads, and/or analytes is sought. A sample may comprise material from natural sources or from man-made sources, such as, tissue cultures, fermentation cultures, bioreactors, and the like.

Samples may comprise animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Samples may include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention. The terms "sample," "biological sample," and "specimen" are used interchangeably.

"Specific" or "specificity" in reference to the binding of one molecule to another molecule means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecules in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least thirty percent. Generally, molecules involved in a specific binding event have areas on their surfaces, and/or in the case of proteins in cavities, giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

"Spectrally resolvable" in reference to a plurality of fluorescent labels, or dyes, means that the fluorescent emission bands of the dyes are sufficiently distinct, e.g. non-overlapping, that binding compounds to which the respective dyes are attached can be distinguished on the basis of the fluorescent signal generated by the respective dyes by conventional photodetection

systems, e.g. employing a standard system of filters, mirrors, dichroics, photomultiplier tubes, photodiodes, or the like, such as described in the following, or like, references: Wheelless et al, Flow Cytometry: Instrumentation and Data Analysis (Academic Press, New York, 1985); Shapiro (cited above).

5 “T lymphocyte-specific marker” means any molecule that is present on or in substantially all T lymphocytes, but is substantially absent from other white blood cell types. Exemplary T lymphocyte-specific markers include, but are not limited to, the following molecules: CD1a, CD1d, CD2, CD3, CD4, CD5, CD7, CD8, CD25, CD38, CD45RO, CD72, CD134, CD150, CRTAM, FOXP3, FT2, GPCA, HML-1, HT23A, Leu-22, Ly-2, Ly-m22,
10 MICG, MRC OX-8, MRC OX-22, OX40, PD-1, RT6, TCR, Thy-1 (CD90), TSA-2, and the like. Preferably, T lymphocyte-specific markers are cell surface molecules. More preferably, T lymphocyte-specific markers are the human equivalent of the markers listed above. Still more preferably, T lymphocyte-specific markers are CD2 or CD3 molecules.

15 The above teachings are intended to illustrate the invention and do not by their details limit the scope of the claims of the invention. While preferred illustrative embodiments of the present invention are described, it will be apparent to one skilled in the art that various changes and modifications may be made therein without departing from the invention, and it is intended in the appended claims to cover all such changes and modifications that fall
20 within the true spirit and scope of the invention.

What is claimed is:

1. A cassette for optical analysis of cells in a sample, the cassette comprising:

5 a sample chamber capable of receiving a sample, the sample chamber being disposed in a body and having an optically transmissive wall and a dimension perpendicular to such wall substantially equivalent to the depth of field of a detector for collecting optical signals generated by probes attached to cells in the sample; and

10 a polymer coating on a surface of the sample chamber substantially parallel to, and co-extensive with, the optically transmissive wall, the polymer coating releasing a composition of probes in a uniform concentration throughout the sample chamber whenever sample is loaded therein, the composition of probes comprising a plurality of analyte-specific probes, each analyte-specific probe comprising an optical label and a binding compound capable of binding specifically to a different cellular analyte of cells in the sample.

15 2. The cassette of claim 1 wherein said optical label of each different probe has a different excitation band and wherein said optical labels of all probes emit optical signals within the same wavelength range.

20 3. The cassette of claim 1 wherein said polymer coating is a polymer film comprising cross-linked hydrophilic polymer having a thickness in the range of from 0.1 to 5.0 mils.

4. The cassette of claim 3 wherein said polymer film has a degree of cross linking that permits free diffusion of said probe composition into said sample upon contact therewith.

25 5. The cassette of claim 4 wherein said polymer film comprises a synthetic polymer.

6. The cassette of claim 5 wherein said synthetic polymer is selected from the group consisting of polyvinyl alcohol, polyvinyl pyrrolidone, Eudragit, and Gantrez AN119.

30 7. The cassette of claim 1 wherein said polymer coating is an array of droplets each comprising cross-linked hydrophilic polymer.

8. A cassette for optical analysis of non-red cells in a sample of whole blood, the cassette comprising:

a sample chamber capable of receiving a sample of whole blood, the sample chamber being disposed in a body and having an optically transmissive wall and a dimension perpendicular to such wall substantially equivalent to the size of a non-red blood cell so that optical signals generated by probes attached to cellular analytes thereof are not obstructed by red blood cells of the sample; and

a polymer coating attached to a surface of the sample chamber substantially parallel to, and co-extensive with, the optically transmissive wall, the polymer coating releasing a composition of probes in a uniform concentration throughout the sample chamber whenever the sample is loaded therein, the composition of probes comprising a plurality of analyte-specific probes, each analyte-specific probe comprising an optical label and a binding compound capable of binding specifically to a different cellular analyte of a non-red blood cell.

9. A system for imaging multiple features of cells in a sample, the system comprising:

one or more light sources capable of successively generating illumination beams each having a distinct wavelength band;

a composition of probes each having a differentially excitable label capable of labeling cells comprising multiple features, such that each different feature is labeled with a different differentially excitable label;

a controller operationally associated with the one or more light sources for successively directing illumination beams onto the sample so that each of the different differentially excitable labels is successively caused to emit an optical signal within the same wavelength band;

an optical system capable of collecting such emitted optical signals and forming successive images corresponding to the labeled features of cells in the sample on a light-responsive surface to form successive sets of image data thereof; and

a cassette operationally associated with the optical system, the cassette comprising (i) a sample chamber capable of receiving the sample, the sample chamber being disposed in a body and having an optically transmissive wall and a dimension perpendicular to such wall substantially equivalent to the depth of field of the optical system for collecting optical

signals generated by probes attached to cells in the sample, and (ii) a polymer coating on a surface of the sample chamber substantially parallel to, and co-extensive with, the optically transmissive wall, the polymer coating releasing the composition of probes in a uniform concentration throughout the sample chamber whenever sample is loaded therein.

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10. A method for analyzing predetermined non-red blood cells in a sample of whole blood, the method comprising the step of:

providing a composition of probes each having a differentially excitable label capable of labeling cells comprising multiple features, such that each different feature is labeled with a different differentially excitable label;

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loading the sample into a cassette comprising (i) a sample chamber capable of receiving the sample, the sample chamber being disposed in a body and having an optically transmissive wall and a dimension perpendicular to such wall substantially equivalent to the depth of field of an optical system for collecting optical signals generated by probes attached to non-red blood cells in the sample, and (ii) a polymer coating on a surface of the sample chamber substantially parallel to, and co-extensive with, the optically transmissive wall, the polymer coating releasing the composition of probes in a uniform concentration throughout the sample chamber;

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successively directing illumination beams onto the sample so that different differentially excitable labels are successively caused to emit an optical signal;

20

collecting such successively emitted optical signals with the optical system and forming successive images corresponding to the labeled features of non-red blood cells in the sample on a light-responsive surface to form successive sets of image data thereof; and

enumerating the non-red blood cells in the sample by analyzing the successive sets of image data.

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11. The method of claim 10 wherein said non-red blood cells include CD4+ lymphocytes.

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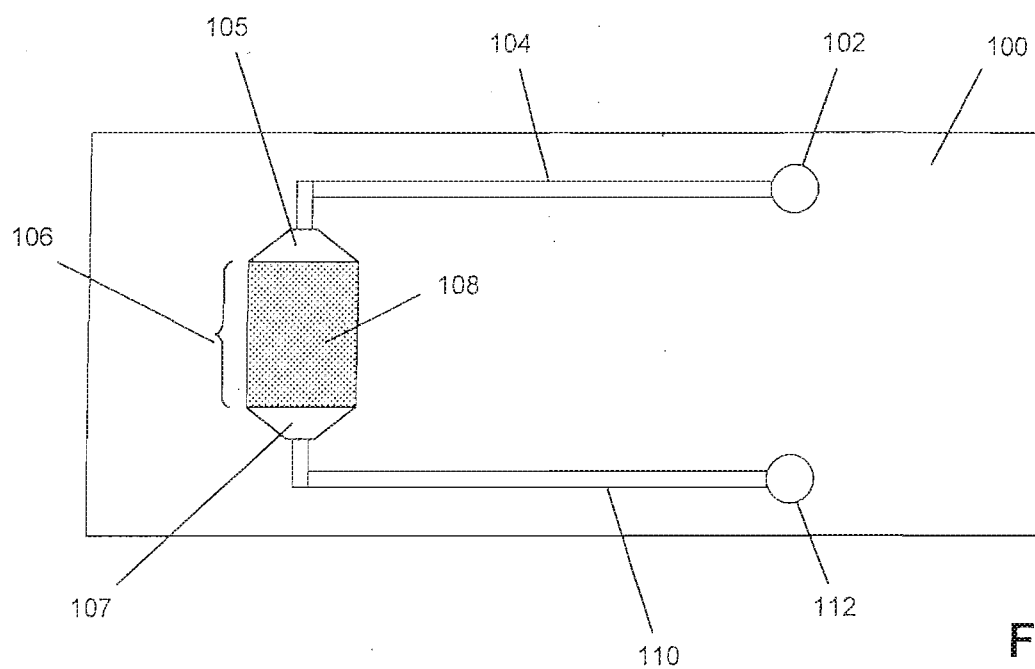


Fig. 1A

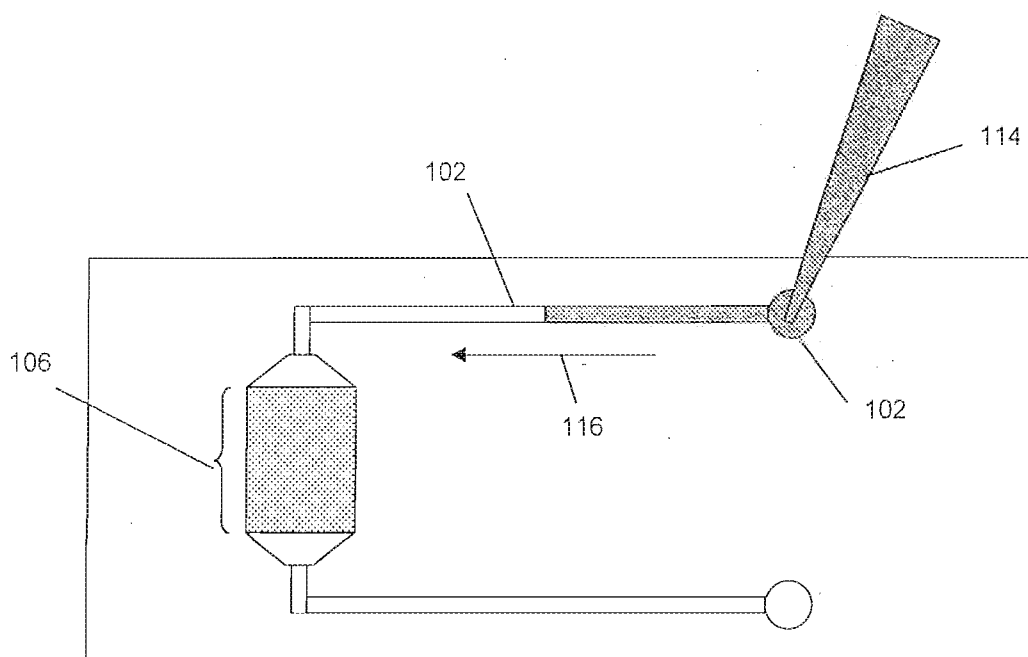


Fig. 1B

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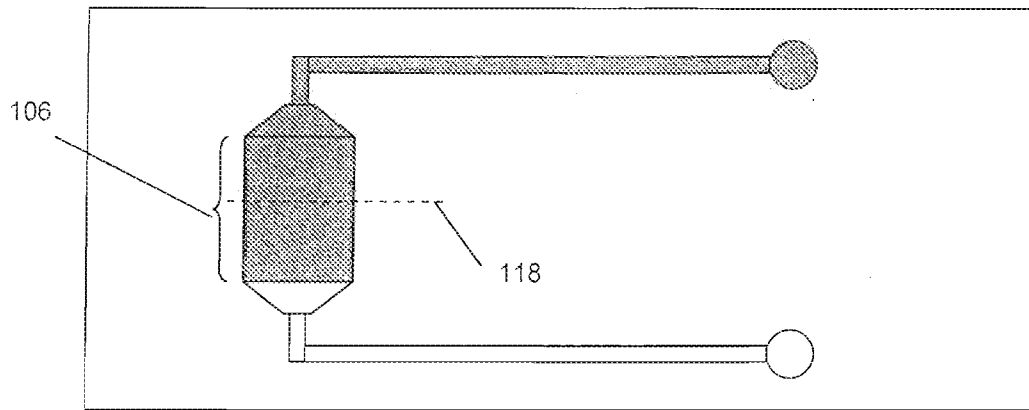


Fig. 1C

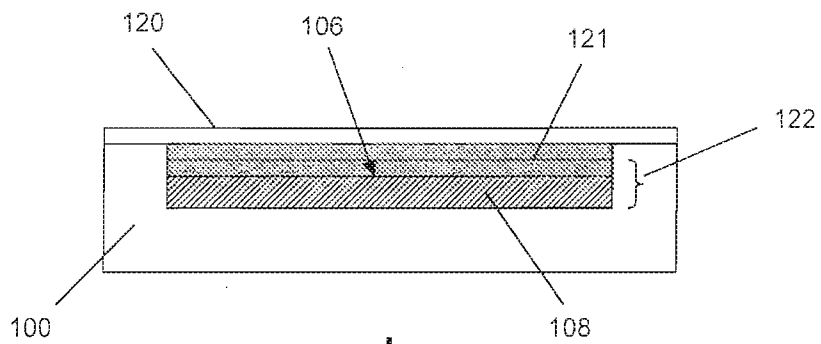


Fig. 1D

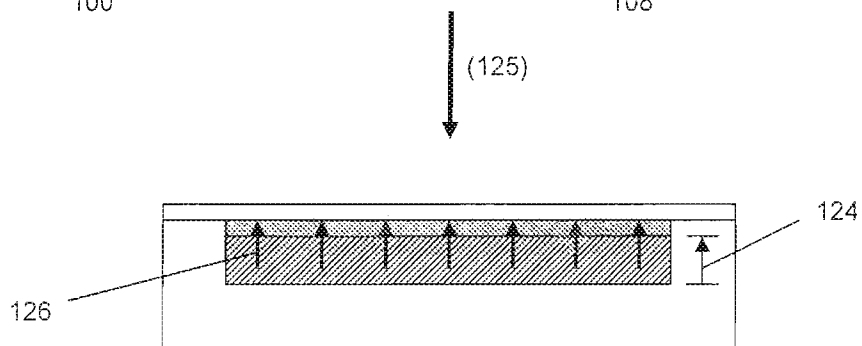


Fig. 1E

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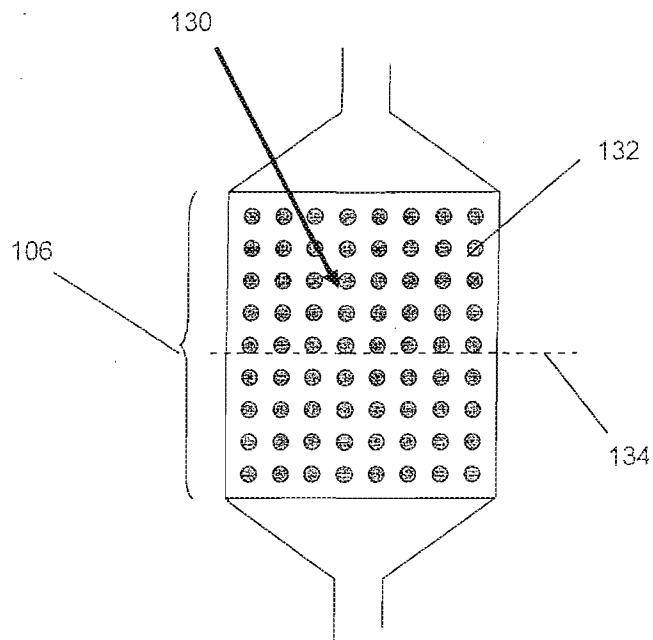


Fig. 1F

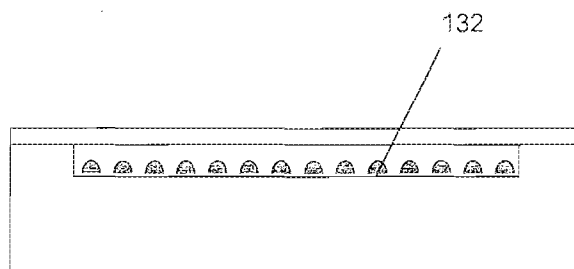


Fig. 1G

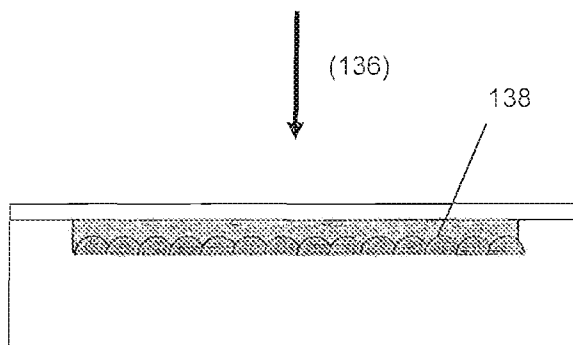


Fig. 1H

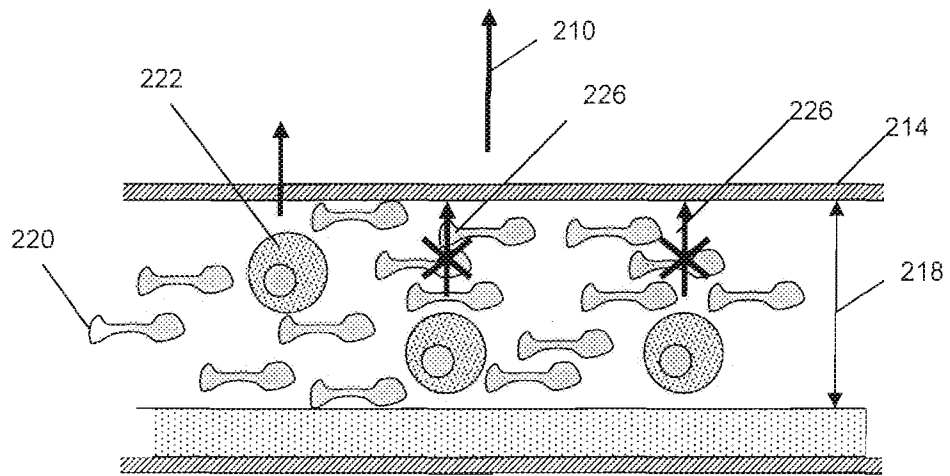


Fig. 2A

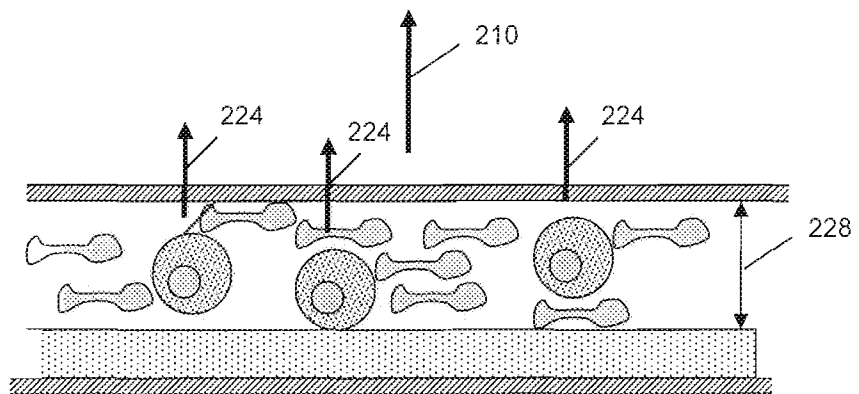
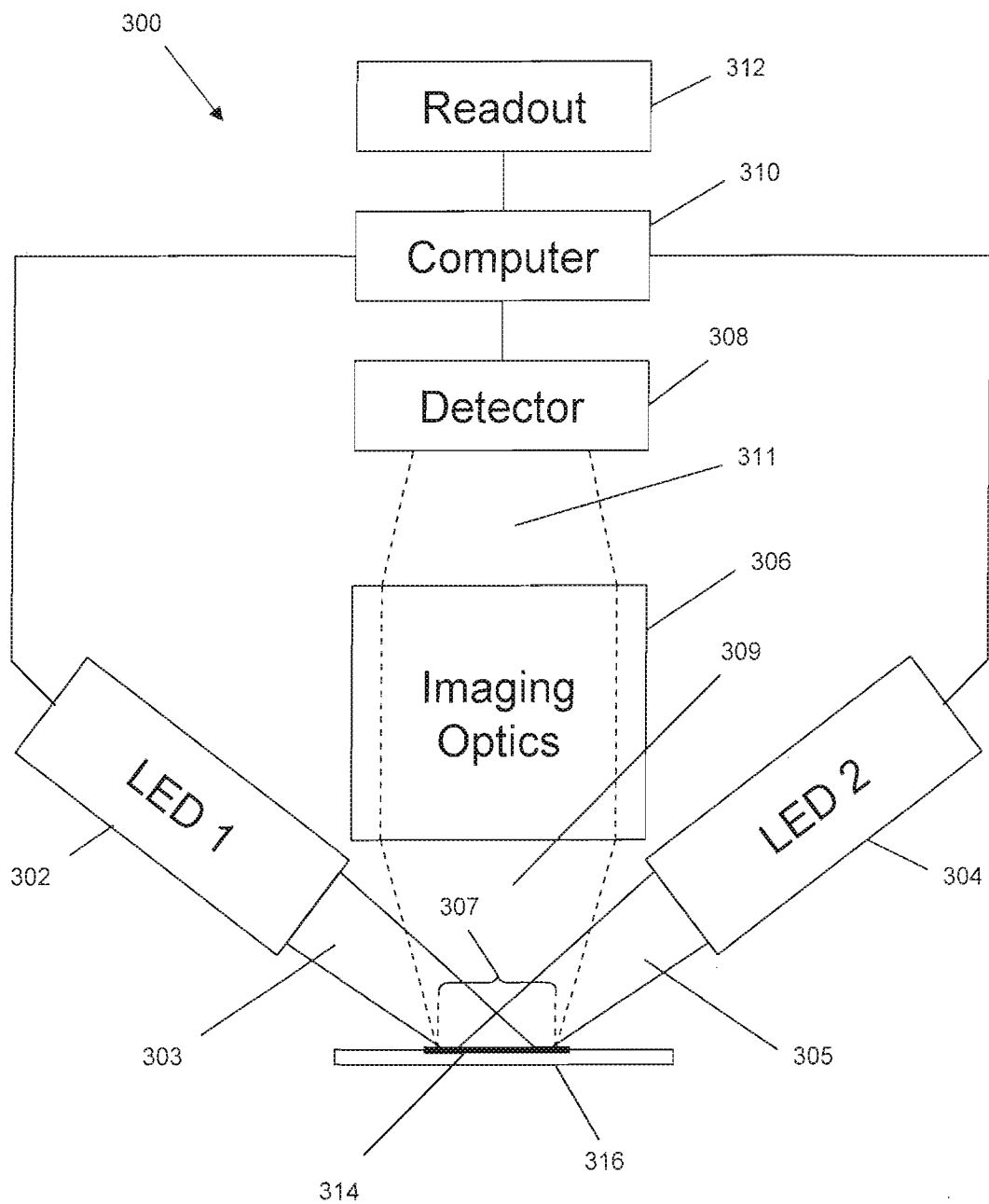


Fig. 2B

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**Fig. 3**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/27613

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61B 5/145 (2010.01)

USPC - 600/368

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)

IPC8 : A61B 5/145 (2010.01)

USPC : 600/368

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 IPC8 : A61B 5/00, A61B 5/1455, A61B 5/157, A61B 5/15; C12M 1/00, C12M 1/34; B81B 7/00, B81B 7/02; G01N 21/00 (2010.01)
 USPC : 600/300, 310, 322, 326, 476, 573, 367, 584; 73/1.02; 435/7.1, 7.24, 283.1, 287.1, 287.3, 288.3, 288.7; 422/68.1, 82.05

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 PubWEST PGPB,USPT,EPAB,JPAB:polymer, several, plural, multiple, optical, transmi\$, transparent, dot, drop, droplet, array, depth, height, size, lymphocyte, rbc, red blood, mems, microfluid\$, excit\$, assay, cd4\$, polyvinyl alcohol, polyvinyl pyrrolidone, Eudragit, Gantrez, releas\$, elut\$, film, coating, layer, hydrophilic, depth of field, chamber,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008/0212069 A1 (GOLDBERG et al) 4 September 2008 (04.09.2008) see especially para [0028]-[0030], [0033]-[0035], [0037], [0038]	1-11
Y	US 2005/0042612 A1 (HUBBARD et al) 24 February 2005 (24.02.2005) see especially para [0020], [0025], [0032], [0037]	1-11
Y	US 2007/0248985 A1 (DUTTA et al) 25 October 2007 (25.10.2007) see especially para [0055]	5, 6
A	US 2006/0014212 A1 (BENKOVIC et al) 19 January 2006 (19.01.2006) see whole document	1-11
A	US 2003/0096302 A1 (YGUERABIDE et al) 22 May 2003 (22.05.2003) see whole document	1-11
A	US 2008/0045884 A1 (LANDHERR et al) 21 February 2008 (21.02.2008) see whole document	1-11
A	US 2007/0254372 A1 (BICKEL et al) 1 November 2007 (01.11.2007) see whole document	1-11

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

29 April 2010 (29.04.2010)

Date of mailing of the international search report

10 MAY 2010

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