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POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) (Names should be indicated as they appear in the Request Form (PCT/RO/101)):

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hereby appoints (appoint) the following person as:



agent



common representative

Name and address

(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

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to represent the undersigned before



all the competent International Authorities



the International Searching Authority only



the Authority specified for supplementary search only: _____
(please indicate the Authority(ies) specified for supplementary search)



the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: FLOW CYTOMETER

Applicant's or agent's file reference: 2294PCT

International application number (if already available):

filed with the following Office _____ RO/US _____ as receiving Office
and to make or receive payments on behalf of the undersigned.

Signature of the applicant(s) (where there are several applicants, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power):


Yong Qin Chen

Date: May 24, 2013

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PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Cooperation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) (Names should be indicated as they appear in the Request Form (PCT/RO/101)):

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in connection with the international application identified below:

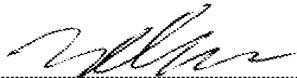
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Yong Qin Chen, President
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File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	RO/101 - Request form for new IA - Conventional	Request.pdf	1377437 3c34422536c798ffec5c2f09071f9acd1cfbe2f8	no	7
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Information:					
2	Specification	Specification.pdf	7764836 ee26df900de424d8628be7e48dc6e74fba84a108	no	48
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Information:					
3	Claims	Claims.pdf	3977434 546e71b11d98bd4377da7221e5554d7b88ace1e1	no	30
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5	Drawings-only black and white line drawings	FormalDwgs.pdf	1892550 1eed637296c9042c01e3dd042508d33c5cd46f79	no	23
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FLOW CYTOMETER

Technical Field

5 The present disclosures relate generally to the technical field of flow cytometry and, more particularly, to the structure and operation of an improved flow cytometer together with various individual subassemblies included therein.

Background Art

10 Flow cytometry is a biophysical technique employed in cell counting, sorting, biomarker detection and protein engineering. In flow cytometry, cells suspended in a stream of liquid pass through an electronic detection apparatus. Flow cytometry allows simultaneous multiparametric analysis of physical and/or
15 chemical characteristics of up to thousands of cells per second.

Flow cytometry has various applications including in the fields of molecular biology, pathology, immunology, plant biology and marine biology. Flow cytometry also has broad
20 application in medicine (especially in transplantation, hematology, tumor immunology and chemotherapy, prenatal diagnosis, genetics and sperm sorting for sex preselection). In marine biology, the autofluorescent properties of photosynthetic plankton can be exploited by flow cytometry in
25 characterizing abundance and community composition. In protein engineering, flow cytometry is used in conjunction with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties. A common variation of flow cytometry is physically sorting
30 particles based on their properties thereby purifying a population of interest.

An entire flow cytometer system includes the following main components.

- 35 1. A flow cell through which a liquid stream, usually called a sheath flow, liquid or fluid, carries and hydrodynamically aligns cells or particles so that they pass single file through the flow cell.

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2. A measuring subsystem system coupled to the flow cell that detects cells or particles passing through the flow cell and is usually either:
 - a. an impedance or conductivity measuring subsystem; or
 - b. an optical illumination subsystem together with an optical sensing subsystem.
3. A conversion subsystem for converting the output signal from the measuring subsystem into computer processable data.
4. A computer for analyzing the data produced by the conversion subsystem.

The optical illumination subsystems provides a collimated and then focused beam of light, usually laser light of a single wavelength, that impinges upon the hydrodynamically-focused stream of liquid passing through the flow cell. Accordingly, the flow cytometer system may have one or more light sources that may include:

1. one or more lamps, e.g. mercury or xenon;
2. one or more high-power water-cooled lasers, e.g. argon, krypton or dye laser;
3. one or more low-power air-cooled lasers, e.g. argon (488 nm), HeNe (red-633 nm), HeNe (green) and HeCd (UV); and/or
4. one or more diode lasers (blue, green, red and violet).

The optical sensing subsystem includes one or more detectors aimed where the focused liquid stream passes through the light beam. Such detectors may include:

1. detectors in line with the light beam (Forward Scatter or FSC);
2. detectors perpendicular to it (Side Scatter or SSC); and
3. fluorescence detectors.

Each suspended particle passing through the beam scatters the light, and fluorescent material present in the particle or attached to the particle excited by the impinging light emit light at a longer wavelength than that of the impinging light.

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Detecting and analyzing brightness changes in a combination of scattered and fluorescent light at each detector (one for each fluorescent emission peak) permits deriving various types of information about the physical and chemical structure of each individual particle. FSC correlates with cell volume. Due to light being scattered off of internal components within a cell, SSC depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers omit a fluorescence detector and detect only scattered light. Other flow cytometers form images of each cell's fluorescence, scattered light, and transmitted light.

The flow cytometer system's conversion subsystem, which may include one or more amplifiers which may be either linear or logarithmic, generally includes one or more Analogue-to-Digital Converters ("ADCs") for converting the measuring subsystem's output signal into data that is then processed by the computer.

Modern flow cytometers usually include up to four (4) lasers and numerous fluorescence detectors. Increasing the number of lasers and detectors permits labeling cells with several different antibodies, and can more precisely identify a target population by their phenotypic markers. Some instruments can even capture digital images of individual cells, allowing for the analysis of a fluorescent signal location within or on the surface of cells.

Sample Illumination

In most of the instruments, particles of interest, such as blood cells or microspheres, are carried by the sheath flow using hydrodynamic focusing into a viewing zone inside a cuvette or jet stream and illuminated there by a focused laser beam. The technique provides the means to accurately identify and count particles of interest without being overwhelmed by background noise occurring outside a registration time window (Practical Flow Cytometry, Howard M. Shapiro, Wiley (2003) ISBN 0471411256). To increase detection sensitivity, the cross section of the focused laser beam is usually elliptical, with

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the minor axis along the direction of flow. In order to maintain the threshold integrity, the laser profile must have a smooth or bell shaped profile along the flow direction. One common method for producing such an beam is to elongate a
5 nearly collimated circular Gaussian beam along the direction of flow with a beam expander made of either prism or cylindrical lens pair, then focus the beam down with a spherical lens. Since the shape of the beam at the focus is the spatial Fourier transform of the beam at far field, this
10 produces a Gaussian shaped elliptical spot with minor axis along the flow.

Conventional lasers are expensive, bulky and power hungry. More recently, laser diodes ("LD") have become available. Differing from conventional lasers, the new generation of LDs
15 is cost effective, compact and power efficient, and shows great promise for new generation of compact biomedical instruments. A LD emits light having an elliptical cross-section with the ellipse's major axis, frequently called the fast axis, perpendicular to the LD's junction, and the ellipse's minor
20 axis, frequently called the slow axis, parallel to the LD's junction. Unfortunately, the beam quality of a typical LD, particularly along its fast axis, leaves much to be desired, preventing its wide acceptance in flow cytometric applications.

In principle, the quality of the LD beam can be
25 significantly improved by spatial filtering. If a small pinhole or a single mode optical fiber is positioned at the focal point of a lens, such that it only accepts the lowest order spatial mode, the beam passing through the pinhole or single mode optical fiber will be of nearly perfect Gaussian
30 shape. United States Patent no. 5,788,927 discloses that such a beam can then be collimated and expanded in the direction of flow through the cytometer, and finally focused down to an elliptical shaped Gaussian beam with minor axis along the flow direction. Unfortunately, the size of desktop instrumentation
35 limits the diameter of a pinhole to less than 5 micron. The core size of a visible wavelength single mode optical fiber also has a similar dimension. The challenge to manufacture such a precision spatial filter and maintain its long-term

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stability not only increases the cost of LD based laser system, but also reduces its reliability.

More recently, in an effort to reduce the possible side lobes due to the edge effect of limited numerical aperture of collimating lens, United States Patent no. 6,713,019 ("the '019 patent") discloses rotating the LD by ninety degrees (90°) such that its slow axis is parallel to the direction of flow. A beam diffusing section, such as a concave cylindrical lens, is then introduced to diffuse the collimated beam in the direction perpendicular to the flow, followed by a beam spot forming section, such as a spherical focusing lens, to form an elliptical spot within the cytometer's particle viewing zone. As described in detail in the '019 patent, the laser beam after the spot forming section is extremely astigmatic. In particular, the width of the beam at the viewing zone in the direction perpendicular to the flow is comparable or even wider than the width of the flow channel. This not only reduces the amount of laser energy impinging upon the particle and consequently the signal intensity, but also increases undesired background scattering from the liquid-flow cell interface. Instead of rotating the LD, United States Patent nos. 7,385,682 and 7,561,267 disclose using a large numerical aperture aspheric lens for LD collimation. Such a design, however, cannot correct the fringe effect inherent in the LD's beam profile. Consequently, there presently exists a need for a simple LD based optical system for use in flow cytometers that can reliably produce a focused elliptical beam with near Gaussian shape along its minor axis and a width along major axis.

Viewing Zone Microscope Objective

Modern flow cytometers include a spatial filter, usually either a mechanical pinhole or a large core optical fiber, located at an image location of an objective lens to prevent undesired background light from entering the cytometer's detector(s). Because particles remain in the cytometer's viewing zone for a few microseconds, microscope objectives with large numerical aperture must be used to maximize light

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collection efficiency. To support multiple spatially separated excitation laser beams in flow cytometers, as disclosed in United States Patent no. 4,727,020, it is also desirable to use an objective with large field of view. In order to achieve these goals, United States Patent nos. 6,510,07 and 7,110,192 disclose an objective design using modified apochromat with a gel-coupled or epoxy bonded near hemisphere lens as the optical element closest to the sample that is followed by multiple meniscus lenses. While such microscope objectives provide both a satisfactory numerical aperture and field of view, they significantly sacrificed image quality thereby:

1. limiting effective use of the spatial filter; and
2. exhibiting poor background light discrimination.

Further, such refractive microscope objectives are bulky, expensive to manufacture and often exhibit severe chromatic aberration. To overcome these limitations Published Patent Cooperation Treaty ("PCT") Patent Application no. WO 01/27590 discloses an alternative objective design based on a spherical concave mirror. The design offers large numerical aperture and good image quality along the optical axis. However, due to its poor off-axis characteristics, such a design is unsuitable for flow cytometers having multiple, spatially separated laser beams.

25 Sheath Liquid Supply

The performance of a flow cytometer depends critically on a stable liquid sheath flow. In particular, flow cytometers that have multiple spatially separated excitation laser beams or perform droplet sorting rely on a constant velocity of the liquid sheath flow for timing synchronization. As disclosed in United States Patent no. 5,245,318, conventional flow cytometers provide a stable liquid sheath flow by using an air-tight fluidic system that either:

1. applies constant air pressure in a sheath liquid reservoir to push the fluid through the flow cell; or
2. by sucking the fluid from the sheath liquid reservoir through the flow cell using a vacuum pump.

These systems are bulky, expensive to manufacture and prone to failure. More recently, United States Patent no. 8,187,888 discloses including a sheath liquid subsystem that pumps the liquid sheath flow from the sheath liquid reservoir into the viewing zone and a waste sheath liquid pump that pumps waste sheath liquid from the viewing zone into the waste tank. Although it appears that the disclosed sheath liquid subsystem has never been used in velocity critical flow cytometers, this patent reports that the disclosed sheath liquid subsystem overcomes most of the drawbacks of conventional sheath liquid flow stabilization by:

1. damping pump pulsations by locating:
 - a. one fluidic capacitor between the sheath liquid pump and the flow cell; and
 - 15 b. another fluidic capacitor between the flow cell and the waste pump; and
2. a pump controller whose operation is responsive to a pressure sensor that measures the pressure differential between the inlet and outlet of the flow cell.

The disclosed sheath liquid subsystem has other limitations. For example, the pressure sensor located near the outlet of the flow cell could be a potential source of contamination.

25 **Peristaltic Pump** **Sample Liquid Supply**

Peristaltic pumps are volumetric pumps in which a set of linearly or circularly moving rollers progressively compresses a compressible tube to propel the fluid through the tube. Peristaltic pumps are widely used particularly to pump clean/sterile or aggressive fluids to avoid cross contamination with exposed pump components.

Conventional peristaltic pump exhibits a pulsation each time a roller rolls off the tube near the pump outlet, caused by the temporary increase of tube volume when the compressed tube expands back to its original shape. The pulsation is undesirable in applications that require smooth flow. Many attempts have been made in the past to reduce the pulsation. For example, United States Patent nos. 3,726,613 and 3,826,593

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introduced a cam operated pusher which synchronously exerts an external pressure on the tube to compensate for the tube expansion. In United States Patent no. 4,834,630, a plurality of tubes mounted on segmented rollers are joined together at the pump inlet and outlet by T-shaped couplers such that pulsations from individual tubes would be reduced by averaging. United States Patent no. 7,645,127 proposed a pump tube with slightly larger inner diameter near the inlet so that the tube decompression near the pump outlet is compensated by the compression of a larger volume tube near the inlet. The various methods either significantly increased the complexity of the peristaltic pump or had limited success in reducing the pulsation effect.

15 **Mmulticolor Fluorescence Detection**

In many multicolor fluorescence detection instrumentations, such as flow cytometers, (Practical Flow Cytometry, Howard M. Shapiro, Wiley (2003) ISBN 0471411256), the fluorescence light emitted from the object of interest is:

- 20 1. collected by a microscope objective;
2. reimaged through a small pinhole or a multimode optical fiber;
3. then collimated and separated into multiple colored bands; and
- 25 4. finally detected by photo detectors such as photomultiplier tube (PMT), PIN photodiode or avalanche photodiode (APD).

A PMT is essentially a special type of electron tube. This "pre-semiconductor age" device is bulky and expensive. In addition, it has poorer quantum efficiency and less reproducible spectral response than silicon based semiconductor detectors, particularly in the biologically important red to near infrared spectral region. Despite the disadvantages, PMT has excellent noise characteristics. For example, the dark current of a typical 13mm PMT (e.g., the R9305 from Hamamatsu Corporation of Japan) is only about 1nA. In contrast, an APD's dark current would be 10 times greater even if its active area were reduced to 1/20th of that of the PMT. As a result, PMT

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has been the de-facto low-level light detector in many commercial fluorescence detection flow cytometers. Only in certain scientific applications where event rate is low and dark current may be discriminated against by expensive photon-counting techniques that the PMT has been replaced by APD detectors. (c.f., High-Throughput Flow Cytometric DNA Fragment Sizing, A.V. Orden, R.A. Keller, and W. P. Ambrose, *Anal. Chem.*, 2000, 72 (1), p 37-41). More recently, a Geiger mode APD array was also promoted as PMT replacement. (For example, the multi-pixel photon counter of Hamamatsu Photonics of Japan and the solid-state photomultiplier of SensL Inc. of Ireland.) These detectors, however, also have high dark current and are nonlinear at high event rate.

The only industry where APD has found wide acceptance is in optical communication. It is known that if the APD's active area is reduced to less than 1mm^2 , the corresponding dark current will be reduced to the same level as a PMT. In optical communication, the light is a laser beam out of single mode optical fiber. Such a beam can be easily collimated then focused down to an area much smaller than 1mm^2 . It should be noted that the color separation devices used in the fluorescence light detection instruments, as described in United States Patent no. 6,683,314 and references therein, are almost identical in function and architecture to the wavelength division multiplexers (WDM) widely used in optical communication, as described in United States Patent nos. 4,482,994, and 5,786,915. A fundamental reasons preventing the use of small area APD in fluorescence detection instrumentation is the well known theorem of etendue conservation: the fluorescence light coming through a pinhole or multimode optical fiber is an extended light source with an etendue hundreds of times greater than that of a laser beam out of a single mode optical fiber. Consequently, as illustrated in FIG. 26, it cannot be collimated over an extended distance unless the diameter of the beam is significantly expanded. Unfortunately, the larger the beam diameter, the greater the technical challenge to focus it down to a small spot. Since efficient color separation can only be accomplished

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economically with collimated light beam, small area APD has not been considered viable for multicolor fluorescence light detection applications. Clearly, a technology capable of collimating a large etendue light beam over an extended distance without significantly expanding the beam diameter would be highly desirable. Such a technology would enable a WDM like device for fluorescence light detection with characteristics comparable to low noise semiconductor detectors.

10

Disclosure

The present disclosure provides an improved flow cytometer together with various improved components included therein.

15 It is an object of the present disclosure to provide a simple and reliable diode laser based optical system capable of delivering a focused laser beam of elliptical cross section with a Gaussian like intensity distribution along its minor axis and a width along major axis optimized for flow cytometric applications.

20 An object of the present disclosure is an imaging quality microscope objective that is easy to manufacture, has long working distance, large numerical aperture, large field of view and minimal chromatic aberration.

25 An object of the present disclosure is a simple fluidics system for flow cytometers that is not only reliable, compact and easy to manufacture, but also capable of supporting velocity critical applications such as in instruments with multiple spatially separated excitation laser beams or in droplet sorters.

30 A object of the present disclosure is simple design for a peristaltic pump can provide a pulseless liquid flow is highly desirable.

An object of the present disclosure to provide a peristaltic pump with minimal pulsation.

35 A object of the present disclosure to provide a peristaltic pump that is simple to manufacture and operate.

an object of the present disclosure to provide a device capable of collimating a light beam from an extended light

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source over an extended distance without significantly expanding the said beam diameter. It is also an object of the present disclosure to provide a WDM system using the said device to separate the said light beam into multiple colored bands.

5 Further, it is an object of the present disclosure to provide such a WDM system that is compatible with low noise semiconductor detectors. In addition, due to the diversity of fluorescent probes, it is an object of the present disclosure to provide such a WDM system that is reconfigurable.

10 Disclosed herein is a flow cytometer that includes:

1. a LD based optical subsystem for impinging a beam of light upon particles passing through a viewing zone;
2. a composite microscope objective for gathering and imaging light scattered from or fluoresced by particles passing through the viewing zone;
- 15 3. a fluidic subsystem for supplying a liquid sheath flow to the viewing zone;
4. a peristaltic pump for injecting into the liquid sheath flow a liquid sample flow carrying particles that passes together with the liquid sheath flow through the viewing zone;
- 20 5. a multimode optical fiber that receives scattered and fluoresced light from the viewing zone that the composite microscope objective gathers and images; and
- 25 6. a wavelength division multiplexer for optically separating into color bands light received via the optical fiber .

The LD based optical subsystem in accordance with the present disclosure for illuminating particles passing through the flow cytometer's viewing zone generally includes:

1. a laser diode oriented with its slow axis parallel to the direction of flow;
2. a collimating lens that converts the diverging beam from the LD into a collimated beam of elliptical shape with its major axis perpendicular to the flow;
- 35

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3. a focusing lens system that reduces the laser beam at the viewing zone to an optimal width in the direction perpendicular to the flow; and
4. finally a high power cylindrical focusing element placed in the proximity of the viewing zone with its axis perpendicular to the direction of flow.

The high power cylindrical focusing element transposes the far field profile of the LD along its slow axis to its Fourier conjugate at the viewing zone along the direction of flow, while maintains the transverse beam profile, such that the laser beam profile at the viewing zone is optimal for flow cytometric applications.

The composite microscope objective in accordance with the present disclosure generally includes:

1. a concave spherical mirror;
2. a transparent aberration compensation plate with the flow cytometer's a viewing zone being located between the mirror and the plate. Scatter and fluorescence light emitted from particles in the viewing zone is collected by the mirror and reflected back toward the compensation plate. Optical aberrations originating from the mirror are significantly reduced after light passes through the compensation plate. In one embodiment of the present disclosure, the viewing zone is located inside a flow cell provided by rectangular glass cuvette with a small rectangular channel through which a particle carrying liquid flows. The concave mirror is made of an optically transparent material, such as glass or optical quality plastics, of piano-convex shape with a highly-reflective coating on the convex side for internal reflection. The piano-side of the mirror is either gel-coupled or bonded to one side surface of the cuvette. The plano-aspheric compensation plate is made of a transparent material, such as glass or optical quality plastics, with the piano side gel-coupled or bonded to the opposite side of the cuvette. The piano-convex shaped mirror and the aspheric compensation plate may also be formed integrally with the cuvette. In yet another embodiment of the present disclosure, the viewing zone is in a jet stream with both the concave

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mirror and the compensation plate being free standing from the viewing zone, and the mirror is preferably a front surface concave mirror.

The fluidic system in accordance with the present disclosure generally includes a sheath liquid reservoir from which a liquid pump draws sheath liquid. Sheath liquid then flows from the liquid pump to an inlet of a T-coupling. One outlet arm of the T-coupling connects to a bypass that returns a fraction of the pumped sheath liquid back to the sheath liquid reservoir with the returned sheath liquid flowing into air within the sheath liquid reservoir. A second outlet arm of the T-coupling connects to a sheath route that includes a reservoir capsule followed by a particle filter and then the flow cell. The sheath liquid exiting the flow cell then goes to the waste tank. The fluidic resistance along the bypass is designed to be lower than the fluidic resistance along the sheath route. Consequently, only a small fraction of the sheath liquid goes through the flow cell. Note that typical sheath flow rate in flow cytometric applications is a few tens of milliliter per minute. The bypass therefore permits using higher flow rate liquid pumps that not only are much less expensive and more reliable, but also operates at higher pulsation frequency which is much easier to attenuate. Since the exit of the bypass route connects to air, it also serves as a large fluidic capacitor for significantly reducing pulsation in sheath liquid flowing along the sheath route. During operation, the inlet portion of the filter cartridge is filled with air. Therefore, the filter cartridge also serves as a fluidic capacitor, for further reducing pulsation in the sheath liquid at the flow cell to negligible level. Due to the large fluidic resistance at the flow cell, the air trapped near the inlet of the filter cartridge becomes compressed. If the liquid pump is turned off, the compressed air in filter cartridge being pushed back towards the sheath liquid reservoir becomes stored in the reservoir capsule whose size is chosen to prevent the trapped air from reaching the T-coupling.

The peristaltic pump in accordance with the present disclosure generally includes a plurality of rollers located

at the periphery of a rotor that moves the rollers circularly inside a housing's arcuate curved track and a compressible tube that the rollers compress against the track. In one embodiment of the present disclosure, the track of the peristaltic pump's housing has one recess so the compressible tube is progressively decompressed to full expansion then compressed to full closure every time one of the rollers moves past the recess. The location and shape of the recess maintains the total volume of liquid within the compressible tube from the recess to the pump's outlet substantially invariant. The effect of tube expansion as a roller moves past the pump's outlet is compensated by the tube compression when a different other roller immediately upstream of the pump outlet moves into the recess' compressing section. In another embodiment of the present disclosure, the track of the pump housing may contain a plurality of recesses, providing for a plurality of roller upstream of the pump outlet to progressively modify the tube compression in multiple sections along the compressible tube. The locations and shapes of the plurality of recesses are designed such that the modification of tube compression at these sections substantially compensates the effect due to the tube expansion near the pump outlet. In yet another embodiment of the present disclosure, the compressible tube is kept fully closed underneath the roller except in the inlet and exit sections. A variable speed motor is used to drive the pump. When a roller reaches the exit section, the motor's rotation programmatically speeds up to compensate for the tube's expansion.

The wavelength division multiplexer ("WDM") in accordance with the present disclosure generally includes at least two optical elements. The first optical element collimate a beam of light received from an extended light source, such as the light from a pinhole or from a multimode optical fiber. The first optical element magnifies the extended light source, for example, as defined by the pinhole, or the core of the multimode optical fiber, to an image having a size similar to the effective cross section of the first optical element thereby creating a collimated light beam between the first

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optical element and its image. A second optical element is positioned near the image, and relays the first optical element with unit magnification down the optical path. In this way the second optical element effectively doubles the collimated path length. Additional optical elements in the same 1:1 image relay configuration may also be included in the present disclosure to further extend the collimated optical path. The cascaded unit-magnification image relay architecture of the present disclosure provides a greatly extend the collimated optical path length without large beam expansion. As a result, WDM techniques well-established in the optical communication industry can be readily adapted for fluorescence light detection. In particular, multiple colored bands present in the beam of light can be separated using dichroic filters located along the optical path with the separated light being tightly focused into small spots compatible with low noise semiconductor photodetectors.

In one embodiment of WDM, the first optical element is a lens and the second element is a concave mirror, although it is apparent to those skilled in the art that other types of refractive and/or reflective optical components may also be used to achieve the same design goal. Similar to its counterpart in optical communication, the optical path in the WDM of the present disclosure may be folded using dichroic filters. In one embodiment of the present disclosure, the light path is folded into a zig-zag configuration. Preferably, to facilitate the flow cytometer's reliable reconfiguration each dichroic filter is bonded to a mechanical holder having a reference surface that is optically parallel to the filter's reflective surface. As a result, all of the WDM's filters can be accurately positioned along the optical path by referencing the filter's holder against a common optical flat.

In another embodiment of the present disclosure, the collimated beam passing through the dichroic filter is further branched out into multiple colored bands using secondary dichroic filters. It is apparent to those skilled in the art that dichroic filters may be inserted anywhere along the long, narrow and collimated beam path afforded by the present

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disclosure's relay imaging to thereby permit delivering a tightly focused beam to photo detectors using a variety of optical configurations, such as the star configuration discussed in United States Patent no. 6,683,314, the branched configuration discussed in United States Patent no. 4,727,020 and other types of WDM optical configuration widely practiced in the optical communication industry. Instead of concave mirrors, the WDM may be replaced by curved dichroic filters to further increase the number of colored bands selected by the WDM.

These and other features, objects and advantages will be understood or apparent to those of ordinary skill in the art from the following detailed description of the preferred embodiment as illustrated in the various drawing figures.

Brief Description of Drawings

FIG. 1 is a diagram schematically illustrating a preferred embodiment of a flow cytometer in accordance with the present disclosure that includes:

- a) a LD based optical illumination subsystem;
- b) a composite microscope objective upon which light emitted from the LD based optical illumination subsystem impinges, the composite microscope objective having a fluid-passing channel formed therethrough with a particle illumination viewing zone located inside a cuvette thereof;
- c) a fluidic system for supplying a pulsation free flow of sheath liquid to the fluid-passing channel formed through the composite microscope objective;
- d) a peristaltic pump for introducing a pulsation free flow of sample liquid that carries cells or particles to be analyzed into the sheath flow of liquid supplied by the fluidic system; and
- e) a wavelength division multiplexer ("WDM") having a zig-zag configuration for separating a beam of light into several different colored bands, the WDM receiving via an optical fiber light that is scattered from cells or particles as they pass

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through the composite microscope objective's fluid-passing channel and are illuminated therein by light emitted from the LD based optical illumination subsystem.

5 FIG. 2 is a schematic view depicting a typical high power edge emitting LD that illustrates the fast and slow axes of light emitted therefrom.

FIG. 2A shows a typical far field profile for a laser beam emitted from the LD chip depicted in FIG. 2.

10 FIG. 3A depicts a 3-dimensional view of a conventional, prior art LD based optical illumination subsystem for flow cytometric instruments together with the system's flow cell.

FIG. 3B depicts a typical time dependent profile of light scattering from a cell or particle passing through the laser beam depicted in FIG. 3A at the focus thereof within the system's flow cell.

15 FIG. 4A is an elevational view of an alternative prior art LD based optical illumination subsystem configuration across the liquid flowing through the fluid-passing channel that improves the beam profile at the focus thereof in the flow cytometer system's viewing zone.

FIG. 4B is a plan view along the liquid flowing through the fluid-passing channel of the alternative prior art LD based optical illumination subsystem depicted in FIG. 4A.

25 FIG. 5A is an elevational view across liquid flowing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1 wherein the LD's slow axis oriented transversely to the liquid flow.

30 FIG. 5B is a plan view along liquid flowing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1 wherein the LD's slow axis oriented transversely to the liquid flow.

35 FIG. 5C depicts a typical time dependent profile of light scattering from a cell or particle passing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1.

FIG. 6 is a perspective view of an alternative embodiment of a LD based optical illumination subsystem in accordance with

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the present disclosure adapted for use in a flow cytometer system in which a jet stream of liquid passes through the viewing zone.

5 FIG. 6A is an enlarged perspective view of the alternative embodiment of a LD based optical illumination subsystem of depicting in greater detail the jet stream of liquid passes through the viewing zone.

10 FIG. 7 is a perspective view of an alternative embodiment LD based optical illumination subsystem in accordance with the present disclosure adapted for use in a flow cytometer system which orients the LD's slow axis parallel to the direction of liquid flowing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1.

15 FIG. 8 is a perspective view of a composite microscope objective in accordance with the present disclosure adapted for use in the flow cytometer system depicted in FIG. 1, the composite microscope objective having a fluid-passing channel formed therethrough with the particle illumination viewing zone located inside a cuvette therein.

20 FIG. 9A is a cross-sectional elevational view of the composite microscope objective taken along the line 9A-9A in FIG. 8 that includes ray traces from three (3) spatially separated locations in the viewing zone to an image plane for the objective illustrating scatter and fluorescence emission
25 propagation.

FIGS. 9B1-9B3 are spot diagrams near the image plane depicted in FIG. 9A for the three (3) spatially separated light emission locations depicted in FIG. 9A.

30 FIG. 10 is a cross-sectional elevational view similar to that of FIG. 9A for an alternative embodiment composite microscope objective in accordance with the present disclosure that includes ray traces from three (3) spatially separated locations in a viewing zone therein to an image plane for the objective illustrating scatter and fluorescence emission
35 propagation.

FIG. 11 is a perspective view of yet another alternative embodiment a composite microscope objective in accordance with the present disclosure adapted for use in the flow cytometer

system depicted in FIG. 1, the alternative embodiment composite microscope objective having a fluid-passing channel formed therethrough with the particle illumination viewing zone located inside a cuvette therein.

5 FIG. 12 is a perspective view of yet another alternative embodiment a composite microscope objective in accordance with the present disclosure adapted for use in the flow cytometer system depicted in FIG. 1, the alternative embodiment composite microscope objective being adapted for a viewing zone that is
10 located inside the jet stream depicted in FIGs. 6 and 6A.

FIG. 13 is a perspective view of yet another alternative embodiment a composite microscope objective in accordance with the present disclosure adapted for use wherein a viewing zone is located on the surface of a microscope slide.

15 FIG. 14 is a schematic diagram depicting a fluidic subsystem in accordance with the present disclosure for supplying stable liquid sheath flow to a flow cytometers flow cell that includes:

1. a small capsule located between a sheath liquid pump
20 and the flow cell; and
2. a particle filter located between the small capsule and the flow cell,

both the particle filter and the small capsule providing air reservoirs for dampening pump pulsations.

25 FIG. 15 is a schematic diagram depicting an alternative embodiment fluidic subsystem similar to that illustrated in FIG 14 that replaces the small capsule with a length of tubing for providing an air reservoir.

FIGs. 16A and 16B are histograms that compare measured
30 particle flight times at the flow cell when the inlet portion of the particle filter has air trapped therein (FIG. 16A), and when there is no air within the fluidic subsystem between sheath liquid pump the and the flow cell (FIG. 16B).

FIG. 17 is a perspective view of a 3-roller peristaltic
35 pump in accordance with the present disclosure depicting the pump's rollers, tube and surrounding pump housing.

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FIGs. 18A through 18D depict simplified views for several states of the 3-roller peristaltic pump depicted in FIG. 17 with the rollers in different locations.

FIGs. 19 is a detailed longitudinal cross-sectional view of the peristaltic pump's tube being partially compressed by the pump's roller.

FIG. 19A and 19B are detailed cross-sectional views orthogonal to the peristaltic pump's tube's length taken along the lines 19A and 19B in FIG. 19 illustrating the tube's partial compression by the roller.

FIGs. 20A and 20B are schematic diagrams illustrating the pump's rollers and tube viewed along the pump's circular coordinates to depicting the pulseless provided by the peristaltic pump.

FIGs. 21 is graph depicting the functional relationship with respect to the roller position when it rolls off the exit section of the compressible tube of:

1. the total volume of liquid in the exit half of the pump; as well as
2. the liquid volumes in the pump's:
 - a. recess section; and
 - b. exit section.

FIGs. 22 is a simplified plan view of a 4-roller peristaltic pump in accordance with the present disclosure.

FIGs. 23 is a simplified plan view of a 6-roller peristaltic pump in accordance with the present disclosure.

FIG. 24A is a longitudinal cross-sectional illustration of rollers and a compressible tube for a pulsation minimizing 3-roller peristaltic pump in accordance with the present disclosure having a rotor with programmable speed.

FIG. 24B is simplified plan view illustrating the pulsation minimizing 3-roller peristaltic pump in accordance with the present disclosure having a rotor with programmable speed.

FIG. 24C is a graph depicting for the pulsation minimizing peristaltic pump depicted in FIG. 24B having a programmable speed rotor:

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1. negative volume change rate with respect to the roller position;
2. rotor speed; and
3. pump flow rate.

5 FIG. 25 is a diagram illustrating optical ray tracing for an exemplary 6 port wavelength division multiplexer ("WDM") using zig-zag configuration in accordance with the present disclosure.

10 FIG. 26 is a diagram illustrating ray tracing of prior art collimating devices showing the device's limitation in collimating an extended light source.

 FIG. 27 is a perspective illustration of an embodiment of a 6 ports WDM using a combination of zig-zag and branched configurations in accordance with the present disclosure.

15 FIG. 28 is a perspective illustration of another embodiment of a WDM having a concave dichroic filters in accordance with the present disclosure.

 FIGs. 29A and 29B are perspective illustrations depicting assembly process for constructing replaceable dichroic filter assembly for an reconfigurable WDM in accordance with the present disclosure.

 FIGs. 29C is a perspective illustration of a replaceable dichroic filter assembly build in accordance with the illustrations of FIGs. 29A and 29B.

25 FIGs. 30A and 30B are perspective illustrations of a WDM in accordance with the present disclosure depicting installing into the WDM the replaceable dichroic filter assembly depicted in FIG. 29C and its removal therefrom.

30 **Best Mode for Carrying Out the Disclosure**

Flow Cytometer

 FIG. 1 depicts a flow cytometer in accordance with the present disclosure identified by the general reference number

35 40. The flow cytometer 40 includes:

1. a LD based optical subsystem 50;
2. a composite microscope objective 60;

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3. a fluidic subsystem 70 for supplying a liquid sheath flow;
4. a peristaltic pump 80 for injecting a liquid sample flow that contains particles to be analyzed into the liquid sheath flow supplied by the fluidic subsystem 70, the liquid sample flow becoming hydrodynamically focused by the liquid sheath flow passes through a viewing zone with the composite microscope objective 60 gathering and imaging light scattered and/or fluoresced by particles in the viewing zone;
5. an optical fiber 852 that receives light scattered and/or fluoresced by particles in the viewing zone that the composite microscope objective 60 gathers and images; and
6. a wavelength division multiplexer 90 ("WDM 90") for optically processing scattered and/or fluoresced light received from the optical fiber 852.

Optical Subsystem 50

The optical subsystem 50 includes a LD 501 that, as depicted in greater detail in FIG. 2, emits a diverging beam of light from an edge thereof. As more graphically depicted in FIGs 2 and 2A, the diverging beam of light has an elliptically shaped cross-sectional profile with both a major axis, a.k.a. the fast axis, and a minor axis, a.k.a. the slow axis. The diverging beam of light emitted from the LD 501 impinges upon a collimating lens 502 which converts the diverging beam of light emitted by LD 501 into a collimated beam of light having an elliptical cross-section. Although not essential, the optical subsystem 50 may also include an optional mirror 503 positioned to direct the collimated elliptical beam of light towards the composite microscope objective 60. A plano convex lens 504, positioned near the composite microscope objective 60, reduces the major axis of the elliptically shaped beam of light that is oriented perpendicular to the direction in which the liquid sample and the surrounding liquid sheath flow through the viewing zone

within the composite microscope objective 60. At the viewing zone, the width of the elliptically shaped beam of light:

1. perpendicular to the direction in which the liquid sample flow passes through the viewing zone is preferably slightly less than the width of the liquid sheath flow; while
2. still sufficiently wide so particles in the sample flow pass through a nearly flat portion of the elliptically shaped beam of light at the beam's maximum intensity.

In accordance with the present disclosure, it is apparent to those skilled in the art that the plano convex lens 504 may be replaced by other types of optical elements such as an achromatic doublet lens or combination of spherical lenses, cylindrical lenses, and/or prism pairs. Alternatively, the mirror 503 and the lens 504 may also be replaced by a concave mirror. For polarization sensitive applications of the flow cytometer 40, an optional polarization conditioning element, such as a half-wave plate, may also be placed in the collimated section of the beam of light extending from the collimating lens 502 to the lens 504. Finally, before passing through the viewing zone the beam of light passes through a high power cylindrical lens 505, positioned adjacent to the viewing zone. As depicted in FIG. 1, the axis of the cylindrical lens 505 is oriented perpendicular to the direction in which the liquid sample flow passes through the viewing zone, and the focal length of the cylindrical lens 505 produces a tight focusing of the beam of light's minor axis at the viewing zone.

An advantage of the optical subsystem 50 in comparison with conventional LD based optical subsystem may be discerned more clearly in FIGs. 2 and 2A. Most commercially available laser diodes suitable for use in a flow cytometer emit a beam of light from an edge thereof. As depicted in FIG. 2, a gain section 509 of such a LD chip 510 is highly confined in the transverse direction indicated by an arrow 511. Consequently, to achieve high output power LD manufacturers often sacrifice beam quality, particularly along the transverse or fast axis direction that is oriented parallel to the arrow 511. FIG. 2A

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shows this characteristic of light emitted from a LD wherein multiple fringes 512 due to gain confinement are clearly visible at the far field in the minor axis direction of the emitted beam of light. It should be noted that the fringes 512 appearing in the illustration of FIG. 2A contain only a minute amount of total energy in the beam of light, and therefore have little impact on the conventional M-square characterization of the corresponding beam profile. However, as discussed in greater detail below, the fringes 512 do have a detrimental effect on the performance of conventional flow cytometers. Alternatively, gain confinement along the slow axis direction of an edge emitting LD that is oriented perpendicular to the arrow 511 is much more relaxed. Consequently, as shown in FIG. 2A, the far field beam profile is much smoother along the slow axis of the LD's beam of light.

FIG. 3A depicts a conventional LD based optical subsystem for a flow cytometer. Those elements depicted in FIG. 3A that are common to the optical subsystem 50 illustrated in FIG. 1 carry the same reference numeral distinguished by a prime (') designation. As depicted in FIG. 3A, the conventional optical subsystem orients the fast axis of the LD 501 parallel to the direction in which the liquid sample flow passes through the viewing zone. In its most simplified configuration, the elliptical beam profile of the LD 501' is directly transposed by the spherical focusing lens 504' into the viewing zone. In an attempt to achieve optimal aspect ratio for the focused beam of light, various different conventional LD based optical subsystems have also included beam shaping optical elements in addition to those depicted in FIG. 3A

The detrimental effect of fringes 512 along the fast axis of the LD 501' for conventional optical subsystem configurations clearly appears in the light scattering time profile depicted in FIG. 3B. Since scattering or fluorescence intensity is directly proportional to the local laser power impinging upon a particle, any fine structure in the beam of light's profile along the direction in which the liquid sample flow passes through the viewing zone will appear in the time profile of the signal produced by the flow cytometer. Such

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structures in the time profile are indistinguishable from signals generated by small particles, and will therefore cause the flow cytometer to trigger falsely and misidentify particles. In addition, the fringes 512 will also lead to
5 uncertainty in the measurement of other cytometric parameters, such as in the area and width of the pulse depicted in FIG. 3B.

FIGs. 4A and 4B depict a yet another prior art optical subsystem for LD based flow cytometric applications disclosed in the '019 patent identified previously. Those elements
10 depicted in FIGs. 4A and 4B that are common to the optical subsystem 50 illustrated either in FIG. 1 or 3A carry the same reference numeral distinguished by a double prime ("') designation. As depicted in FIGs. 4A and 4B, by orienting the
15 slow axis of the LD 501" parallel to the direction in which the liquid sample flow passes through the viewing zone the optical subsystem depicted in FIGs. 4A & 4B effectively overcomes the problem caused by the fringes 512 as described above. Unfortunately, the beam-diffusing element 513" placed before
20 the spherical focusing lens 504" in FIGs. 4A and 4B to diffuse the beam of light perpendicular to the direction in which the liquid sample flow passes through the viewing zone produces a highly astigmatic beam of light near the viewing zone. Specifically, focusing this astigmatic beam of light at the
25 viewing zone in the direction in which the liquid sample flow passes through the viewing zone increases the width of the beam of light perpendicular to the direction in which the liquid sample flow passes through the viewing zone so the beam's width become similar to or even wider than the sheath flow. Consequently, the optical subsystem depicted in FIGs. 4A and
30 4b not only diminished the amount of light energy impinging upon particles flowing through the viewing zone, the optical subsystem also increases undesirable scattering of light from the interface between the liquid sheath flow and adjacent parts of the composite microscope objective 60.

35 FIG. 5 highlights the main differences between the optical subsystem disclosed in the '019 patent and the optical subsystem 50 depicted in FIG. 1. Instead of placing an out-of-plane beam-diffusing element 513" before the spherical beam

focusing lens 504 as shown in FIG. 4, the high power cylindrical lens 505, depicted in FIGs. 5A and 5B as a cylindrical plano-convex lens, is placed along the beam of light after the spherical beam focusing lens 504 and is preferably juxtaposed with the composite microscope objective 60. As shown in FIGs. 5A and 5B, the cylindrical lens 505 focuses the minor axis of the beam of light in the viewing zone while leaving the major axis of the beam of light essentially unchanged. Consequently, the optical subsystem 50 depicted in FIGs. 1, 5A and 5B establishes a beam of light profile at the viewing zone which is elliptical with:

1. a tightly focused minor axis that spans across the combined liquid sample and sheath flows; and
2. a smooth minor axis profile in the direction of the combined liquid sample and sheath flows that is the Fourier conjugate of the far field beam profile along the slow axis of LD 501.

Meanwhile, as shown in FIG. 5B, the out-of-plane beam width is unaffected by the cylindrical lens 505. FIG. 5C shows a measured time profile of light scattered from a micro particle using the optical subsystem 50 depicted in FIGs. 1, 5A and 5B. The LD 501 used in making the measurement presented in FIG. 5C is the same as that used in generating the measured time profile of light scattered from a micro particle appearing in FIG. 3B. As shown in FIG. 5C, the side lobes caused by the fringes 512 along the fast axis of the LD 501 no longer have any material effect on performance of the flow cytometer 40.

FIG. 6 depicts yet another alternative diode laser based optical subsystem in accordance with of the present disclosure adapted for use in a flow cytometer. Those elements depicted in FIGs. 6 and 6A that are common to the optical subsystem 50 illustrated in FIGs. 1, 5A and 5B carry the same reference numeral distinguished by a triple prime ("") designation. The optical subsystem 50''' depicted in FIGs. 6A and 6B is almost identical to that shown in FIGs. 1, 5A and 5B except that the viewing zone occurs without a composite microscope objective 60 because it occurs in a free-flowing jet stream 519 that includes both the sample and sheath flows that is emitted from

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a nozzle 518. Consequently, for the configuration of the optical subsystem 50'" depicted in FIGs. 6A and 6B the high power cylindrical lens 505 is detached from the viewing zone that is located within the jet stream 519.

5 In the exemplary embodiments of the present disclosure depicted in FIGs. 1, 5A, 5B, 6A and 6B, the minor axis, i.e. the slow axis, of the LD 501 is oriented perpendicular to the direction in which the liquid sample flow passes through the viewing zone. However, it will be apparent to those skilled
10 in the art that using an alternative optical configuration the major axis, i.e. the fast axis, of the LD 501 may be reoriented to be perpendicular to the direction in which the liquid sample flow passes through the viewing zone. FIG. 7 depicts one example of such an alternative configuration of optical
15 elements. Those elements depicted in FIG.7 that are common to the optical subsystem 50 illustrated in FIGs. 1, 5A, 5B, 6A and 6B carry the same reference numeral distinguished by a quadruple prime ("") designation. As shown, the slow axis of the LD 501"" is oriented in the z-direction. The beam of light
20 emitted from the LD 501"" is then rotated to the in-plane y-direction by a pair of ninety degrees (90°) reflection mirrors 523a and 523b. In the illustration of FIG. 7, a normal to the first elliptically-shaped light beam reorienting mirror 523a is oriented in the x-y plane at forty-five degrees (45°)
25 to the x-axis, and a normal to the second elliptically-shaped light beam reorienting mirror 523b is oriented in the y-z plane at forty-five degrees (45°) to the z-axis.

Composite Microscope Objective 60

30 FIG. 8 depicts one embodiment in accordance with the present disclosure for the composite microscope objective 60 depicted in FIGs. 1, 5A, 5B and 7. As depicted in FIG. 8, the composite microscope objective 60 images a viewing zone that is located inside a prismatically-shaped glass cuvette 603
35 within a small flow channel 604, that preferably has a rectangular cross-sectional shape, through which passes the particle carrying combined liquid sample and sheath flows. A piano-concave back-surface mirror 601 included in the composite

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microscope objective 60 is made of an optically transparent material preferably having a refractive index similar to that of the glass cuvette 603, such as glass or optical quality plastics. To minimize optical loss, the back-surface mirror 5 601 includes a flat front surface that is optically coupled to an abutting flat surface of the prismatically-shaped cuvette 603. Optical coupling of the back-surface mirror 601 to the cuvette 603 may employ an index-matching gel, optical adhesive or direct optical bonding. Alternatively, the back-surface 10 mirror 601 may also be formed integrally with the cuvette 603.

The composite microscope objective 60 also includes a plano-aspheric corrector plate 602 that is also made of an optically transparent material preferably having a refractive index similar to that of the glass cuvette 603, such as glass 15 or optical quality plastics. To reduce optical loss, a flat surface of the corrector plate 602 may be optically coupled to an abutting flat surface of the prismatically-shaped cuvette 603 on a face thereof that is diametrically opposite to the back-surface mirror 601. Optical coupling of the corrector 20 plate 602 to the cuvette 603 may employ an index-matching gel, optical adhesive or direct optical bonding. The aspheric surface of the corrector plate 602 furthest from the corrector plate 602 may carry an anti-reflective coating to reduce optical transmission loss, although such a coating is not a 25 mandatory requirement for a composite microscope objective 60 in accordance with the present disclosure. The shape of the aspheric surface of the corrector plate 602 is similar to that in a classical Schmidt camera, (Schmidt, B., *Mitt. Hamburg Sternwart* 7 (36) 1932). As known by those skilled in the art, 30 the corrector plate of a Schmidt camera includes a circularly shaped neutral zone where the corrector plate does not deviate rays of light passing through the plate. For use in the composite microscope objective 60, outside of the neutral zone of the corrector plate 602, where the plate thickness is 35 thinnest, the corrector plate 602 has negative optical power while inside the neutral zone the corrector plate 602 has positive optical power. The exact shape of the aspheric corrector plate 602 may be readily obtained using any

commercially available optical ray tracing tool by any person having ordinary skill in the art. Note that in the flow cytometer 40 the beam of light generated by the optical subsystem 50 depicted in FIGs. 1, 5A, 5B and 7 enters the
5 cuvette 603 perpendicularly to the flow channel 604 through one (1) of the two (2) faces of the cuvette 603 that do not abut the back-surface mirror 601 or corrector plate 602.

FIG. 9A depicts the result of ray tracing for the embodiment of composite microscope objective 60 illustrated in
10 FIG. 8. As depicted in FIG. 9A, scatter and fluorescence emission from three (3) spatially separated locations in the flow channel 604 near the center of the cuvette 603:

1. initially propagate toward back-surface mirror 601 to be internally reflected by the back-surface
15 mirror 601;
2. then pass first through the cuvette 603;
3. subsequently pass through the aspheric corrector plate 602; and
4. finally forms three (3) distinct images near an
20 image plane 605.

Note that rays traversing the composite microscope composite microscope objective 60 depicted in FIG. 9A are nearly optically-uniform and that light emitted near the center of the cuvette 603 traverses the corrector plate 602 at near normal
25 incidence. Consequently, the composite microscope objective 60 introduces very little chromatic dispersion in light emitted near the center of the cuvette 603.

Further, it is well known in the astrophysics community that Schmidt camera offers the unparalleled combination of a
30 fast focal ratio and a large field of view with near diffraction limited optical performance. The principal drawback in a conventional Schmidt camera is that the image surface lies inside the instrument. For the composite microscope objective 60, light near the center of the cuvette
35 603 propagates opposite to that of a conventional Schmidt camera and therefore the image surface lies outside the composite microscope objective 60. Consequently, the present disclosure takes full advantage of the optical performance of

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the Schmidt camera without experiencing its limitation. FIGs. 9B1 through 9B3 depict spot diagrams near the image plane 605 for three (3) emission locations in viewing zone within the flow channel 604 that are separated 150 micron from each other.

5 The diameters of all images depicted in FIGs. 9B1 through 9B3 are less than 35 microns.

Light emitted from the viewing zone within the flow channel 604 of the composite microscope objective 60 depicted in FIGs. 8 and 9A that traverses the aspheric corrector plate

10 602 suffers from a small amount of chromatic aberration. FIG. 10 depicts an alternative embodiment for the composite microscope objective 60 depicted in FIGs. 1, 5A, 5B and 7 in accordance with the present disclosure. Those elements depicted in FIG. 10 that are common to the composite microscope

15 objective 60 illustrated in FIGs. 8 and 9A carry the same reference numeral distinguished by a prime (') designation. The shapes of the back-surface mirror 601' and the aberration corrector plate 602' depicted in FIG. 10 are modified slightly to produce collimated afocal images of the emission locations

20 near the viewing zone within the flow channel 604'. In FIG. 10 the composite microscope objective 60' also includes a chromatic compensating doublet lens 609 inserted between the corrector plate 602' and the image plane 605'. In addition to focusing the light emitted from the corrector plate 602' onto

25 the image plane 605', the doublet lens 609 also serves to further reduce the residual chromatic aberration introduced by the aspheric corrector plate 602'.

It is not essential that the flat surface of the corrector plate 602 to be optically coupled to the cuvette 603. FIG. 11

30 depicts an alternative embodiment of the composite microscope objective 60 of the present disclosure. Those elements depicted in FIG. 11 that are common to the composite microscope objective 60 illustrated in FIGs. 8 and 9A carry the same reference numeral distinguished by a double prime (")

35 designation. FIG. 11 depicts the aberration corrector plate 602" optically decoupled from the cuvette 603". Although not essential for operation of the composite microscope objective 60", to improve the light transmission efficiency both surfaces

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of the corrector plate 602" and the exposed flat surface of the cuvette 603" may carry an anti-reflectively coating. It is understood that the corrector plate 602" shown in FIG. 11 is held in fixed relationship to the combined back-surface mirror 601 and cuvette 603 by a mechanical support not depicted in FIG. 11. Similar to the composite microscope objective 60 and 60' depicted respectively in FIGs. 9A and 10, the composite microscope objective 60" with detached corrector plate 602" may be configured to provide either finite focal length image, or an afocal system which in turn is focused to a finite distance image plane by addition of the chromatic compensating doublet lens 609.

FIG. 12 depicts yet another alternative embodiment of the composite microscope objective 60. Those elements depicted in FIG. 12 that are common to the composite microscope objective 60 illustrated in FIGs. 8, 9A and 11 carry the same reference numeral distinguished by a triple prime ("') designation. The composite microscope objective 60'" depicted in FIG. 12 is adapted for collecting scatter and fluorescence emission from cells or other microscopic particles carried in the jet stream 519 emitted by the nozzle 518. The composite microscope objective 60'" consists of a concave, spherically shaped, front surface mirror 610 and an aberration corrector plate 612. The front surface mirror 610 may be made of glass or other types of hard material with a highly reflective coating on the concave surface 611. or made of metal with polished concave surface 611. Similar to the corrector plate 602, the plano-aspheric corrector plate 612 is made of a thin piece of transparent material, such as glass or optical quality plastics. The aspheric surface may be formed on either side of the corrector plate 612. Preferably, both surfaces of the corrector plate 612 are coated with an anti-reflective coating to reduce optical transmission loss, although such a coating is not a mandatory requirement for a corrector plate 612 in accordance with the present disclosure. It is understood that the front surface mirror 610 and the corrector plate 612 are held in fixed relationship to each other by a mechanical support not depicted in FIG. 12. Scatter and fluorescence

light emitted from cells or other types of microscopic particles in the viewing zone inside the jet stream 519 is reflected by the concave surface 611 of the front surface mirror 610. The aberration due to reflection from the concave surface 611 is corrected by the corrector plate 612 after light
5 traverses through the corrector plate 612. It is understood by those having skill in the art that the composite microscope objective 60'" may be configured to provide either a finite-focused image similar to that depicted in FIG. 9A, or a
10 collimated afocal image which is focused at finite distance from the composite microscope objective 60'" by a chromatic aberration correction doublet similar to the doublet lens 609 depicted in FIG. 10.

FIG. 13 depicts an adaptation of the composite microscope
15 objective 60 for imaging specimens fixed to the surface of a transparent substrate such as a glass slide. Those elements depicted in FIG. 13 that are common to the composite microscope objective 60 illustrated in FIGs. 8, 9A and 11 carry the same reference numeral distinguished by a quadruple prime ("")
20 designation. The composite microscope objective 60"" depicted in FIG. 13 includes two (2) optical elements, one a plano-concave back surface mirror 617 made of a transparent material such as glass or optical quality plastics, and an aberration corrector plate 618. As depicted in FIG. 13, the specimen to
25 be imaged is fixed to a front surface 615 of a transparent, usually glass slide 616. The slide 616 is optically coupled, preferably using a thin layer of index matching fluid, to the flat surface of the back surface mirror 617. Scatter and fluorescence light emitted by the specimen:

- 30 1. initially propagates through the slide 616 and the back surface mirror 617;
2. is internally reflected by the back surface mirror 617 back through the slide 616;
3. then through the corrector plate 618; and
- 35 4. finally forms an image at an image plane that is located beyond the corrector plate 618.

Fluidic Subsystem 70

FIG. 15 depicts a fluidic subsystem 70 in accordance with the present disclosure that includes a sheath liquid reservoir 702 and a liquid pump 701 that draws sheath liquid from the sheath liquid reservoir 702. The liquid pump 701 may be a diaphragm pump, or a peristaltic pump, or a piston pump or any types of continuous fluid pump. An outlet of the liquid pump 701 connects to an inlet of a T-coupling 703 that receives sheath liquid from the liquid pump 701. The T-coupling 703 has two (2) outlets a first one of which connects to a bypass conduit 710 for returning a fraction of the sheath liquid received by the T-coupling 703 from the liquid pump 701 back to the sheath liquid reservoir 702. Returning a fraction of the sheath liquid received by the T-coupling 703 from the liquid pump 701 back to the sheath liquid reservoir 702 is advantageous for two (2) reasons.

1. As depicted in FIG. 1, the bypass conduit 710 is left open to the surrounding atmosphere which effectively dampens pulsation to thereby significantly reducing the pulsation inherent in the operation of the liquid pump 701.
2. Returning a fraction of the sheath liquid received by the T-coupling 703 from the liquid pump 701 back to the sheath liquid reservoir 702 also effectively reduces the throughput of the liquid pump 701 thereby allowing the use of comparatively high flow rate, low cost pumps in the flow cytometer 40.

Denote the flow resistance of the bypass conduit 710 as "r" and the flow resistance of path from the T-coupling 703 to the flow channel 604 of the cuvette 603 as "R." The output resistance to the sheath pump is then

$$R_p = \frac{rR}{r+R} \quad (1)$$

Since $R \gg r$, the behavior of the liquid pump 701 is therefore dominated by the resistance of the bypass conduit 710 whose fluid dynamic properties are temperature insensitive. Thus, the configuration of the fluidic subsystem 70 depicted in FIG.

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15 also provides a simple mechanism for achieving a temperature insensitive sheath liquid flow to the flow channel 604.

As depicted in FIG. 15, a second outlet of the T-coupling 703 connects to the to the flow channel 604 that extends
5 through the cuvette 603 preferably first via a small reservoir capsule 704 and then via a filter cartridge 705. As depicted in FIG. 16, a piece of tubing 704' approximately 4 ft. long may be substituted for the small reservoir capsule 704. During initialization of the fluidic subsystem 70, some air becomes
10 trapped in the filter cartridge 705 near its inlet which as depicted in FIG. 15 is located above an outlet of the filter cartridge 705. The air trapped in the filter cartridge 705 acts as an additional fluidic capacitor effectively reducing to negligible level the pulsation in sheath liquid emitted into
15 the flow channel 604. Due to the large fluidic resistance at the flow channel 604, the air trapped inside the filter cartridge 705 becomes compressed. When the liquid pump 701 is turned off, a trapped in the filter cartridge 705 is pushed back towards the T-coupling 703 analogous to a discharging
20 capacitor. Without the small reservoir capsule 704, some air ejected from the filter cartridge 705 reaches the bypass conduit 710 due to its low fluidic resistance, and will be pushed out of the fluidic subsystem 70 once the liquid pump 701 is turned on again. Without additional air supply, such a
25 scenario will repeat until most of the air becomes purged from the fluidic subsystem 70 causing the filter cartridge 705 to lose its effectiveness as a pulsation damper. The purpose of the small reservoir capsule 704 or the piece of tubing 704' is therefore to provide a reservoir for isolating the filter
30 cartridge 705 from the bypass conduit 710 ensuring that air trapped inside the filter cartridge 705 remains within the fluidic subsystem 70 despite repeated on-off operations of the liquid pump 701.

The pulsation damping effect of the trapped air near the
35 inlet of the filter cartridge 705 is clearly evident in the histograms depicted in FIG. 16A and 16B. FIG. 16A depicts measured particle flight times at the flow channel 604 when a pocket of air is trapped near the inlet of the filter cartridge

705. FIG. 16B depicts measured particle flight times at the flow channel 604 when the trapped air is purged from the fluidic subsystem 70. The result depicted in the histograms of FIGs. 16A and 16B is made using two (2) knife edge shaped laser beams focused near the center of the flow channel 604 that are spaced approximately 200 micrometers apart. The horizontal axis of the FIGs. 16A and 16B is the flight time a particle takes from one laser beam to the other measured by recording the peak arrival time of light scattered from the particle at ninety degrees (90°) from the excitation beams. In both cases, the average flight time for particles to cross the two laser beams is the same. As shown in FIG. 16A, when the filter cartridge 705 retains some air, all particles take about the same amount of time to cross the two laser beams. If the filter cartridge 705 retains no air, as shown in FIG. 16B, the distribution of flight times not only broadens, but also becomes bimodal. In other words, some particles take less time while others take longer than average amount of time to cross the two laser beams, a phenomenon that can be easily attributed to sheath liquid velocity pulsation at the flow channel 604.

In the embodiments of the present disclosure discussed so far, the fluidic resistance along bypass conduit 710 as well as between the T-coupling 703 and the flow channel 604 is not adjustable. As should be apparent to those ordinary skilled in the art, flow restrictors such a fixed restrictor or adjustable valves 711, 711' and 712, 712' may be advantageously inserted in the bypass conduit 710 and between the T-coupling 703 and the flow channel 604 to permit adjusting the flow rate through the flow channel 604. Alternatively, the velocity of sheath liquid flowing through the flow channel 604 may also be adjusted using a liquid pump 701 that is driven by a variable speed brushless DC motor.

35 Peristaltic Pump 80

One embodiment of an peristaltic pump 80 in accordance with the present disclosure appears in FIG. 17. The pump includes a housing 809 with arcuate curved track 808, three

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rollers 810, 811 and 812 attached to a rotor 816 rotatable within the housing 809, and a compressible tube 807 sandwiched between the compressible tube 807 of the housing 809 and the rollers 810, 811 and 812. As depicted schematically in FIGs. 5 18A through 18D, the rollers 810, 811 and 812 of the peristaltic pump 80 are spaced at substantially equal angular distances, separations or spacings from each other around the perimeter of the rotor 816. For simplicity, it is assumed in the following discussions that the rotor 816 rotates 10 counterclockwise, although it is to be understood that the discussions apply equally well to a peristaltic pump with clockwise rotating rotor. The compressible tube 807 of the housing 809 may be divided into several sections:

1. an open section between point 801 and point 806 15 where the experiences no compression;
2. a pump inlet section between point 801 and point 802 where the is progressively compressed until fully closed when a roller rolls over the section;
3. two pumping sections between point 802 and point 20 803, as well as between point 804 and point 805 wherein the is fully closed by the roller;
4. a recess section between point 803 and point 804 in which the progressively expands from fully closed to fully open as a roller rolls through the 25 expansion part of the recess section from point 803 to point 813;
5. then the is progressively compressed to fully closed as a roller rolls through a compression part of the recess section from point 813 to point 804; 30 and
6. the exit section between point 805 and point 806 where the progressively expands from fully closed to fully open as a roller rolls through the section.

In other words, when a roller rolls anticlockwise over the 35 from inlet point 801 to outlet point 806, the inner gap of the :

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1. progressively decreases from fully open at point 801, to fully closed at point 802 and remain closed until point 803;
2. then progressively expands back to fully open at point 813;
3. then progressively decreases to fully closed at point 804, and remain closed until the roller reaches point 805; and
4. finally progressively expands back to fully open at point 806.

The size of the gap inside the is schematically illustrated in FIGs. 18A through 18D as the spacing between dashed circle and the solid compressible tube 807. As illustrated in FIGs. 18A through 18D, in this embodiment of the peristaltic pump 80 the angular distances, separations or spacings between points 801 and 803, points 802 and 813, point 813 and 805, as well as between point 804 and 866 are identical to the angle between adjacent rollers. As a result, when the roller 810 rolls through the pumping section from point 804 to point 805, as depicted in FIGs. 18A through 18B, its interaction with the completely determines the fluid flow rate of the peristaltic pump 80. Once the roller 810 reaches the exit section between point 805 and 806, as shown in FIG. 18C, the underneath the roller 810 starts to progressively expand and a gap starts to grow. Meanwhile, the roller 811 arrives at the compression part of the recess section and starts to progressively compress the . In the peristaltic pump 80, the shape of the compression part of the recess section between point 813 and point 804 along the compressible tube 807 is such that the volume of liquid pushed out by the compression of the underneath roller 811 in the compression part of the recess section between point 813 and point 804 substantially fills the volume created by the expansion underneath roller 810 in the exit section between point 5 and point 6. During this period, the compressible is partially open underneath both rollers 810 and 811 and completely closed underneath roller 12. Consequently, the pumping action is mainly delivered by roller 12. In particular, since by design the total volume of liquid in the

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section of the between point 13 and point 6 remains substantially constant during this period, the flow rate of the peristaltic pump 80 in the state shown in FIG. 18C remains substantially the same as that in the state shown in FIGs. 18A and 18B. Once the roller 810 passes point 806, roller 811 reaches the pumping section between point 804 and point 805. Note there is no physical difference between the rollers 810, 811 and 812, the flow rate of the peristaltic pump 80 therefore remains substantially constant throughout the entire process.

The mechanism of the pulseless peristaltic pump of the present disclosure may be understood more clearly if it is viewed along a circular coordinate following the movement of the rollers. Referring to FIG. 19, denote as V the volume of the fluid inside a compressible tube 819 from the outlet to a nearest roller 820 that closes off the compressible tube 819, i.e., the amount of fluid represented by the hatched area 818 hatched area 818 shown in FIG. 19. Clearly, V depends on the angular position, θ , of the roller 20, as well as δ , the amount of tube compression exerted by all other downstream rollers.

$$V = V(\theta, \delta_1, \delta_2, \dots) \quad (1)$$

Consequently, the flow rate, F , of a peristaltic pump is related to the time derivative of V , by:

$$-F = \frac{dV}{dt} = \frac{\partial V}{\partial \theta} R + \sum_i \frac{\partial V}{\partial \delta_i} \frac{d\delta_i}{dt} \quad (2)$$

Here R is the rotational speed of the rotor and the subscripts are used to identify multiple downstream rollers. The first term on the right hand side of Eqn. (2) represents the contribution from the roller that closes off the tube. The partial derivative $\frac{\partial V}{\partial \theta}$ is therefore independent of θ . The summation term represents contributions from all other downstream rollers partially compressing the compressible tube 819. Now let ΔS be the cross sectional area change due to the compression of the compressible tube 819 by the , and L the length of tube where its cross sectional shape is affected by the tube compression. Then, it is obvious to a person skilled in the art that L is proportional to the tube compression δ , and ΔS proportional to its square, δ^2 . Consequently, ΔV , the

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volume of fluid lost due to the compression of the tube by the roller, follows Eqn. (3):

$$\Delta V \propto L \cdot \Delta S \propto \delta^3 = (D - G)^3 \quad (3)$$

5 where D is the inner diameter of the compressible tube and G the minimum gap indicated in FIGs. 19, 19A and 19B which is also represented in FIGs. 18A through 18D by the spacing between the dashed circle and the solid compressible tube 807 of the housing 809. Now referring to FIGs. 20A AND 20B, in the
10 circular coordinate system FIG. 20A corresponds to the state of pump shown in FIGs. 18A and 18B. During this period, there is no roller downstream of the roller 810' and the summation term in Eqn. (2) vanishes. FIG. 20B corresponds to the state of the pump shown in FIG. 18C. The is closed off by roller
15 12' and partially compressed by the rollers 810' and 811'. However, volumetric changes introduced by the two rollers 810' and 811' substantially cancel each other. Consequently, the summation term in Eqn. (2) vanishes as well. As a result, the flow rate of the peristaltic pump 80 remains substantially
20 constant regardless of roller positions.

The shape of the compressible tube 807 satisfying the above requirement can be readily derived from Eqn. (3). Referring to FIG. 18C, if the gaps of the arcuate compressible tube 807 in the compression part of the recess section between
25 point 813 and point 804, $G_{13,4}$, and in the exit section between point 805 and point 806, $G_{5,6}$, follow the equation

$$(D - G_{13,4})^3 + (D - G_{5,6})^3 = D^3 \quad (4)$$

then the total fluid volume in the two sections remains
30 substantially constant, as shown in FIG. 21. In the peristaltic pump 80, the shape of the pump housing 809 is symmetrical with respect to its center line, such that the entrance half of the pump housing 809 is the mirror image of the exit half of the housing 809, as shown in FIG. 17. The
35 peristaltic pump 80 can therefore be operated both in counterclockwise and clockwise rotation very little pulsation, although it is understood that the symmetry is not required to realize a pulseless peristaltic pump in accordance with the

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present disclosure. For example, as long as the gaps of the arcuate compressible tube 807 in the section between point 13 and point 3, $G_{13,3}$, and in the section between point 2 and point 1, $G_{2,1}$, follow Eqn. (5),

$$(D - G_{13,3})^3 + (D - G_{2,1})^3 = D^3 \quad (5)$$

a peristaltic pump in accordance with the present disclosure will exhibit little pulsation when the rotor 816 rotates clockwise.

FIG. 22 depicts an alternative embodiment of a peristaltic pump in accordance with the present disclosure. Those elements depicted in FIG. 22 that are common to the peristaltic pump 80 illustrated in FIG. 17 carry the same reference numeral distinguished by a prime (') designation. The peristaltic pump 80' includes a compressible tube 807' having two (2) recesses 820 and 821, and four (4) rollers 822, 823, 824 and 825. In the embodiment depicted in FIG. 22, the fluid volume loss due to the tube expansion near the outlet of the pump is compensated by the combined effect of the compression of the compressible tube by rollers 822 and 823 near the two recesses 820 and 821.

FIG. 23 depicts yet another alternative embodiment of a peristaltic pump in accordance with the present disclosure. Those elements depicted in FIG. 23 that are common to the peristaltic pump 80 illustrated in FIG. 17 and the peristaltic pump 80' illustrated in FIG. 22 carry the same reference numeral distinguished by a double prime (") designation. The peristaltic pump 80" includes six (6) rollers and an arcuate compressible tube 807" having two recesses 818" and 819". In the peristaltic pump 80", the fluid volume loss due to the tube expansion near the outlet of the pump is compensated by the action of the roller immediately upstream of the one recess 818" or 819" near the pump outlet.

Pulsation due to the expansion of a compressed compressible tube near the outlet of a peristaltic pump may also be overcome by a peristaltic pump having a programmable rotor speed. FIGs. 24A through 24C illustrate pertinent aspects of an alternative embodiment mechanism for minimizing

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peristaltic pump pulsation in accordance with the present disclosure for a 3-roller peristaltic pump. As depicted in FIG. 24B, the track 828 is substantially circular between the pump inlet and pump exit section. Consequently, as indicated by the spacing between the dashed circle 829 and the solid curve of the track 828 the compressible tube is completely closed by various ones of the pump's three (3) rollers 826, 827 and 829 between the pump inlet and pump outlet. FIG. 24A illustrates schematically in a circular coordinate system the roller positions for the peristaltic pump depicted in FIG. 24B. Since there is only one roller downstream of the one that closes off the tube, Eqn. (2) is much simplified:

$$-F = R \left(\frac{\partial V_c}{\partial \theta} + \frac{\partial V_c}{\partial \delta} \frac{d\delta}{d\theta} \right) \quad (6)$$

Here the tube compression $\delta(\theta)$ is explicitly expressed as a function of roller position θ . The terms inside the parentheses represent the change rate of fluid volume with respect to roller position. The first term is the contribution from the roller that closes off the tube, i.e., roller 827 in FIG. 24A, and the second term the contribution from the roller in the exit section. Note that by definition, the volume change rate is negative and the second term inside the parentheses vanishes when there is no roller in the exit section. The dotted curve in FIG. 24C is a representative plot of the negative volume change rate with respect to the position of the roller. The bump along the curve, due to the tube expansion when a roller rolls off the tube near the pump outlet, is the cause for pulsation in conventional peristaltic pumps having a constant rotor speed. However, for the peristaltic pump depicted in FIGs. 24A through 24C the rotor speed, R , shown as dashed curve in FIG. 24C, is set to vary in synchronism with the rotor position and inversely proportional to the change rate of the fluid volume. Consequently, the flow rate of the pump, which is the product of the rotor speed and the change rate of the fluid volume, remains constant, as indicated by the solid line at the top of FIG. 24C. Note the terms inside the parentheses of Eqn. (6) are uniquely determined by the mechanical structure of the pump. The rotor

speed profile can therefore be readily generated from the shape of the track 828 in accordance with Eqn. (3). To those skilled in the art, there are many ways to realize a programmable rotor, for example, with stepping motor or DC servo motor.

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WDM Device 90

FIG. 25 shows the optical ray trace for an exemplary 6 ports wavelength division multiplexer of the present disclosure using zig-zag configuration. As shown in FIG. 25, fluorescence light going through a pinhole or emitted from the facet of a multimode optical fiber, such as the optical fiber 852 depicted in FIG. 1, forms an extended object or light source at location 901, i.e. the optical input of the WDM 90. The size of the object is defined by the diameter of the pinhole or the core diameter of the multimode optical fiber. Note that the practical size of the pinhole or the core diameter of the multimode optical fiber is measured in millimeters, in contrast to the diameter of single mode optical fibers that are measured in micrometers. Consequently, the etendue of the fluorescence light source, defined as the product of beam size and its divergence angle, is hundreds times greater than its counterpart in optical communication. According to the theorem of the conservation of etendue (Julio Chaves, *Introduction to Nonimaging Optics*, CRC Press, 2008 [ISBN 978-1420054293]), light from such an extended source, similar to that from a flash light, can only be kept collimated for a very limited distance, particularly when the diameter of the collimated portion needs to be small.

As depicted in FIG. 25, a collimating optical element, in this case an achromatic doublet lens 902, captures the light from source 901, and projects a magnified image of the object near a final focusing lens 905. The size of the image near 905 is kept approximately the same as the effective size of the collimating optical element 902. Consequently, beam of light propagating between lens 902 and the lens 905 is effectively collimated. As shown in FIG. 25, so long as the magnification factor is kept small, for example, less than around 10, using a simple singlet lens 905 the collimated beam of light can

readily focused down to a spot smaller than that of the beam of light received by the WDM 90 at location 901. The ability to focus the beam of light down to such a small size permits placing a small area semiconductor detector at a focal point
5 906 of the focusing lens 905 for efficient photo detection.

A dichroic filter 903, oriented at a slanted angle, is inserted into the optical path approximately midway between the collimating optical element 902 and the lens 905. The dichroic filter 903 passes the color band of interest and reflects the
10 remaining colors in the beam of light for further processing within the WDM 90. An optional band pass filter 904 may be inserted following the dichroic filter 903 to further improve the color isolation capability of the WDM 90.

Light reflected from the dichroic filter 903 impinges upon
15 a second optical element 907, preferably a concave mirror. The concave mirror 907 has a radius of curvature approximately equal to the distance between the collimating optical element 902 and the image near focusing lens 905. The concave mirror 907 therefore creates a second image of the collimating lens
20 902 near a second focusing lens 908. The light beam between the concave mirror 907 and the second image at the lens 908 has substantially the same diameter as the beam of light between the collimating lens 902 and the first image near the focusing lens 905. The relay imaging concave mirror 907 therefore
25 effectively doubles the collimated beam path without expanding the beam's diameter. Again, the extended yet collimated beam can be easily focused down to a spot smaller than that of the light source at 901. A second dichroic filter 909 is then inserted approximately midway between the relay imaging concave
30 mirror 790 and the second image near focusing lens 908. The second dichroic filter 909 passes another band of color in the beam of light received by the WDM 90 at location 901 and reflects the remainder of the impinging beam of light for further processing.

35 As shown in FIG. 25, additional relay collimating optical elements 910, 911, 912, 913 and dichroic filters 914, 915, 916, 917 can be cascaded in the same way to produce multiple images near focusing lenses 918, 919, 920 and 921, each of these

images corresponding to a specific color band of light received by the WDM 90 at location 1. As shown in FIG. 25, due to the present disclosure's 1:1 imaging relay architecture, the spots of light produced by focusing lenses 906, 908, 918, 919, 920 and 921 are all smaller than the source of the beam of light, and therefore can be easily captured by small area APD's.

Although FIG 25 illustrates a 6-port wavelength division multiplexer for a beam of light from the extended light source, it is readily apparent to those skilled in the art that WDM's having different numbers of ports can be easily built in accordance with the present disclosure. It is also apparent to those skilled in the art that although the WDM 90 preferably uses achromatic doublets as the first collimating optical element, singlet lens can also be used since the images created before the focusing lenses 906, 908, 918, 919, 920 and 921 are all nearly monochromatic. Instead of using concave mirrors for relaying the beam of light reflected from the dichroic filters, one may also use refractive optics as relay element to extend the path of the collimated beam of light. A clear advantage of the zig-zag architecture used in the WDM 90, however, is the possibility for using array detectors, which would lead to a more compact WDM suitable for portable instrumentation.

FIG. 26 illustrates an optical ray trace for a prior art collimating device. The technique depicted in FIG. 26 is extensively used in conventional multicolor fluorescence instruments, for example, in United States Patent no. 6,683,314. As shown in FIG. 26, the beam of light diverges rapidly beyond the image 924 created by the collimating optical element 923. Consequently, the only option for constructing a multi-color device is to insert dichroic filters in between the collimating element 923 and its image 924.

Due to the constraint of etendue conservation, the diameter of the collimated beam must be significantly expanded to accept multiple dichroic filters in the section. The expanded beam creates serious challenge to refocusing the collimated beam down to small spots suitable for small area semiconductor detectors. To overcome these difficulties, some instrument manufacturers have chosen to use Mt exclusively for

fluorescence detection such as in the main stream flow cytometers manufactured by Becton-Dickinson, Becton Coulter and Partec 's and the MegaBACE series of DNA sequencers by GE Amersham. Other instruments, such as the Luminex multiplexed
5 bead analyzers, have selected certain color bands with known bright fluorescence, and uses large area APD for detecting light in the selected color bands.

FIG. 27 illustrates a perspective view of an alternative embodiment for a 6-port WDM 90 using a combination of zig-zag and branched configurations. The design is a modification of
10 zig-zag configuration depicted in FIG. 25. In the alternative embodiment depicted in FIG. 27, band pass filter 904 of FIG. 25 is replaced by a dichroic filter 904'. The filter 904' is positioned to let one color pass through and reflects other
15 colors at ninety degrees (90°). The optical path length of the beam of light passing through the dichroic filter 904' and that being reflected from the 904' are substantially the same, such that one arm is focused by lenses 905 and the other by lens 905' to small spots compatible with small area semiconductor
20 detectors placed at focal location 906 and 906'. As shown in FIG. 25, the remaining color of the light reflected by dichroic filter 903 is relay imaged by a concave mirror 907 and the configuration including optical elements 903, 904', 905 and 905' is cascaded two (2) more times to form a 6 port-WDM.

25 FIG. 28 illustrates a perspective view of an alternative embodiment for a 8-port WDM 90. By replacing the concave relay imaging mirrors 907 and 910 in FIG. 27 with concave shaped dichroic filters 907' and 910', the WDM depicted in FIG. 28 provides 2 more color bands in comparison with the WDMs
30 depicted in FIGs. 25 and 27.

Numerous fluorescence probes for use in flow cytometry have been developed over the years. More recently, multiple fluorescence proteins have also become an important tool in biomedical studies. To accommodate different types of
35 fluorescence probe, various techniques have been developed to enable user selection of dichroic filters suitable for their particular needs. A significant challenge for replaceable dichroic filters is avoiding direct contact of the coated

filter surface with any hard flow cytometer reference frame. Repeated direct contact between the coated filter surface and any hard reference frame may damage a replaceable dichroic filter. Presently, most conventional solutions addressing this problem use precision-machined mechanical spacers for holding replaceable dichroic filters in place. One example of such a solution appears in United States Patent no. 6,683,314. However, such a solution becomes unreliable if the detector's active area is smaller than 1.0 mm².

FIGs. 29A and 29B depict fabricating a replaceable dichroic filter assembly 934 illustrated in FIG. 29C suitable for small area detectors. Assembly of the replaceable dichroic filter assembly 934 begins in FIG. 29A which depicts constructing a reference template for its fabrication. The reference template is a staircase made of two (2) optically parallel glass plates 925 and 926. Bonding the two (2) glass plates 925 and 926 together in optical contact ensures that a surface 929 of the glass plate 925 becomes optically parallel to a surface 930 of the glass plate 926. A front surface 932 of a replaceable dichroic filter 927 is then pressed against the surface 929 of the template. A filter holder 928, which loosely fits the dichroic filter 927, includes a reference surface 931 and a filter slot 933. During assembly of the replaceable dichroic filter, the filter slot 933 is partially filled with epoxy adhesive and the reference surface 931 of the filter holder 928 is pressed against the surface 930 of the template while filter holder 928 slides toward the dichroic filter 927. While the epoxy adhesive sets, part of the dichroic filter 927 remains seated within the filter slot 933 while pressure is applied against the dichroic filter 927 and filter holder 928. It should be apparent to those skilled in the art that the epoxy adhesive may be either UV or thermally curable, or made by blending together components of an A/B mixture. FIG. 29C depicts the E934~ fabricated as depicted in FIGs. 29A and 29B and described above. The assembly process depicted in FIGs. 29A and 29B and described above ensures that front surface 932 of the replaceable dichroic filter assembly 934 will be optically parallel to the reference surface 931,

and indented with respect to the latter at a spacing accurately determined by the thickness of the glass plate 925.

FIGs. 30A and 30B depict an embodiment of the present disclosure where the fore-mentioned replaceable dichroic filter assembly 934 is used in the WDM 90 for optically processing a beam of light from an extended light source. A notable feature of the WDM 90 is a glass reference block 935 having an optically flat surface. As will be apparent to those skilled in the art, the glass reference block 935 may be made of other materials. As shown in FIG 30B, when installing a dichroic filter 927 the reference surface 931 of the replaceable dichroic filter assembly 934 slides against the flat surface of the glass reference block 935 and is kept in contact therewith by a spring loaded screw 936. Consequently, the coated front surface 932 of the replaceable dichroic filter assembly 934 remains optically parallel to the optical flat and accurately located. In the meantime, the indentation of front surface 932 with respect to the reference reference surface 931 protects it from in physical contact with any object during filter replacement.

It is apparent to those skilled in the art that many modifications and variations of the described embodiments of the replaceable dichroic filter assembly 934 are possible. For example, an alternative embodiment of the present disclosure is a pedestal assembled using a first and a second round optical flat. When assembling the replaceable dichroic filter assembly 934, the reference surface of a filter holder rests against a surface of the first optical flat and the coated surface of the dichroic filter rests against the flat surface of the second optical flat. Epoxy bonding then holds the coated surface of the dichroic filter optically parallel to the reference surface of a filter holder, yet indented at a distance accurately determined by the thickness of the second optical flat.

Industrial Applicability

Although an embodiment of the present disclosure of an LD based optical system for flow cytometric application has been

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described in some detail, and equally advantageous embodiments have also been described for a stream based flow cytometric instrument, it will be apparent to those of ordinary skill in the art that many modifications and variations of the described
5 embodiment are possible in the light of the above teachings without departing from the principles and concepts of the disclosure as set forth in the claims.

Although an embodiment of the present disclosure of wavelength division multiplexing device for separating light
10 beam from an extended light source into multiple color bands has been described in some detail, and several other equally advantageous embodiments have also been described, it will be apparent to those ordinary skilled in the art that many modifications and variations of the described embodiments are
15 possible in the light of the above teachings without departing from the principles and concepts of the disclosure as set forth in the claims.

Although the present invention has been described in terms of the presently preferred embodiment, it is to be understood
20 that such disclosure is purely illustrative and is not to be interpreted as limiting. Consequently, without departing from the spirit and scope of the disclosure, various alterations, modifications, and/or alternative applications of the disclosure will, no doubt, be suggested to those skilled in the
25 art after having read the preceding disclosure. Accordingly, it is intended that the following claims be interpreted as encompassing all alterations, modifications, or alternative applications as fall within the true spirit and scope of the disclosure.

The Claims

What is claimed is:

1. A flow cytometer (40) comprising:
 - a) a laser diode ("LD") based optical subsystem (50) for directing a beam of light into a viewing zone of said flow cytometer (40) through which a sample liquid carrying particles flows, the sample liquid being hydrodynamically focused within the viewing zone by a liquid sheath flow that also flows through the viewing zone, the optical subsystem (50) including:
 - i. a LD for emitting a diverging beam of light from an edge thereof, the diverging beam of light having an elliptically shaped cross-sectional profile with both a major axis and a minor axis;
 - ii. a collimating lens for converting the diverging beam of light emitted from said LD into a collimated elliptical beam of light, wherein the minor axis of said collimated elliptical beam of light is oriented parallel to a direction in which particles pass through the viewing zone;
 - iii. a beam compressing optical element for reducing the size of said elliptical beam of light at the viewing zone whereby a width of said major axis of said elliptical beam of light oriented perpendicular to the direction in which particles pass through the viewing zone is less than a width of said liquid sheath flow; and
 - v. a cylindrical focusing element positioned adjacent to the viewing zone with an axis of said cylindrical focusing element being oriented perpendicular to the direction in which particles pass through the viewing zone whereby:

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- 35 1) said minor axis of said beam of light
 becomes focused at the viewing zone; and
- 2) the size of said major axis of said
 elliptical beam of light at the viewing
 zone remains essentially unchanged;
- 40 b) a composite microscope objective (60) for imaging
 light scattered from and fluoresced by a particle
 present within the viewing zone, the composite
 microscope objective (60) including:
 - 45 i. a concave mirror upon which scattered and
 fluoresced light impinges;
 - ii. an aberration corrector plate made of optically
 transparent material wherein said aberration
 corrector plate is an aspheric lens that has:
 - 50 1) a first zone of said aberration corrector
 plate outside a neutral zone thereof where
 said aberration corrector plate:
 - A. is thinnest; and
 - B. has negative optical power; and
 - 55 2) a second zone of said aberration corrector
 plate inside the neutral zone has positive
 optical power
 light reflected from the composite microscope
 objective (60) passing through said aberration
 corrector plate,
- 60 the viewing zone of said flow cytometer (40) being
 located between said concave mirror and said
 aberration corrector plate;
- c) a fluidic subsystem (70) for supplying the liquid
 sheath flow to the viewing zone, the liquid sheath
65 flow lacking pulsations, the fluidic subsystem (70)
 including:
 - 70 1. a liquid pump for supplying liquid drawn from
 a reservoir;
 - ii. a T-coupling having at least one (1) inlet and
 two (2) outlets,
 - 1) the inlet of said T-coupling receiving
 liquid from said liquid pump;

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- 75 2) a first fraction of the liquid received by
 the inlet flowing via a first one of the
 outlets and via a bypass conduit back to
 the reservoir; and
- 80 3) a second fraction of the liquid received
 by the inlet flowing via a second one of
 the outlets and via a particle filter to
 the viewing zone of said flow cytometer
 (40);
- 85 d) a peristaltic pump 80 for supplying the sample
 liquid carrying the particles, the sample liquid
 being hydrodynamically focused within the viewing
 zone by the liquid sheath flow, the peristaltic pump
 80 comprising:
- 90 i. a pump housing having a arcuate curved track
 formed therein that extends between a pump
 inlet and a pump outlet;
- 95 ii. a plurality of rollers that are attached to a
 rotor, the rollers having a substantially equal
 angular spacing between each pair of
 immediately adjacent rollers, the rotor being
 rotatable together with the rollers attached
 thereto inside said pump housing; and
- 100 ii a compressible tube sandwiched between said
 rollers and the arcuate curved track of said
 pump housing, the arcuate curved track
 including:
- 105 1) an exit section wherein, as a roller rolls
 through the exit section, said
 compressible tube adjacent to said roller
 progressively expands from fully closed at
 a beginning of said exit section to fully
 open at the pump outlet where said roller
 breaks contact with said compressible
 tube; and
- 110 2) at least one pumping section along the
 arcuate curved track between the pump
 inlet and the pump outlet wherein said

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compressible tube is compressed to fully closed by at least one of said rollers; and

- 115 e) a wavelength division multiplexer 90 ("WDM (90)") for separating into multiple colored bands a beam of light emitted initially from the viewing zone and imaged by the composite microscope objective (60) into an optical fiber for transmission to the WDM (90), the WDM (90) comprising:
- 120 i. a collimating optical element that magnifies an to produce an image of substantially the same size as the effective size of said collimating optical element;
- 125 ii. at least one dichroic filter located between said collimating optical element and said image, wherein said dichroic filter separates the collimated beam of light into two (2) branches of distinctive colors;
- 130 iii. a focusing optical element located in one of said branches, wherein the beam of light in said branch is focused to a spot having a diameter of less than 1.0 mm by said focusing optical element; and
- 135 iv. an image relay optical element located near the image produced by said collimating optical element in the other branch, wherein said image relay optical element produces an image of said collimating optical element at substantially unit magnification.

2. The flow cytometer (40) of claim 1 wherein said cuvette has a rectangularly-shaped , and wherein the viewing zone of the flow cytometer (40) is located within a channel having a rectangularly-shaped cross-section that is located within said cuvette.

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3. The flow cytometer (40) of claim 1 wherein said cuvette has a tubularly-shaped cross-section, and wherein the viewing zone of the flow cytometer (40) is located within a channel having a circularly-shaped cross-section that is located within said cuvette.

4. The flow cytometer (40) of claim 1 wherein the sample liquid and the liquid sheath flow form a jet stream in which the viewing zone of the flow cytometer (40) is located.

5. The flow cytometer (40) of claim 2 wherein said cylindrical focusing element is in optical contact with an entrance face of said rectangularly-shaped cuvette.

6. The flow cytometer (40) of claim 2 wherein said cylindrical focusing element is separated from said rectangularly-shaped cuvette.

7. The flow cytometer (40) of claim 3 wherein said cylindrical focusing element is separated from said tubularly-shaped cuvette.

8. The flow cytometer (40) of claim 4 wherein said cylindrical focusing element is separated from said jet stream.

9. The flow cytometer (40) of claim 1 further comprising a polarization conditioning element through which said collimated elliptical beam of light passes.

10. The flow cytometer (40) of claim 1 wherein an optical image of the viewing zone is formed outside the composite microscope objective (60).

11. The flow cytometer (40) of claim 10 wherein the viewing zone is located within a flow channel included in a rectangularly-shaped cuvette made of optically transparent material.

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12. The flow cytometer (40) of claim 11 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

13. The flow cytometer (40) of claim 12 wherein the piano-surface of said piano-concave back surface mirror is optically coupled to a flat surface of said cuvette.

14. The flow cytometer (40) of claim 13 wherein an optical adhesive material accomplishes the optical coupling.

15. The flow cytometer (40) of claim 13 wherein an index matching gel accomplishes the optical coupling.

16. The flow cytometer (40) of claim 13 wherein an index matching fluid accomplishes the optical coupling.

17. The flow cytometer (40) of claim 13 wherein optical contact bonding accomplishes the optical coupling.

18. The flow cytometer (40) of claim 13 wherein the piano-concave back surface mirror formed integrally with said cuvette means.

19. The flow cytometer (40) of claim 11 wherein said aberration corrector plate is a piano-aspherical lens.

20. The flow cytometer (40) of claim 19 wherein a piano-surface of said aberration corrector plate is optically coupled to a flat surface of said cuvette opposite of said piano-concave back surface mirror.

21. The flow cytometer (40) of claim 20 wherein an index matching gel accomplishes the optical coupling.

22. The flow cytometer (40) of claim 20 wherein an index matching fluid accomplishes the optical coupling.

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23. The flow cytometer (40) of claim 20 wherein optical contact bonding accomplishes the optical coupling.

24. The flow cytometer (40) of claim 20 wherein the piano-aspherical lens is formed integrally with said cuvette.

25. The flow cytometer (40) of claim 19 wherein said aberration corrector plate is detached from said cuvette.

26. The flow cytometer (40) of claim 10 wherein the viewing zone is inside a jet stream.

27. The flow cytometer (40) of claim 25 wherein said concave mirror is a front surface mirror.

28. The flow cytometer (40) of claim 10 wherein the viewing zone is located on a surface of a flat, transparent substrate.

29. The flow cytometer (40) of claim 28 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

30. The flow cytometer (40) of claim 29 wherein the piano-surface of said piano-concave back surface mirror is optically coupled to said flat, transparent substrate.

31. The flow cytometer (40) of claim 30 wherein an optical adhesive material accomplishes the optical coupling.

32. The flow cytometer (40) of claim 30 wherein an index matching gel accomplishes the optical coupling.

33. The flow cytometer (40) of claim 30 wherein an index matching fluid accomplishes the optical coupling.

34. The flow cytometer (40) of claim 30 wherein optical contact bonding accomplishes the optical coupling.

35. The flow cytometer (40) of claim 29 wherein said piano-concave back surface mirror is formed integrally with said flat, transparent substrate.

36. The flow cytometer (40) of claim 28 wherein said aberration corrector plate is detached from said flat, transparent substrate.

37. The flow cytometer (40) of claim 1 wherein the particle filter has inlet thereto for receiving liquid from the T-coupling, the inlet of the particle filter being disposed so that air becomes trapped within the particle filter at the inlet thereto.

38. The flow cytometer (40) of claim 37 wherein when said liquid pump is turned off air cannot enter into the bypass conduit.

39. The flow cytometer (40) of claim 38 further comprising a small capsule disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

40. The flow cytometer (40) of claim 38 further comprising a length of tubing disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

41. The flow cytometer (40) of claim 38 further comprising an adjustable valve located in the bypass conduit between the first one of the outlets of the T-coupling and the reservoir for restricting liquid flow therebetween.

42. The flow cytometer (40) of claim 38 further comprising an adjustable valve located between the second one

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of the outlets of the T-coupling and the viewing zone for restricting liquid flow therebetween.

43. The flow cytometer (40) of claim 38 wherein the throughput of the liquid pump is adjustable.

44. The flow cytometer (40) of claim 1 wherein the arcuate curved track of said pump housing includes at least two (2) pumping sections, the arcuate curved track further including at least one recess section located between said
5 pumping sections along the arcuate curved track, and wherein said compressible tube at said recess section becomes decompressed to full expansion then compressed to fully closed when one (1) of said rollers rolls through said recess section.

45. The flow cytometer (40) of claim 44 includes a plurality of recess sections along said arcuate curved track upstream of the pump outlet, the angular spacing between the compression part of said recess section adjacent to the pump
5 outlet and said exit section of said arcuate curved track being substantially the same as the angular spacing between each pair of immediately adjacent rollers.

46. The flow cytometer (40) of claim 44 wherein said compression part of said recess section adjacent to the pump outlet has a shape complementing a shape of said exit section of said arcuate curved track to maintain the total fluid volume
5 inside a section of said compressible tube extending from said recess section to the pump outlet substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

47. The flow cytometer (40) of claim 44 having a plurality of recess sections respectively interspersed between immediately adjacent pairs of a plurality of pumping sections.

48. The flow cytometer (40) of claim 46 wherein both:

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- a) angular spacing between adjacent pairs of recess sections; and
 - b) angular spacing between said exit section of said arcuate curved track and an adjacent recess section to said exit section
- are substantially the same as the angular spacing between each pair of immediately adjacent rollers.

49. The flow cytometer (40) of claim 47 wherein shapes of a plurality of recess sections of said arcuate curved track complement a shape of said exit section of said arcuate curved track to maintain a fluid volume in sections of said compressible tube at the plurality of recess sections and said exit section substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

50. The flow cytometer (40) of claim 1 wherein a speed of said rotor is programmably controlled to vary substantially in inverse proportion to the fluid volume change rate in said compressible tube due to its changing compression near the exit section of said arcuate curved track.

51. The flow cytometer (40) of claim 1 wherein at least one additional dichroic filter is located between said image relay optical element and the image produced by said image relay optical element, wherein said dichroic filter produces two (2) branches of the beam of light having distinctive colors.

52. The flow cytometer (40) of claim 51 wherein another focusing optical element is located in one of said branches and focuses the beam of light in the branch into a spot having a diameter of less than 1.0 mm.

53. The flow cytometer (40) of claim 52 wherein successive combinations of said image relay optical element, dichroic filter and focusing optical element are cascaded to

produce additional focused spots having a diameter of less than 1.0 mm for multiple colored bands of said beam of light.

54. The flow cytometer (40) of claim 52 wherein the dichroic filter is assembled using a template that include two (2) optically flat glass plates bonded together in optical contact, and wherein the dichroic filter is bonded to a filter holder using the template such that a coated filter surface of the dichroic filter is indented and optically parallel to a reference surface of the filter holder.

55. The flow cytometer (40) of claim 54 wherein the reference surface of the filter holder rests against an optically flat surface of an reference block included in the WDM (90) thereby providing consistent optical alignment when installing the dichroic filter into the WDM (90).

56. A LD based optical subsystem (50) for impinging a beam of light into a viewing zone in which a particle is present, the optical subsystem (50) comprising:

- a) a LD for emitting a diverging beam of light from an edge thereof, the diverging beam of light having an elliptically shaped cross-sectional profile with both a major axis and a minor axis;
- b) a collimating lens for converting the diverging beam of light emitted from said LD into a collimated elliptical beam of light, wherein the minor axis of said collimated elliptical beam of light is oriented parallel to a direction in which particles pass through the viewing zone;
- c) a beam compressing optical element for reducing the size of said elliptical beam of light at the viewing zone whereby a width of said major axis of said elliptical beam of light oriented perpendicular to the direction in which particles pass through the viewing zone is less than a width of said liquid sheath flow; and

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- 25 d) a cylindrical focusing element positioned adjacent to the viewing zone with an axis of said cylindrical focusing element being oriented perpendicular to the direction in which particles pass through the viewing zone whereby:
- i. said minor axis of said beam of light becomes focused at the viewing zone; and
 - ii. the size of said major axis of said elliptical beam of light at the viewing zone remains essentially unchanged.

57. The optical subsystem (50) of claim 56 further comprising a cuvette having a rectangularly-shaped cross-section, and wherein the viewing zone is located within a channel having a rectangularly-shaped cross-section that is located within said cuvette.

58. The optical subsystem (50) of claim 56 further comprising a cuvette having a tubularly-shaped cross-section, and wherein the viewing zone is located within a channel having a circularly-shaped cross-section that is located within said cuvette.

59. The optical subsystem (50) of claim 56 wherein the sample liquid and the liquid sheath flow form a jet stream in which the viewing zone is located.

60. The optical subsystem (50) of claim 57 wherein said cylindrical focusing element is in optical contact with an entrance face of said rectangularly-shaped cuvette.

61. The optical subsystem (50) of claim 57 wherein said cylindrical focusing element is separated from said rectangularly-shaped cuvette.

62. The optical subsystem (50) of claim 58 wherein said cylindrical focusing element is separated from said tubularly-shaped cuvette.

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63. The optical subsystem (50) of claim 59 wherein said cylindrical focusing element is separated from said jet stream.

64. The optical subsystem (50) of claim 56 further comprising a polarization conditioning element through which said collimated elliptical beam of light passes.

65. A method for delivering an elliptically shaped beam of light using a LD based optical subsystem (50), the beam of light having a smooth profile at a focus of a minor axis thereof that is located at a viewing zone through which a sample liquid flows, the sample liquid being hydrodynamically focused within the viewing zone by a liquid sheath flow that also flows through the viewing zone, the method comprising the steps of:

- 10 a) providing a LD that emits a diverging beam of light from an edge thereof, the diverging beam of light having an elliptically shaped cross-sectional profile with both a major axis and a minor axis;
- 15 b) impinging the diverging beam of light emitted by the LD upon a collimating lens for converting the diverging beam of light emitted therefrom into a collimated elliptical beam of light wherein the minor axis of said collimated elliptical beam of light is oriented parallel to a direction in which sample liquid passes through the viewing zone;
- 20 c) after passing through said collimating lens, impinging the collimated elliptical beam of light upon an beam compressing optical element for reducing the size of said elliptical beam of light at the viewing zone whereby a width of said major axis of said elliptical beam of light oriented perpendicular to the direction in which sample liquid passes through the viewing zone becomes less than a width of said liquid sheath flow; and
- 25 d) after passing through said beam compressing optical element, impinging the beam of light upon a
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cylindrical focusing element positioned adjacent to the viewing zone with an axis of said cylindrical focusing element being oriented perpendicular to the direction in which sample liquid passes through the viewing zone whereby:

- i. said minor axis of said beam of light becomes focused at the viewing zone; and
- ii. the size of said major axis of said elliptical beam of light at the viewing zone remains essentially unchanged.

66. The method of claim 65 wherein the viewing zone is located within a channel having a rectangularly-shaped cross-section that is located within a cuvette.

67. The method of claim 65 wherein the viewing zone is located within a channel having a circularly-shaped cross-section that is located within a cuvette.

68. The method of claim 65 wherein the viewing zone is located within a jet stream.

69. The method of claim 66 further comprising a step of establishing an optical contact between said cylindrical focusing element and an entrance face of said cuvette.

70. The method of claim 66 further comprising a step of establishing a spacing between said cylindrical focusing element and said cuvette.

71. The method of claim 67 further comprising a step of establishing a spacing between said cylindrical focusing element and said cuvette.

72. The method of claim 68 further comprising a step of establishing a spacing between said cylindrical focusing element and said jet stream.

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73. The method of claim 65 further comprising a step of inserting a polarization conditioning element between the collimating lens and the beam compressing optical element whereby the collimated elliptical beam of light passes through
5 the polarization conditioning element.

74. A composite microscope objective (60) adapted for imaging light scattered from and fluoresced by a particle present within a viewing zone comprising:

- 5 a) a concave mirror upon which scattered and fluoresced light impinges;
- b) an aberration corrector plate made of optically transparent material wherein said aberration corrector plate is an aspheric lens that has:
 - 10 i. a first zone of said aberration corrector plate outside a neutral zone thereof where said aberration corrector plate:
 - 1) is thinnest; and
 - 2) has negative optical power; and
 - 15 ii. a second zone of said aberration corrector plate inside the neutral zone has positive optical power,
light reflected from the composite microscope objective (60) passing through said aberration corrector plate,
 - 20 the viewing zone being located between said concave mirror and said aberration corrector plate.

75. The composite microscope objective (60) of claim 74 wherein an optical image of the viewing zone is formed outside the composite microscope objective (60).

76. The composite microscope objective (60) of claim 75 wherein the viewing zone is located within a flow channel included in a rectangularly-shaped cuvette made of optically transparent material.

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77. The composite microscope objective (60) of claim 76 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

78. The composite microscope objective (60) of claim 77 wherein a piano-surface of said piano-concave back surface mirror is optically coupled to a flat surface of said cuvette.

79. The composite microscope objective (60) of claim 78 wherein an optical adhesive material accomplishes the optical coupling.

80. The composite microscope objective (60) of claim 78 wherein an index matching gel accomplishes the optical coupling.

81. The composite microscope objective (60) of claim 78 wherein an index matching fluid accomplishes the optical coupling.

82. The composite microscope objective (60) of claim 78 wherein optical contact bonding accomplishes the optical coupling.

83. The composite microscope objective (60) of claim 78 wherein the piano-concave back surface mirror formed integrally with said cuvette means.

84. The composite microscope objective (60) of claim 76 wherein said aberration corrector plate is a piano-aspherical lens.

85. The composite microscope objective (60) of claim 84 wherein a piano-surface of said aberration corrector plate is optically coupled to a flat surface of said cuvette opposite of said piano-concave back surface mirror.

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86. The composite microscope objective (60) of claim 85 wherein an index matching gel accomplishes the optical coupling.

87. The composite microscope objective (60) of claim 85 wherein an index matching fluid accomplishes the optical coupling.

88. The composite microscope objective (60) of claim 85 wherein optical contact bonding accomplishes the optical coupling.

89. The composite microscope objective (60) of claim 85 wherein the piano-aspherical lens is formed integrally with said cuvette.

90. The composite microscope objective (60) of claim 84 wherein said aberration corrector plate is detached from said cuvette.

91. The composite microscope objective (60) of claim 75 wherein the viewing zone is inside a jet stream.

92. The composite microscope objective (60) of claim 90 wherein said concave mirror is a front surface mirror.

93. The composite microscope objective (60) of claim 75 wherein the viewing zone is located on a surface of a flat, transparent substrate.

94. The composite microscope objective (60) of claim 93 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

95. The composite microscope objective (60) of claim 94 wherein a piano-surface of said piano-concave back surface mirror is optically coupled to said flat, transparent substrate.

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96. The composite microscope objective (60) of claim 95 wherein an optical adhesive material accomplishes the optical coupling.

97. The composite microscope objective (60) of claim 95 wherein an index matching gel accomplishes the optical coupling.

98. The composite microscope objective (60) of claim 95 wherein an index matching fluid accomplishes the optical coupling.

99. The composite microscope objective (60) of claim 95 wherein optical contact bonding accomplishes the optical coupling.

100. The composite microscope objective (60) of claim 94 wherein said piano-concave back surface mirror is formed integrally with said flat, transparent substrate.

101. The composite microscope objective (60) of claim 93 wherein said aberration corrector plate is detached from said flat, transparent substrate.

102. A method for characterizing microscopic species using a microscope objective device comprising:

- a) A concave mirror;
- 5 b) An aberration corrector plate made of optically transparent material wherein said aberration corrector plate is an aspheric lens with the zone of said plate means outside of the neutral zone wherein the thickness of said plate means is thinnest, having negative optical power, and the zone inside
10 the neutral zone having positive optical power
- c) A viewing zone located in between said concave mirror and said aberration corrector plate.

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103. The method of claim 102 wherein an optical image of the viewing zone is formed outside the device.

104. The method of claim 103 wherein the viewing zone is located within a flow channel contained in a rectangularly-shaped cuvette means made of optically transparent material.

105. The method of claim 104 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

106. The method of claim 105 wherein a piano-surface of said piano-concave back surface mirror means is optically coupled to a flat surface of said cuvette means.

107. The method of claim 106 wherein an optical adhesive material accomplishes the optical coupling.

108. The method of claim 106 wherein an index matching gel accomplishes the optical coupling.

109. The method of claim 106 wherein an index matching fluid accomplishes the optical coupling.

110. The method of claim 106 wherein optical contact bonding accomplishes the optical coupling.

111. The method of claim 106 wherein the piano-concave back surface mirror is formed integrally with said cuvette.

112. The method of claim 104 wherein said aberration corrector plate is a plano-aspherical lens.

113. The method of claim 112 wherein a piano-surface of said aberration corrector plate is optically coupled to a flat surface of said cuvette means opposite of said concave mirror.

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114. The method of claim 113 wherein an index matching gel accomplishes the optical coupling.

115. The method of claim 113 wherein an index matching fluid accomplishes the optical coupling.

116. The method of claim 113 wherein optical contact bonding accomplishes the optical coupling.

117. The method of claim 113 wherein the piano-aspherical lens is formed integrally with said cuvette.

118. The method of claim 112 wherein said aberration corrector plate is detached from said cuvette.

119. The method of claim 102 wherein the viewing zone is inside a jet stream.

120. The method of claim 119 wherein said concave mirror is a front surface mirror.

121. The method of claim 102 wherein the viewing zone is located on a surface of a flat, transparent substrate.

122. The method of claim 121 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

123. The method of claim 122 wherein a piano-surface of said piano-concave back surface mirror means is optically coupled to said flat, transparent substrate.

124. The method of claim 123 wherein an optical adhesive material accomplishes the optical coupling.

125. The method of claim 123 wherein an index matching gel accomplishes the optical coupling.

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126. The method of claim 123 wherein an index matching fluid accomplishes the optical coupling.

127. The method of claim 123 wherein optical contact bonding accomplishes the optical coupling.

128. The method of claim 122 wherein said piano-concave back surface mirror is formed integrally with said flat, transparent substrate.

129. The method of claim 128 wherein said aberration corrector plate is detached from said flat, transparent substrate.

130. A fluidic subsystem (70) for supplying a liquid flow pulsation free to an outlet of the fluidic subsystem (70), the fluidic subsystem (70) comprising:

- 5 a) a liquid pump for supplying liquid drawn from a reservoir;
- b) a T-coupling having at least one (1) inlet and two (2) outlets,
 - 10 i. the inlet of said T-coupling receiving liquid from said liquid pump;
 - ii. a first fraction of the liquid received by the inlet flowing via a first one of the outlets and via a bypass conduit back to the reservoir; and
 - 15 iii. a second fraction of the liquid received by the inlet flowing via a second one of the outlets and via a particle filter to the outlet of the fluidic subsystem (70).

131. The fluidic subsystem (70) of claim 130 wherein the particle filter has inlet thereto for receiving liquid from the T-coupling, the inlet of the particle filter being disposed so that air becomes trapped within the particle filter at the inlet thereto.

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132. The fluidic subsystem (70) of claim 131 wherein when said liquid pump is turned off air cannot enter into the bypass conduit.

133. The fluidic subsystem (70) of claim 132 further comprising a small capsule disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

134. The fluidic subsystem (70) of claim 132 further comprising a length of tubing disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

135. The fluidic subsystem (70) of claim 132 further comprising an adjustable valve located in the bypass conduit between the first one of the outlets of the T-coupling and the reservoir for restricting liquid flow therebetween.

136. The fluidic subsystem (70) of claim 132 further comprising an adjustable valve located between the second one of the outlets of the T-coupling and the outlet of the fluidic subsystem (70) for restricting liquid flow therebetween.

137. The fluidic subsystem (70) of claim 132 wherein the throughput of the liquid pump is adjustable.

138. A method for supplying a liquid flow pulsation free to an outlet of the fluidic subsystem (70), the method comprising:

- 5 a) a liquid pump for supplying liquid drawn from a reservoir;
- b) a T-coupling having at least one (1) inlet and two (2) outlets,
 - i. the inlet of said T-coupling receiving liquid from said liquid pump;

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- 10 ii. a first fraction of the liquid received by the
 inlet flowing via a first one of the outlets
 and via a bypass conduit back to the reservoir;
 and
 iii. a second fraction of the liquid received by the
15 inlet flowing via a second one of the outlets
 and via a particle filter to the outlet of the
 fluidic subsystem (70).

139. The method of claim 138 wherein during normal operation certain amount of air is trapped near the inlet portion of said filter cartridge means.

140. The method of claim 139 wherein said reservoir means holds sufficient amount of liquid such that when said pump means is turned off, portion of the tubing between said Tee means and said reservoir means is still filled with liquid,
5 preventing said trapped air from leaking into said bypass means.

141. The method of claim 140 wherein said reservoir means is a capsule.

142. The method of claim 140 wherein said reservoir means is a piece of tubing.

143. The method of claim 140 wherein an adjustable flow restrictor means is placed in the bypass route.

144. The method of claim 140 wherein an adjustable flow restrictor means is placed in the sheath route.

145. The method of claim 140 wherein the throughput of the sheath pump is adjustable.

146. A peristaltic pump 80 comprising:

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- a) a pump housing having a arcuate curved track formed therein that extends between a pump inlet and a pump outlet;
- 5 b) a plurality of rollers that are attached to a rotor, the rollers having a substantially equal angular spacing between each pair of immediately adjacent rollers, the rotor being rotatable together with the rollers attached thereto inside said pump housing;
- 10 and
- c) a compressible tube sandwiched between said rollers and the arcuate curved track of said pump housing, the arcuate curved track including:
 - 15 i. an exit section wherein, as a roller rolls through the exit section, said compressible tube adjacent to said roller progressively expands from fully closed at a beginning of said exit section to fully open at the pump outlet where said roller breaks contact with
 - 20 said compressible tube; and
 - ii. at least one pumping section along the arcuate curved track between the pump inlet and the pump outlet wherein said compressible tube is compressed to fully closed by at least one of said rollers.

147. The peristaltic pump 80 of claim 146 wherein the arcuate curved track of said pump housing includes at least two (2) pumping sections, the arcuate curved track further including at least one recess section located between said
5 pumping sections along the arcuate curved track, and wherein said compressible tube at said recess section becomes decompressed to full expansion then compressed to fully closed when one (1) of said rollers rolls through said recess section.

148. The peristaltic pump 80 of claim 147 includes a plurality of recess sections along said arcuate curved track upstream of the pump outlet, the angular spacing between the compression part of said recess section adjacent to the pump

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- 5 outlet and said exit section of said arcuate curved track being substantially the same as the angular spacing between each pair of immediately adjacent rollers.

149. The peristaltic pump 80 of claim 147 wherein said compression part of said recess section adjacent to the pump outlet has a shape complementing a shape of said exit section of said arcuate curved track to maintain the total fluid volume
5 inside a section of said compressible tube extending from said recess section to the pump outlet substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

150. The peristaltic pump 80 of claim 147 having a plurality of recess sections respectively interspersed between immediately adjacent pairs of a plurality of pumping sections.

151. The peristaltic pump 80 of claim 149 wherein both:
a) angular spacing between adjacent pairs of recess sections; and
b) angular spacing between said exit section of said
5 arcuate curved track and an adjacent recess section to said exit section
are substantially the same as the angular spacing between each pair of immediately adjacent rollers.

152. The peristaltic pump 80 of claim 150 wherein shapes of a plurality of recess sections of said arcuate curved track complement a shape of said exit section of said arcuate curved track to maintain a fluid volume in sections of said
5 compressible tube at the plurality of recess sections and said exit section substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

153. The peristaltic pump 80 of claim 146 wherein a speed of said rotor is programmably controlled to vary substantially in inverse proportion to the fluid volume change rate in said

compressible tube due to its changing compression near the exit section of said arcuate curved track.

154. A method for delivering liquid using a peristaltic pump 80 comprising:

- a) a pump housing with arcuate curved track;
- b) A plurality of rollers attached to a rotor means rotatable inside said pump housing;
- c) Said plurality of rollers spaced at substantially equal angular spacing from each other;
- d) A compressible tube sandwiched between said rollers and the arcuate curved track of said pump housing;
- e) Said arcuate curved track of said pump housing including an exit section, wherein said compressible tube being progressively decompressed to full expansion when one of said rollers rolls off the compressible tube at the pump outlet;
- f) At least one pumping section along the arcuate curved track of said pump housing between the pump inlet and pump outlet wherein said compressible tube is compressed to full closure by one of said rollers.

155. The method of claim 154 wherein the arcuate curved track of said pump housing includes at least two (2) pumping sections, the arcuate curved track further including at least one recess section located between said pumping sections along the arcuate curved track, and wherein said compressible tube at said recess section becomes decompressed to full expansion then compressed to fully closed when one (1) of said rollers rolls through said recess section.

156. The method of claim 155 wherein the peristaltic pump 80 includes a plurality of recess sections along said arcuate curved track upstream of the pump outlet; The angular spacing between the compression part of said recess section adjacent to the pump outlet and said exit section of said arcuate curved

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track being substantially the same as the angular spacing between each pair of immediately adjacent rollers.

157. The method of claim 155 wherein said compression part of said recess section adjacent to the pump outlet has a shape complementing a shape of said exit section of said arcuate curved track to maintain the total fluid volume inside a section of said compressible tube extending from said recess
5 section to the pump outlet substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

158. The method of claim 155 having a plurality of recess sections respectively interspersed between immediately adjacent pairs of a plurality of pumping sections.

159. The method of claim 157 wherein both:
a) angular spacing between adjacent pairs of recess sections; and
b) angular spacing between said exit section of said
5 arcuate curved track and an adjacent recess section to said exit section
are substantially the same as the angular spacing between each pair of immediately adjacent rollers.

160. The method of claim 158 wherein shapes of a plurality of recess sections of said arcuate curved track complement a shape of said exit section of said arcuate curved track to maintain a fluid volume in sections of said compressible tube
5 at the plurality of recess sections and said exit section substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

161. The method of claim 154 wherein a speed of said rotor of the peristaltic pump 80 is programmably controlled to vary substantially in inverse proportion to the fluid volume change rate in said compressible tube due to its changing compression near the exit section of said arcuate curved track.

162. A WDM (90) for separating a beam of light emitted from an into multiple colored bands, the WDM (90) comprising:

- 5 a) a collimating optical element that magnifies an to produce an image of substantially the same size as the effective size of said collimating optical element;
- 10 b) at least one dichroic filter located between said collimating optical element and said image, wherein said dichroic filter separates the collimated beam of light into two (2) branches of distinctive colors;
- 15 c) a focusing optical element located in one of said branches, wherein the beam of light in said branch is focused to a spot having a diameter of less than 1.0 mm by said focusing optical element; and
- 20 d) an image relay optical element located near the image produced by said collimating optical element in the other branch, wherein said image relay optical element produces an image of said collimating optical element at substantially unit magnification.

163. The WDM (90) of claim 162 wherein at least one additional dichroic filter is located between said image relay optical element and the image produced by said image relay optical element, wherein said dichroic filter produces two (2) branches of the beam of light having distinctive colors.

164. The WDM (90) of claim 163 wherein another focusing optical element is located in one of said branches and focuses the beam of light in the branch into a spot having a diameter of less than 1.0 mm.

165. The WDM (90) of claim 164 wherein successive combinations of said image relay optical element, dichroic filter and focusing optical element are cascaded to produce

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additional focused spots having a diameter of less than 1.0 mm for multiple colored bands of said beam of light.

166. The WDM (90) of claim 164 wherein the dichroic filter is assembled using a template that include two (2) optically flat glass plates bonded together in optical contact, and wherein the dichroic filter is bonded to a filter holder using
5 the template such that a coated filter surface of the dichroic filter is indented and optically parallel to a reference surface of the filter holder.

167. The WDM (90) of claim 166 wherein the reference surface of the filter holder rests against an optically flat surface of an reference block included in the WDM (90) thereby providing consistent optical alignment when installing the dichroic filter into the WDM (90).

168. A method for separating beam of light emitted from an into colored bands using a WDM (90) comprising

- a) a collimating optical element that magnifies an to produce an image of substantially the same size as
5 the effective size of said collimating optical element;
- b) at least one dichroic filter located between said collimating optical element and said image, wherein said dichroic filter separates the collimated beam
10 of light into two (2) branches of distinctive colors;
- c) a focusing optical element located in one of said branches, wherein the beam of light of said branch is focused to a spot having a diameter of less than
15 1.0 mm by said focusing optical element; and
- d) an image relay optical element located near the image produced by said collimating optical element in the other branch, wherein said image relay optical element produces an image of said
20 collimating optical element at substantially unit magnification.

169. The method of claim 168 wherein at least one additional dichroic filter is located between said image relay optical element and the image produced by said image relay optical element, wherein said dichroic filter produces two (2) branches of beam of light having distinctive colors.

170. The method of claim 169 wherein another focusing optical element is located in one of said branches and focuses the beam of light in the branch into a spot having a diameter of less than 1.0 mm.

171. The method of claim 170 wherein successive combinations of said image relay optical element, dichroic filter and focusing optical element are cascaded to produce additional focused spots having a diameter of less than 1.0 mm for multiple colored bands of said beam of light.

172. The method of claim 170 wherein the dichroic filter is assembled using a template that include two (2) optically flat glass plates bonded together in optical contact, and wherein the dichroic filter is bonded to a filter holder using
5 the template such that a coated filter surface of the dichroic filter is indented and optically parallel to a reference surface of the filter holder.

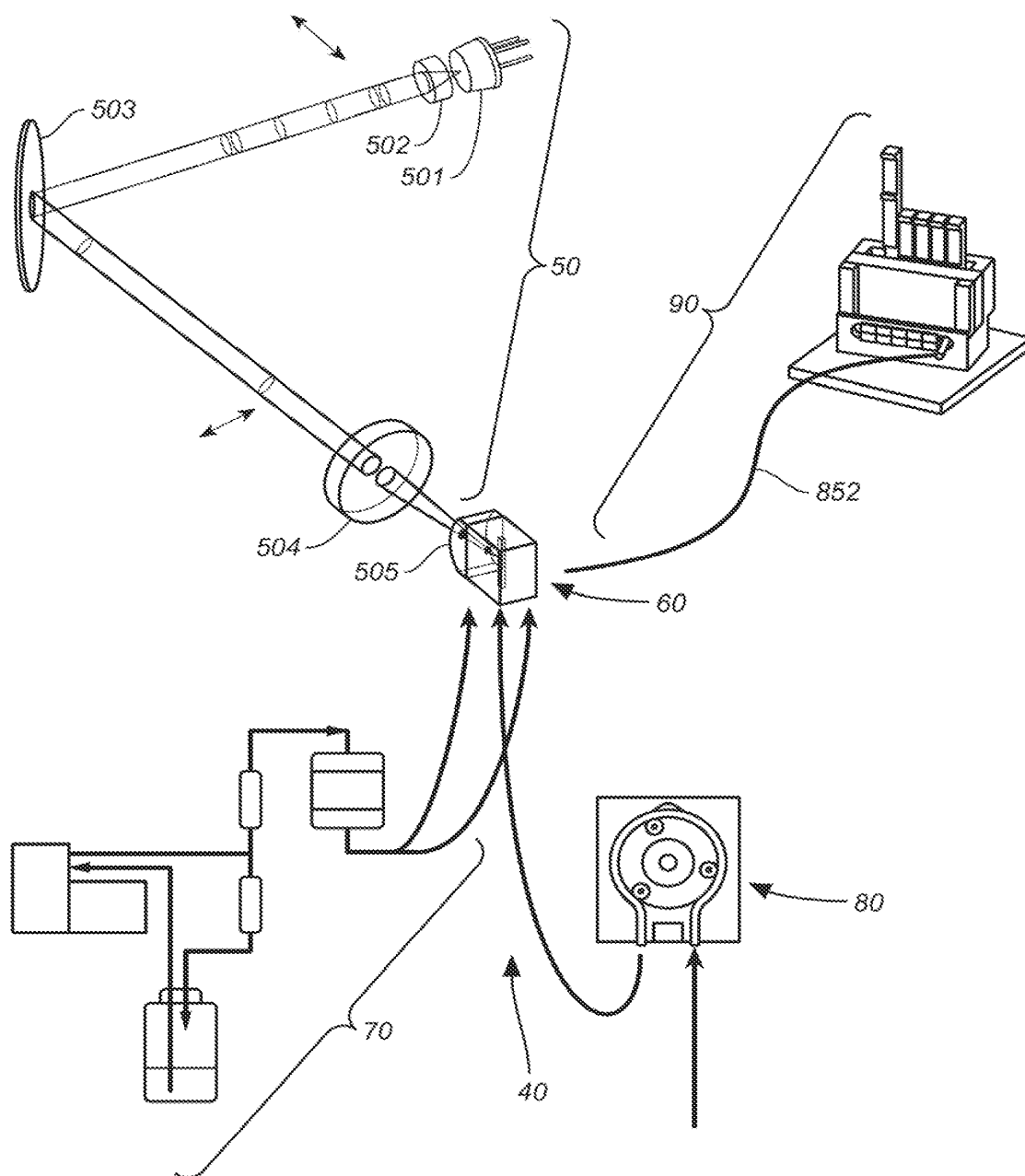
173. The method of claim 172 wherein the reference surface of the filter holder rests against an optically flat surface of an reference block included in the WDM (90) thereby providing consistent optical alignment when installing the dichroic filter into the WDM (90).

ABSTRACT

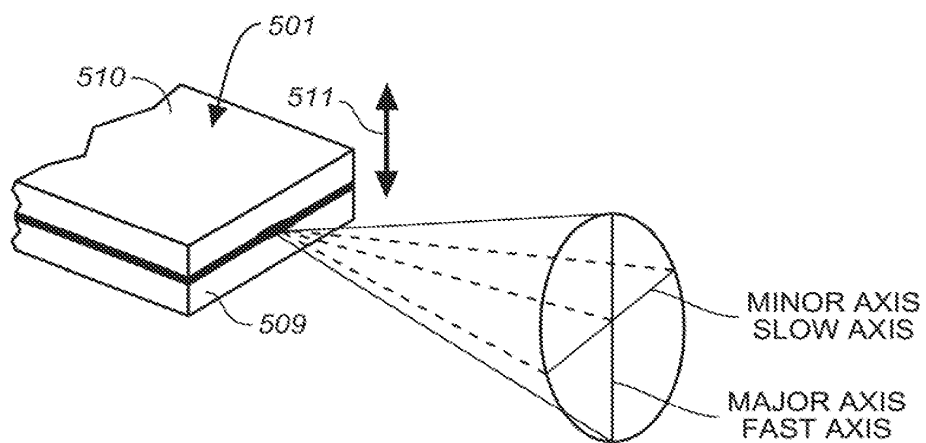
The disclosed flow cytometer (40) includes:

1. a laser diode ("LD") based optical subsystem (50) for impinging a beam of light upon particles passing through a viewing zone;
2. a composite microscope objective (60) for gathering and imaging light scattered from or fluoresced by particles passing through the viewing zone;
3. a fluidic subsystem (70) for supplying a liquid sheath flow to the viewing zone;
4. a peristaltic pump (80) for injecting into the liquid sheath flow a liquid sample flow carrying particles that passes together with the liquid sheath flow through the viewing zone;
5. a multimode optical fiber (852) that receives scattered and fluoresced light from the viewing zone that the composite microscope objective (60) gathers and images; and
6. a wavelength division multiplexer (90) for optically separating into color bands light received via the optical fiber (852).

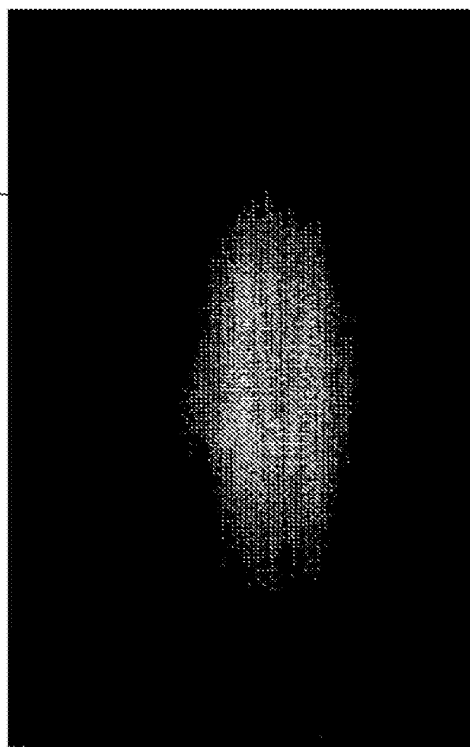
1 / 23

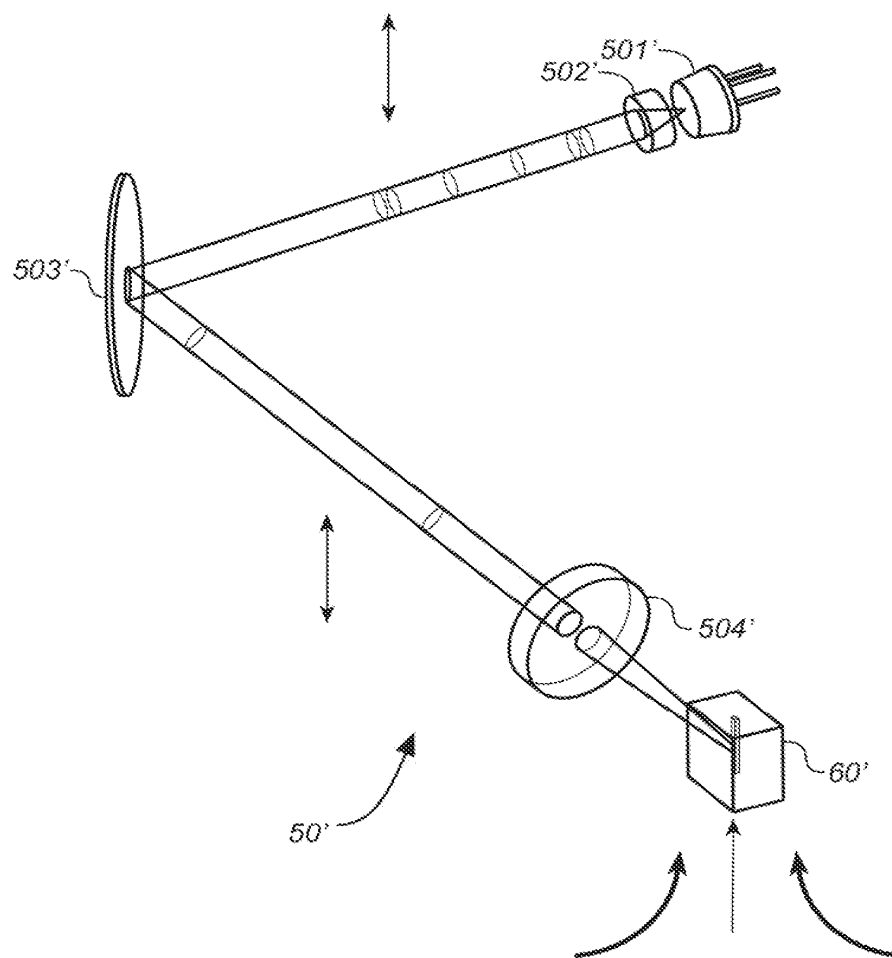
**FIG. 1**

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**FIG. 2**

512

FIG. 2A

**FIG. 3A**

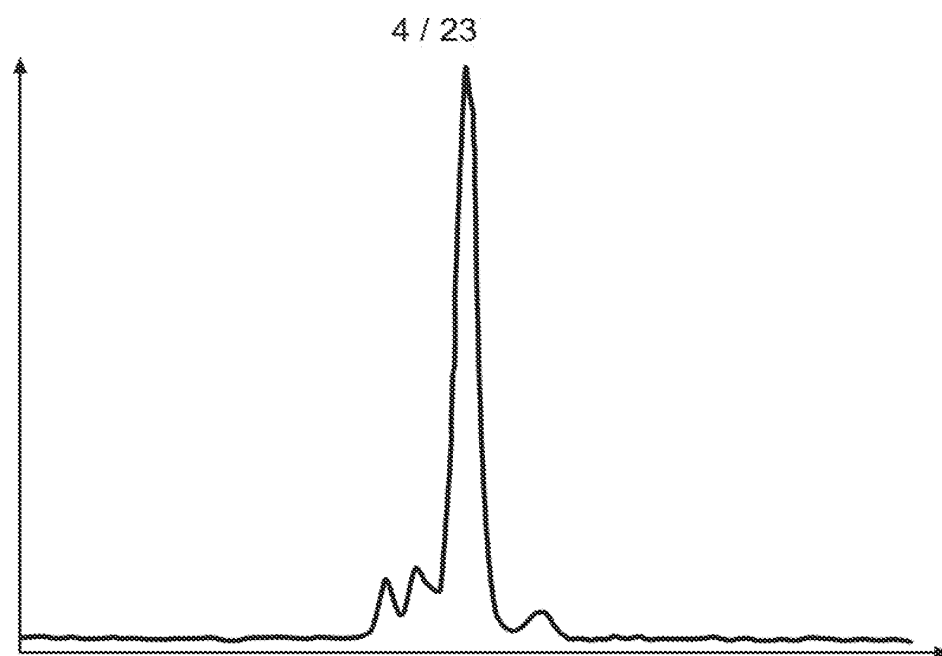


FIG. 3B

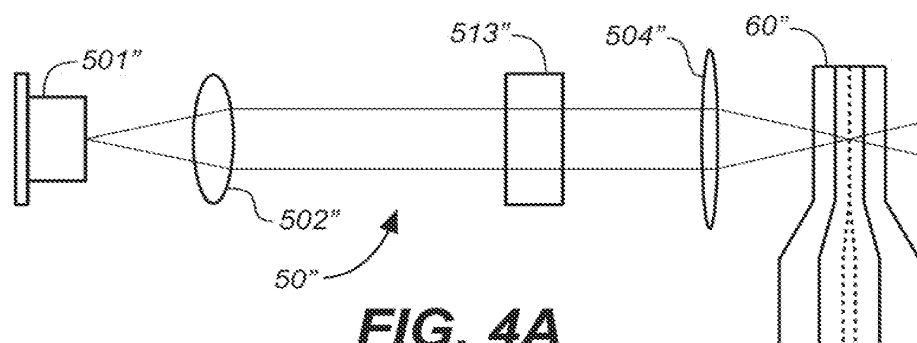


FIG. 4A

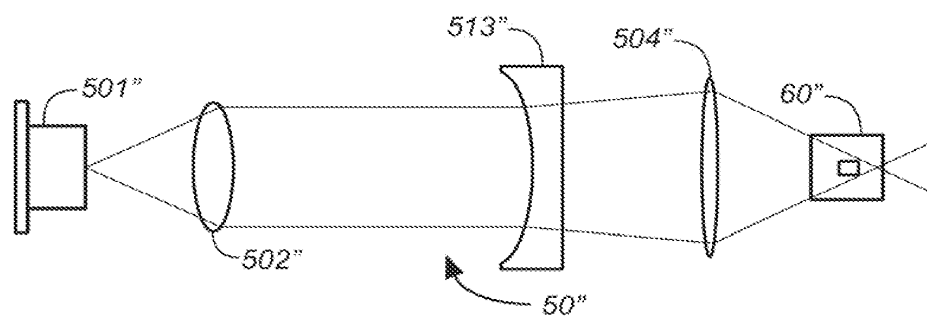
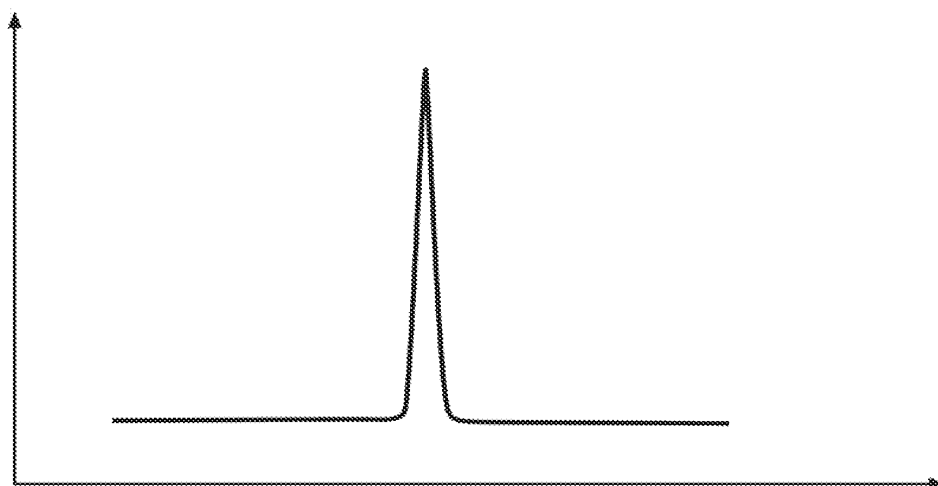
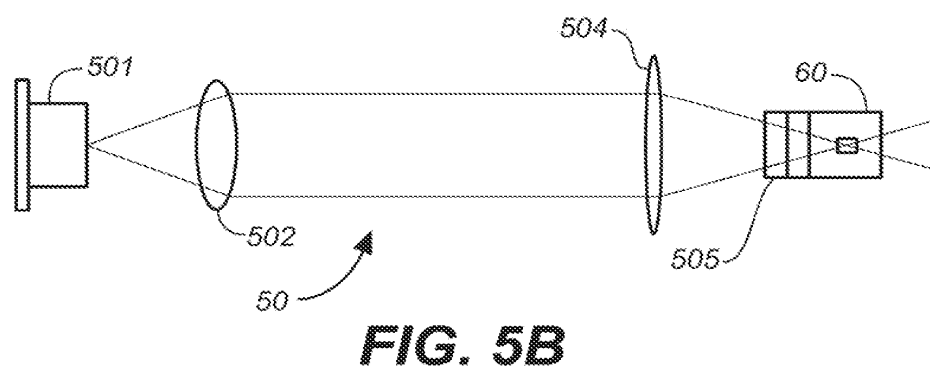
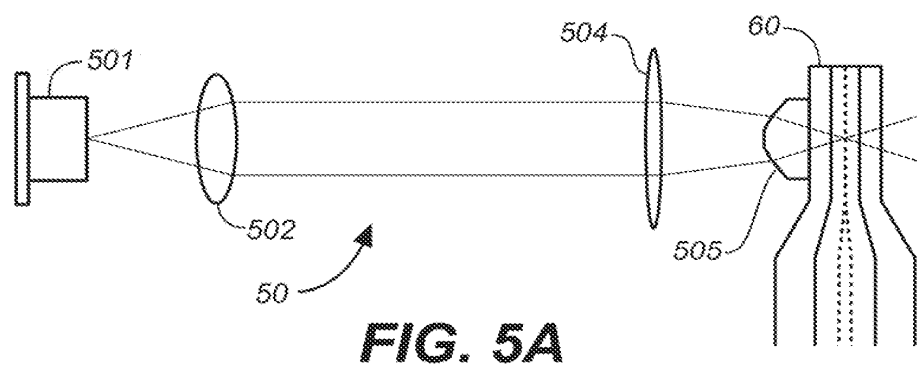
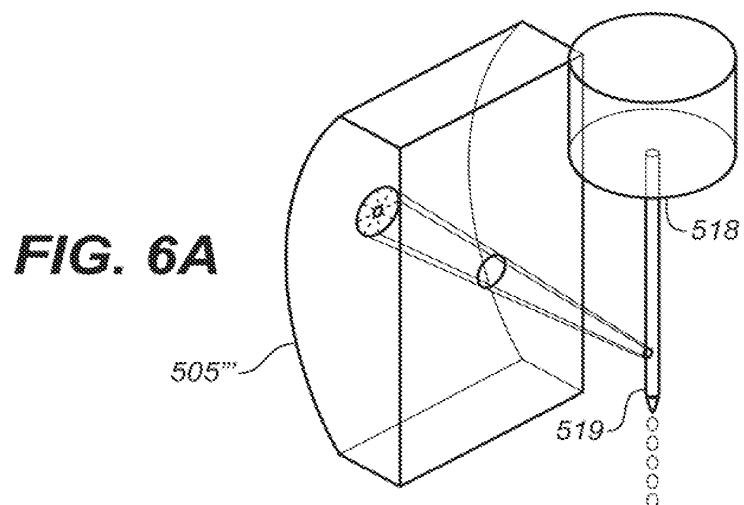
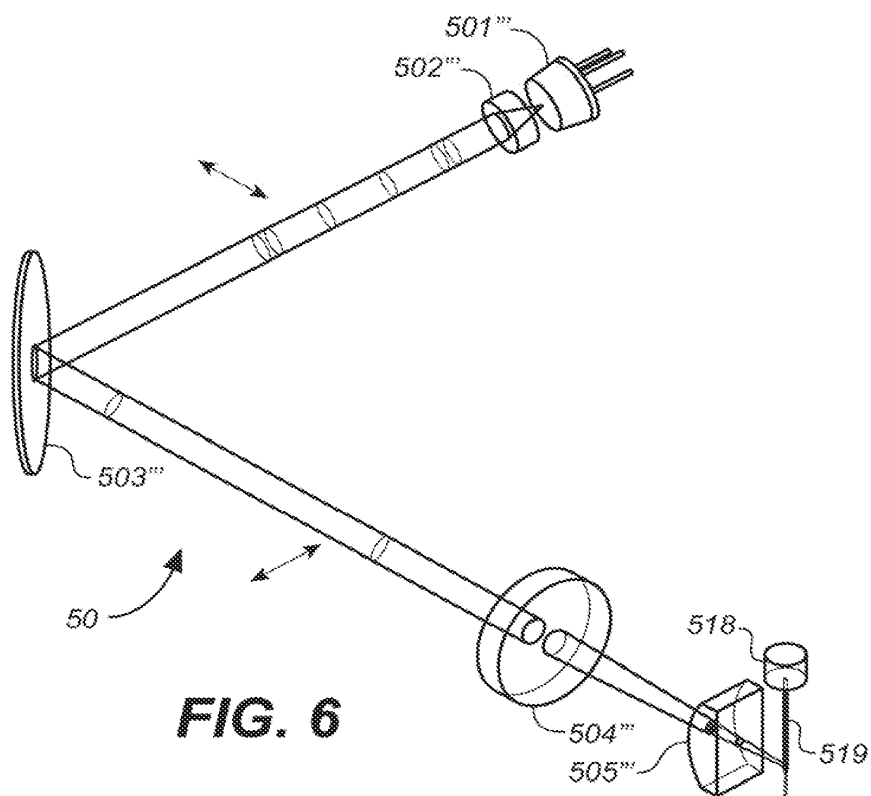
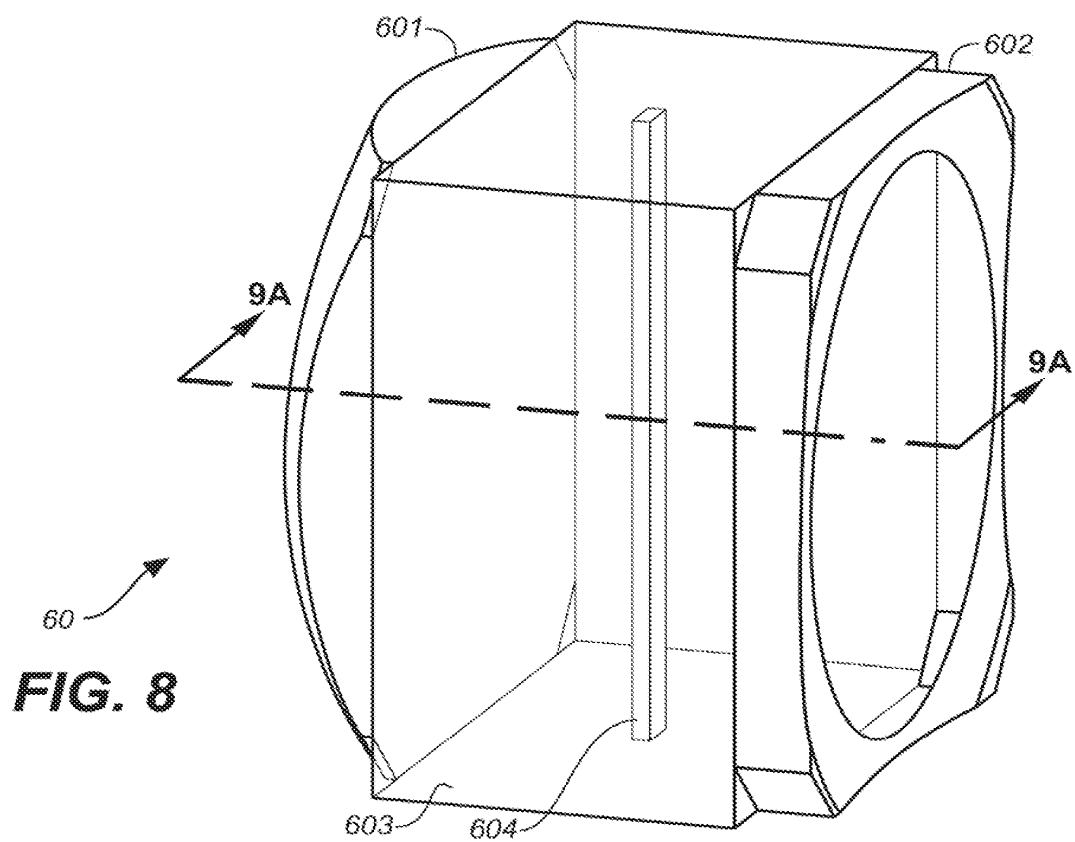
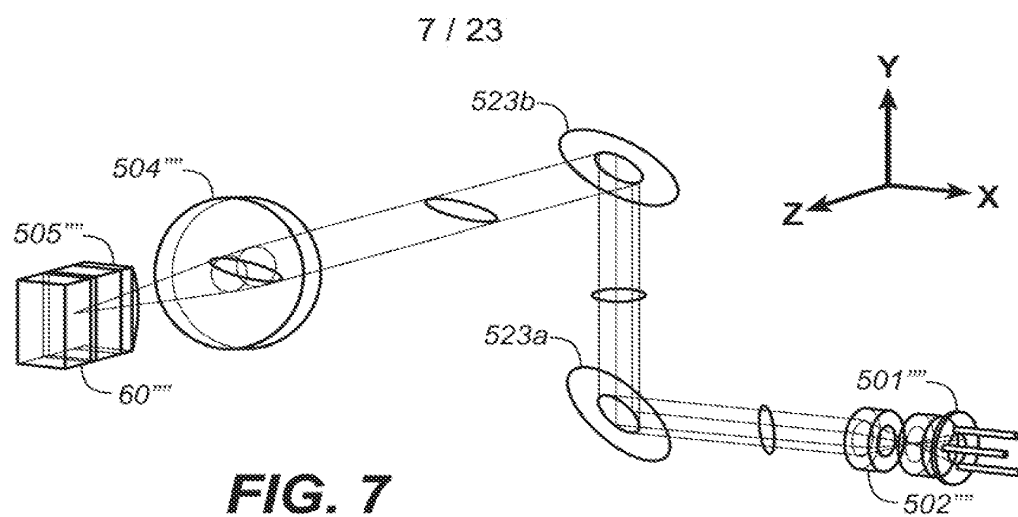


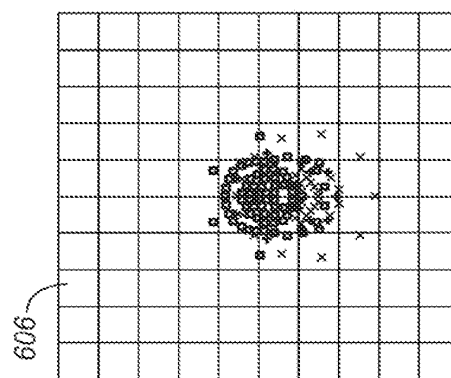
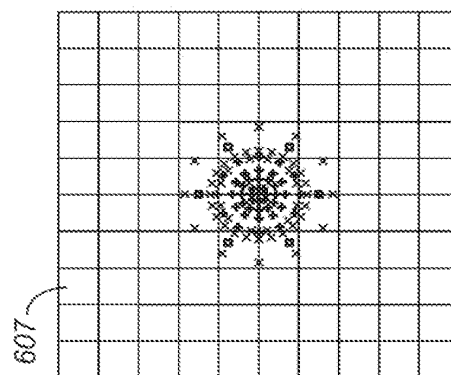
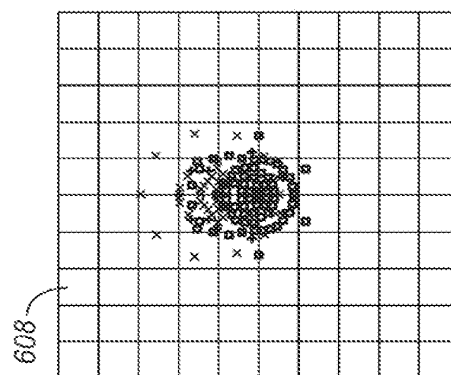
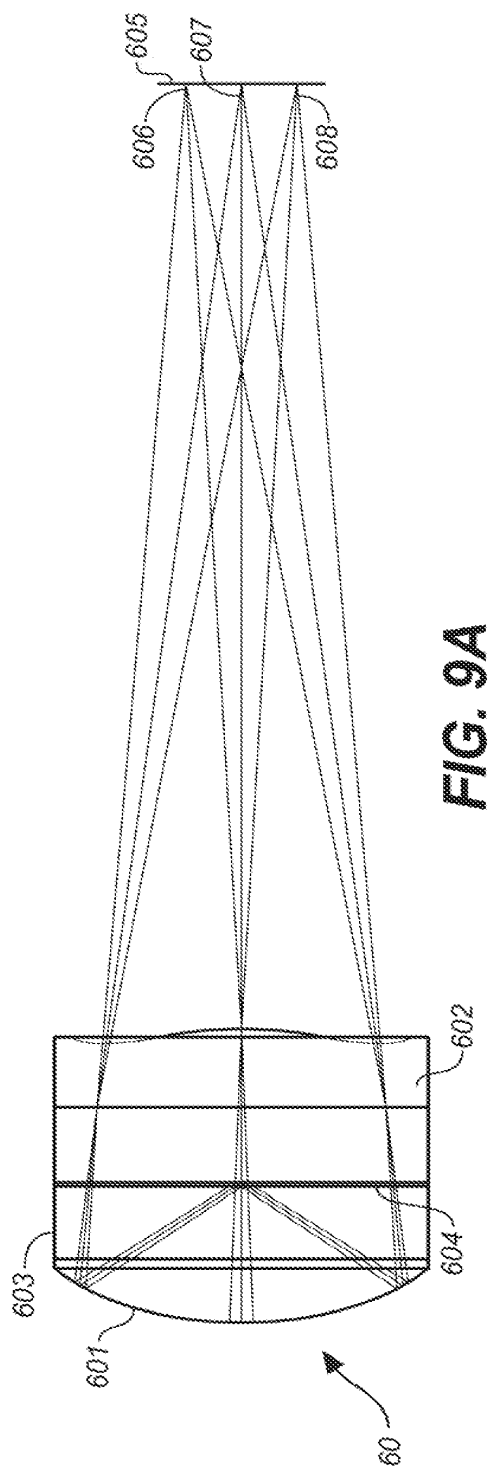
FIG. 4B

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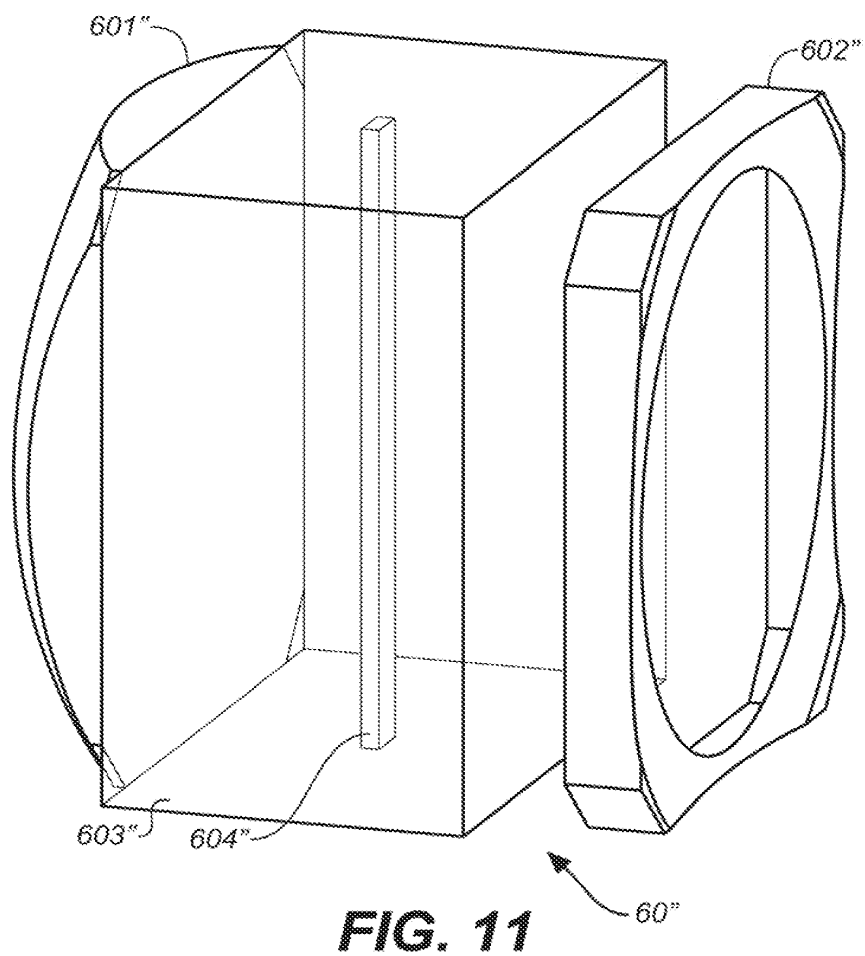
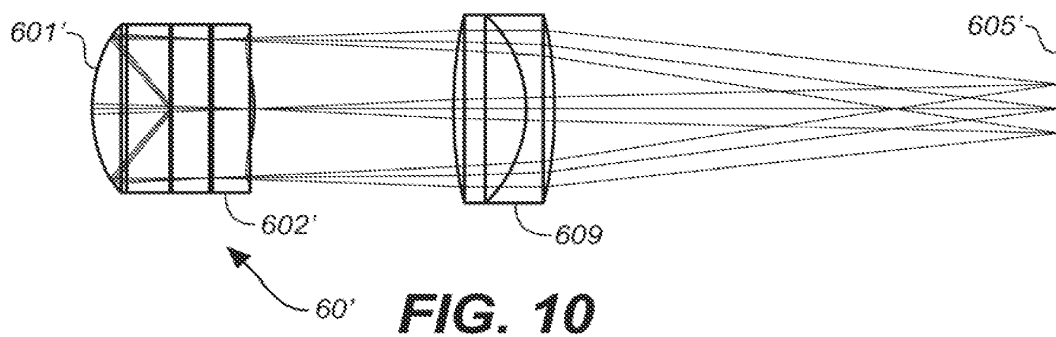
**FIG. 5C**







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FIG. 12

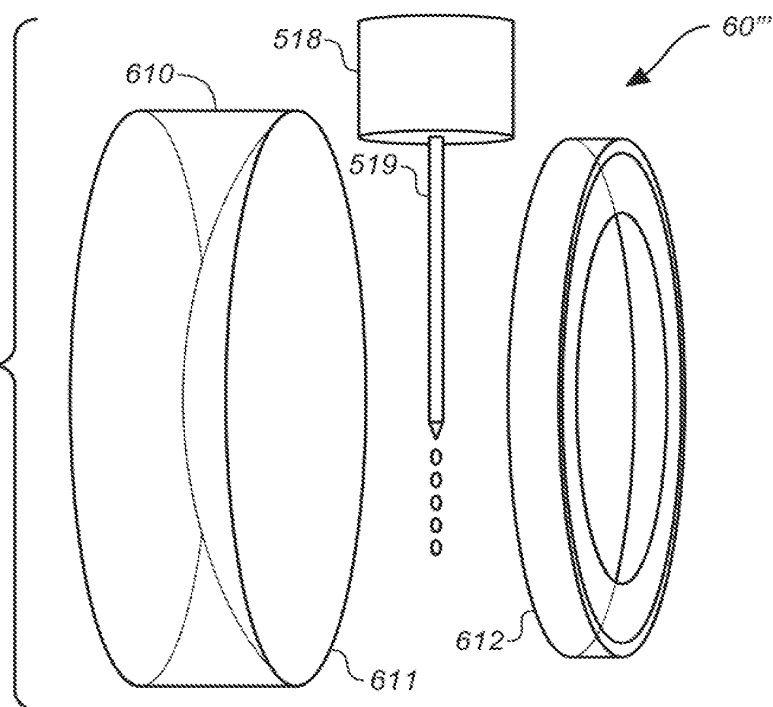
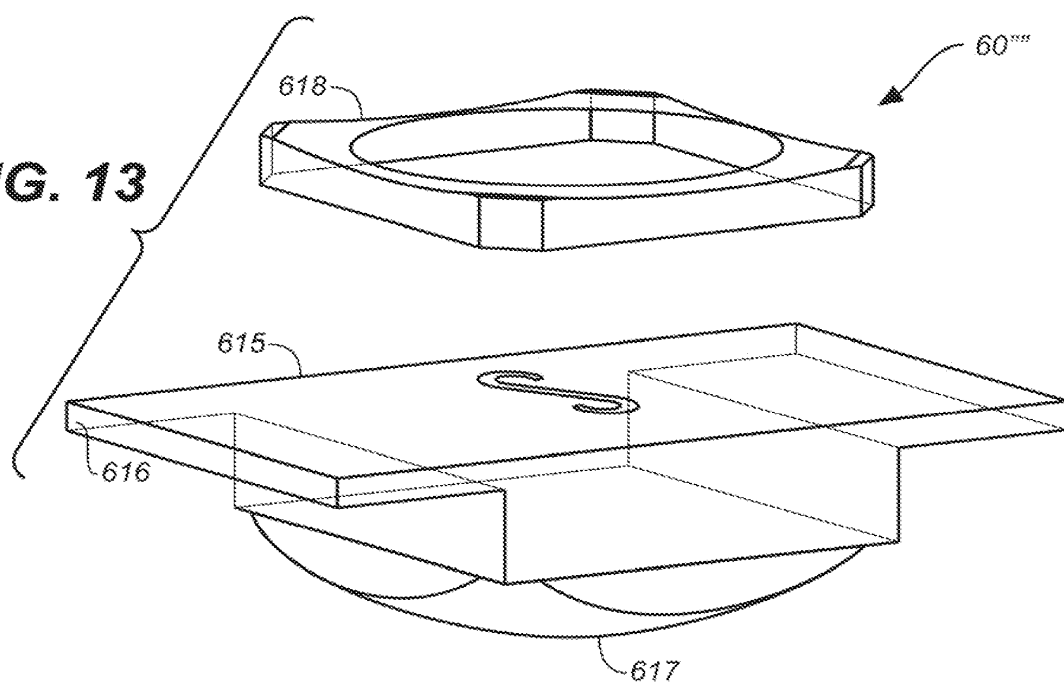
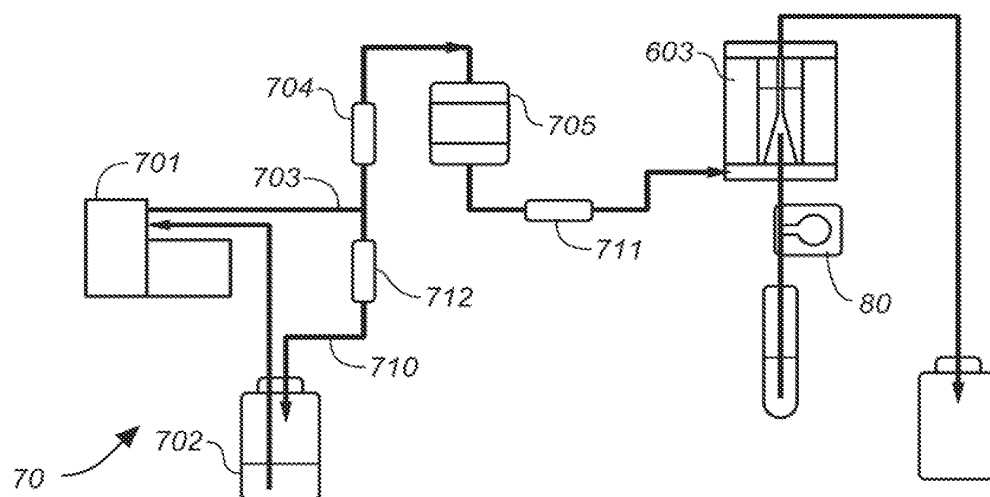
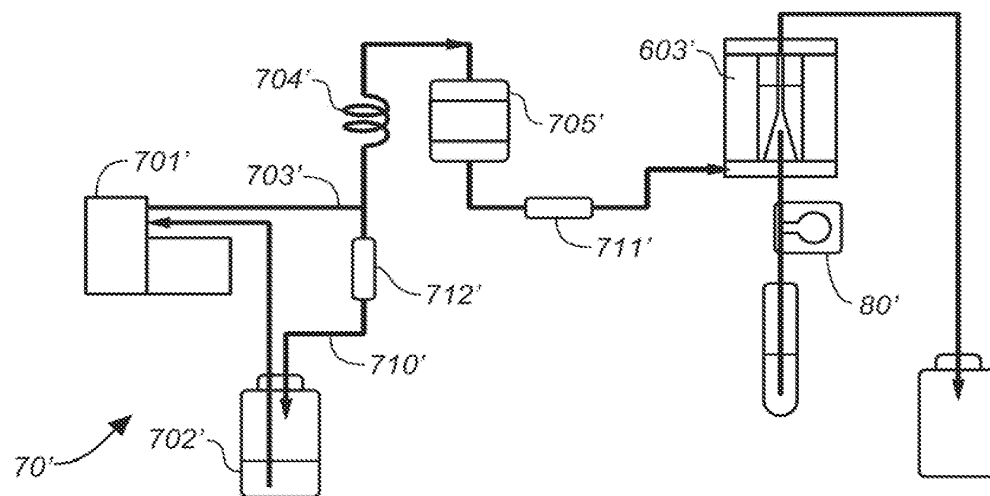


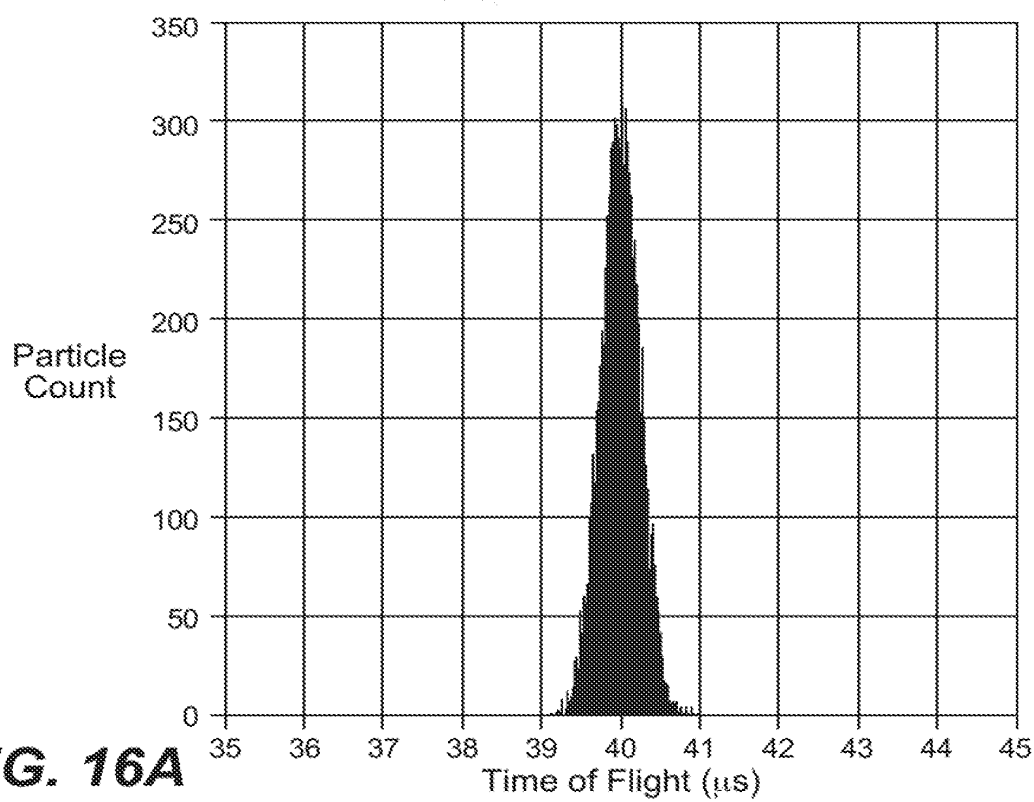
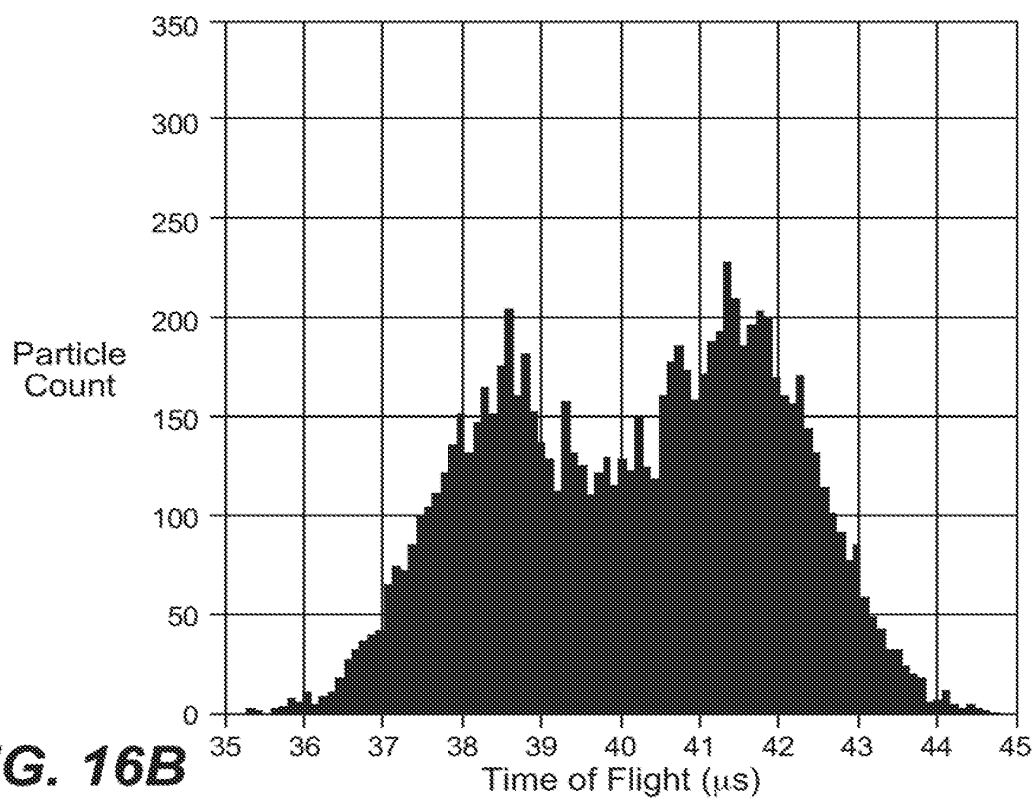
FIG. 13



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**FIG. 14****FIG. 15**

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**FIG. 16A****FIG. 16B**

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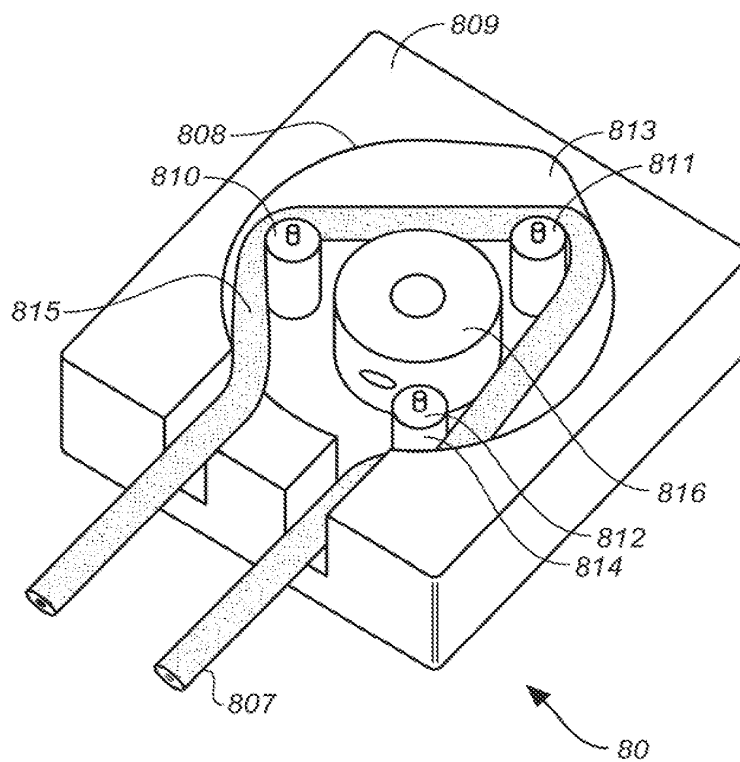


FIG. 17

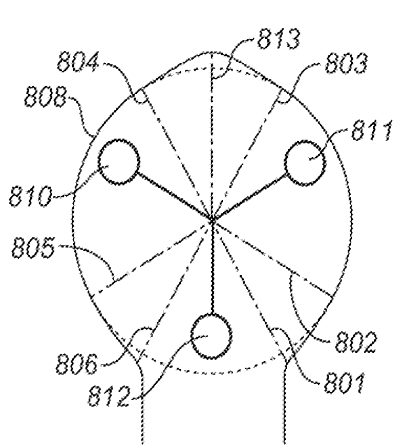


FIG. 18A

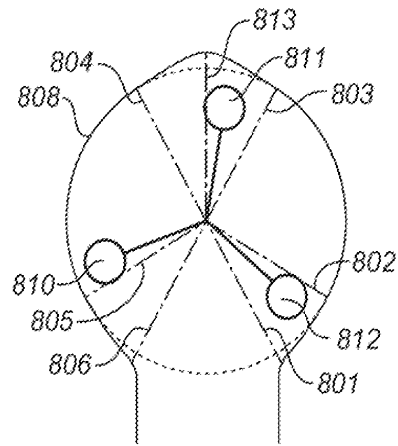


FIG. 18B

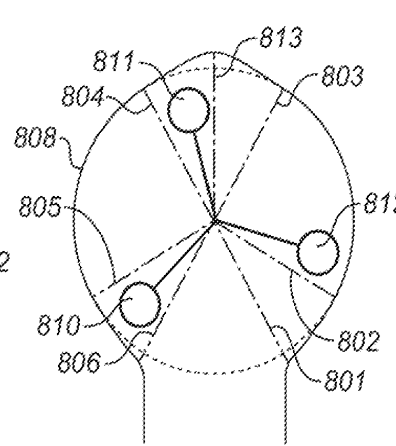


FIG. 18C

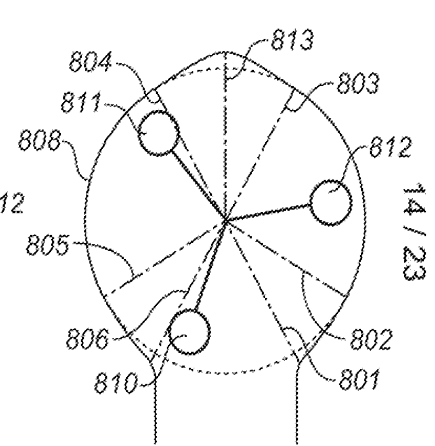


FIG. 18D

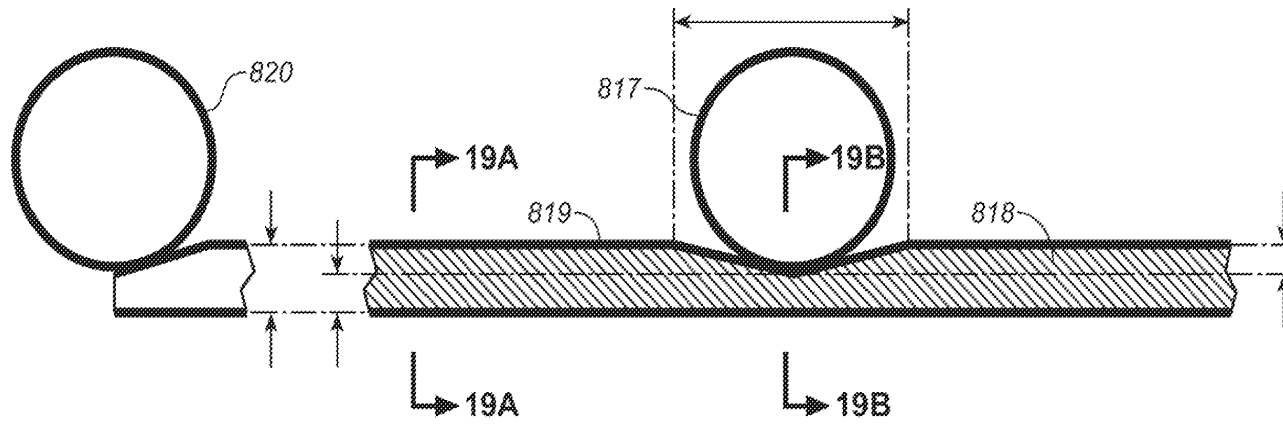


FIG. 19

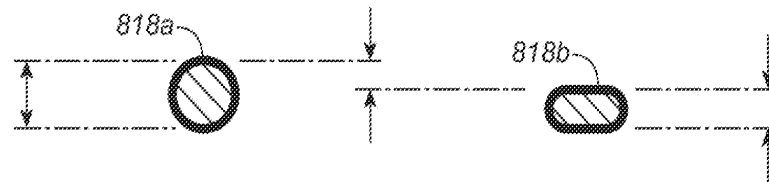
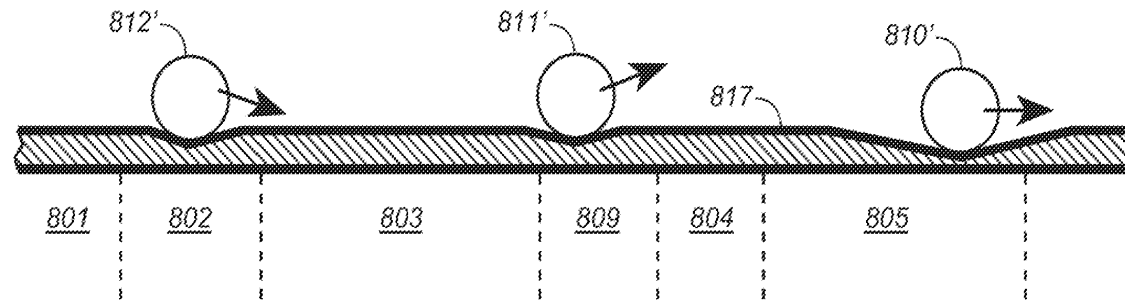
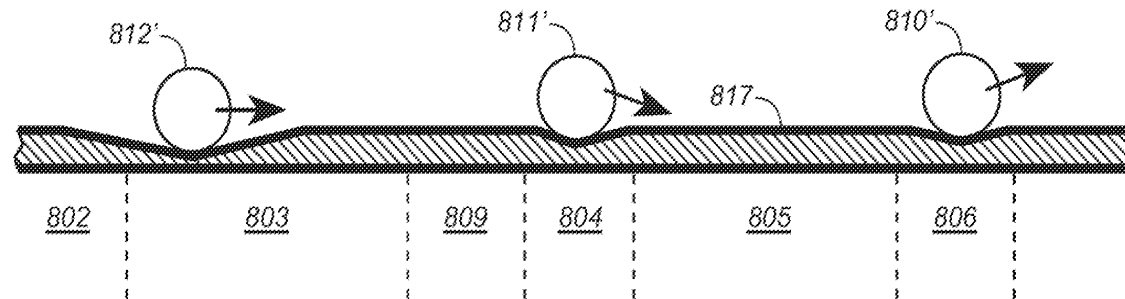
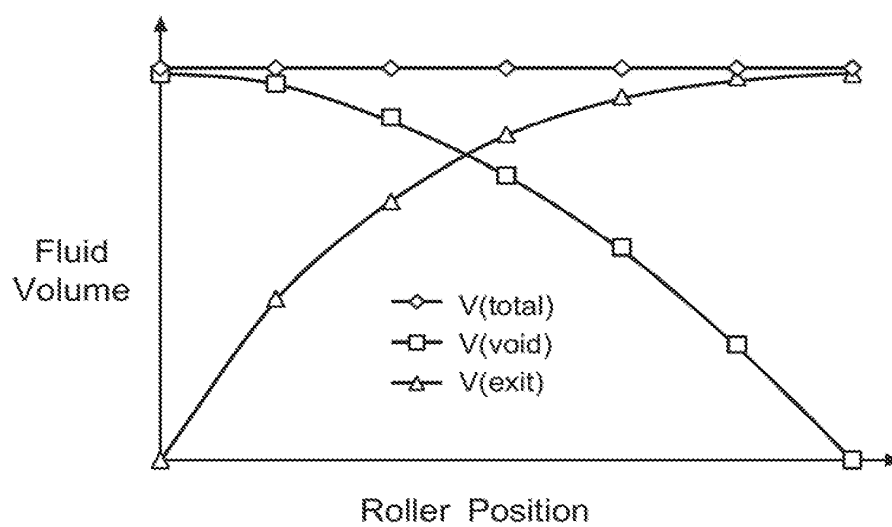
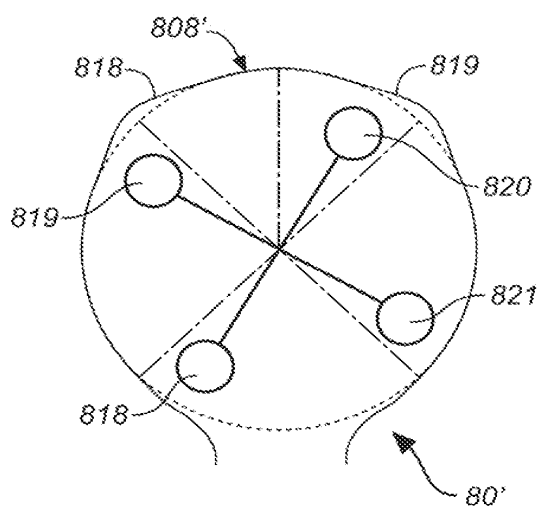
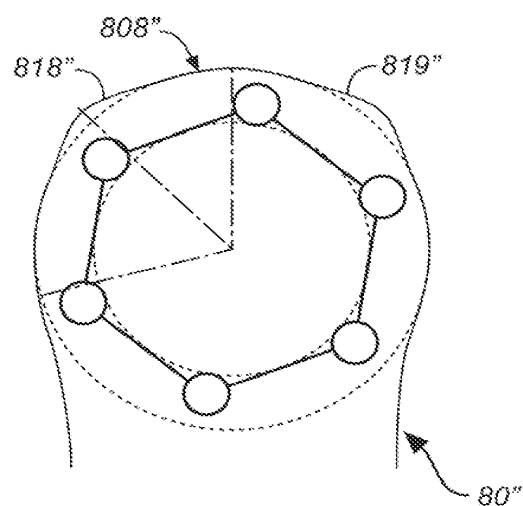


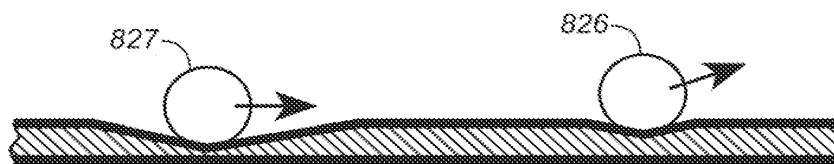
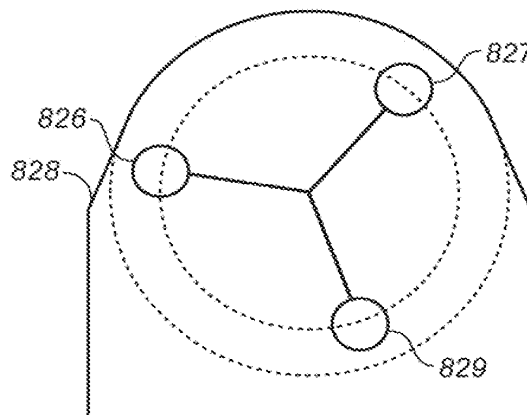
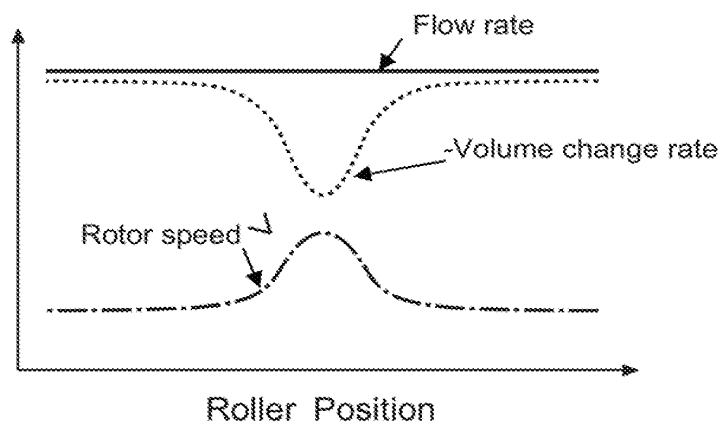
FIG. 19A

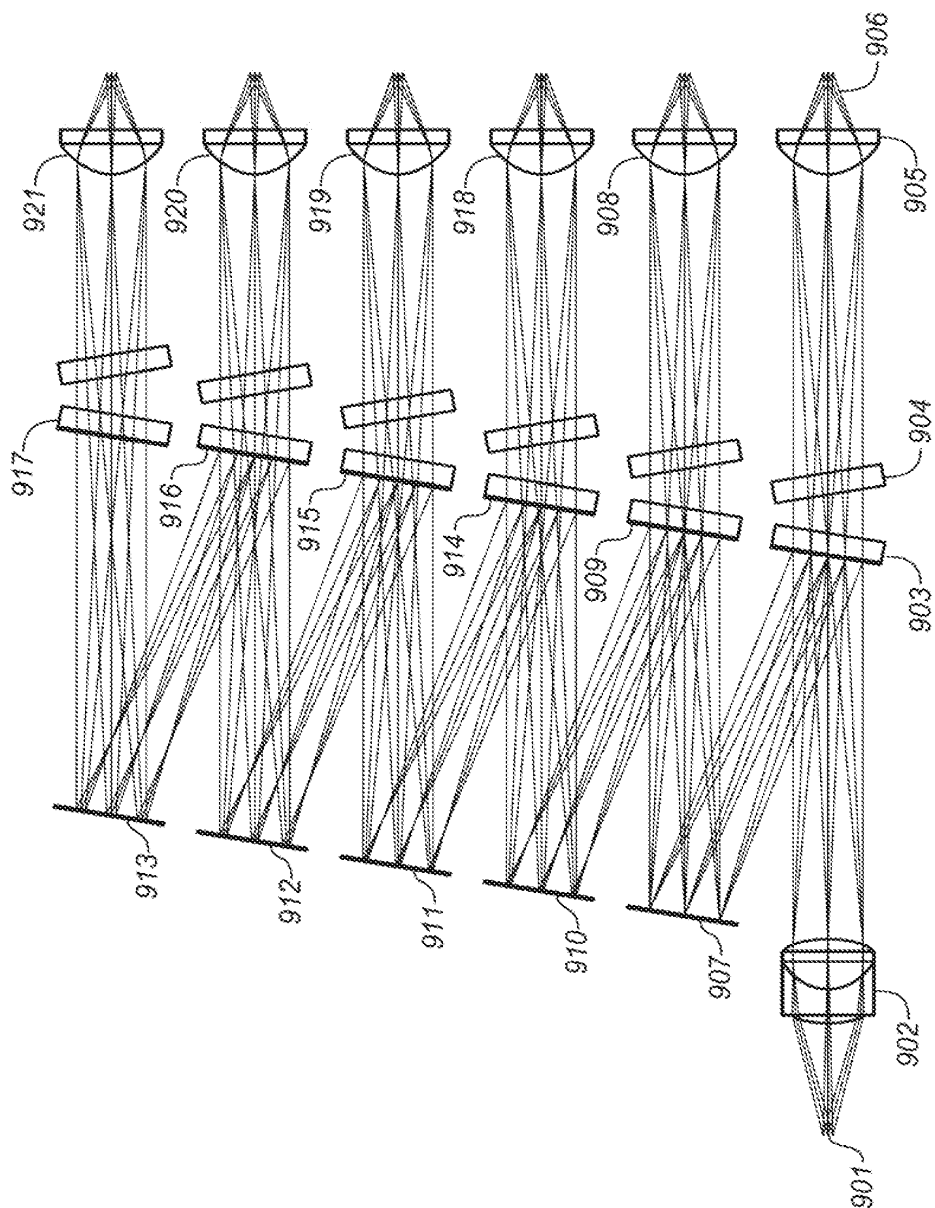
FIG. 19B

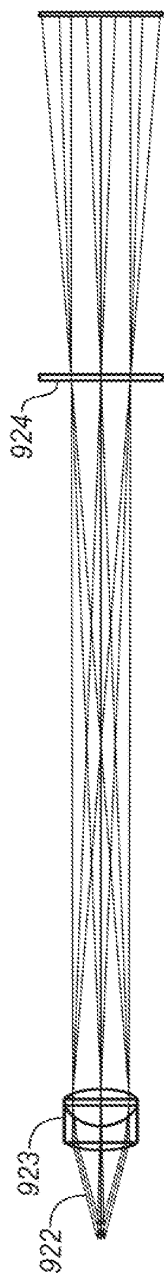
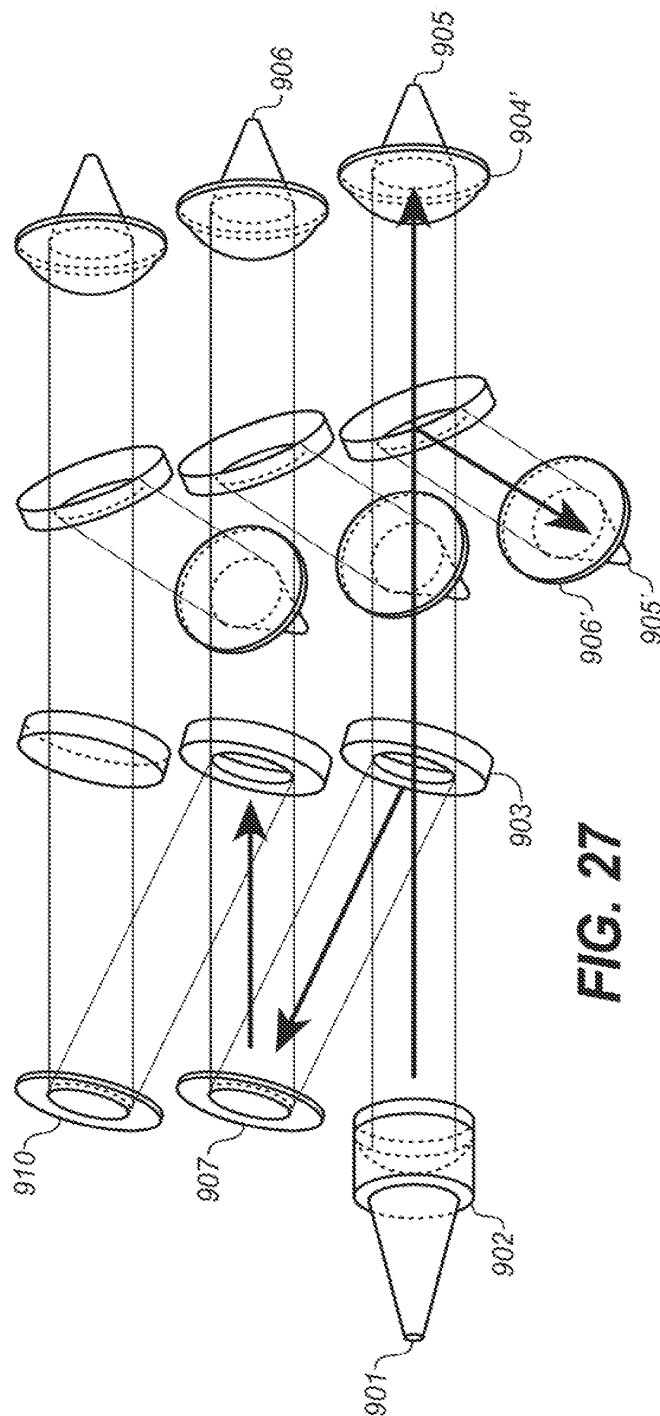
FIG. 20A**FIG. 20B**

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**FIG. 21****FIG. 22****FIG. 23**

**FIG. 24A****FIG. 24B****FIG. 24C**

**FIG. 25**

**FIG. 26****FIG. 27**

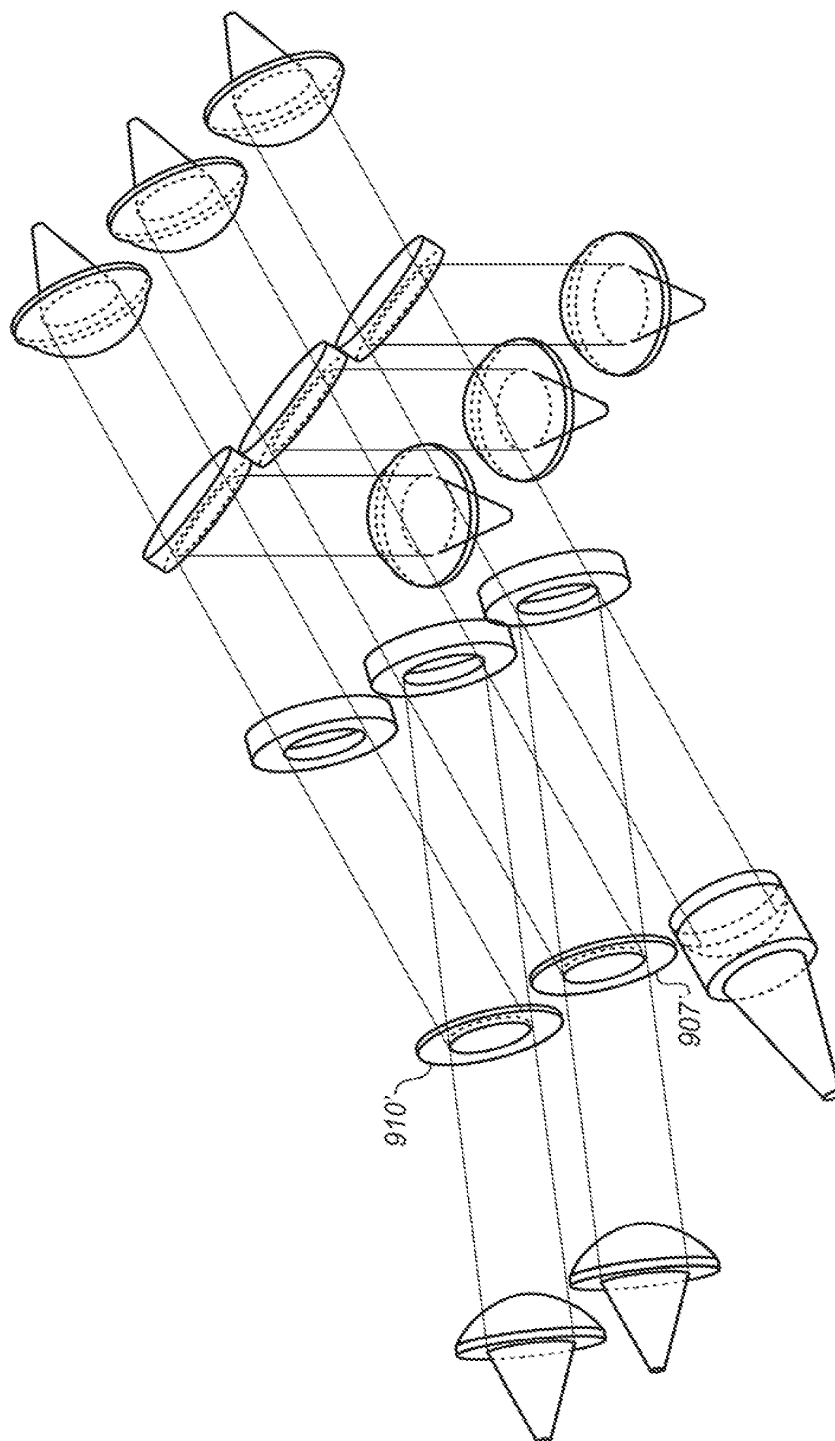


FIG. 28

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FIG. 29A

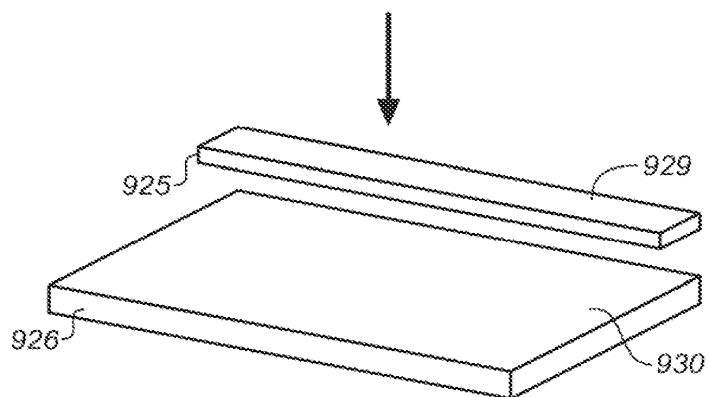


FIG. 29B

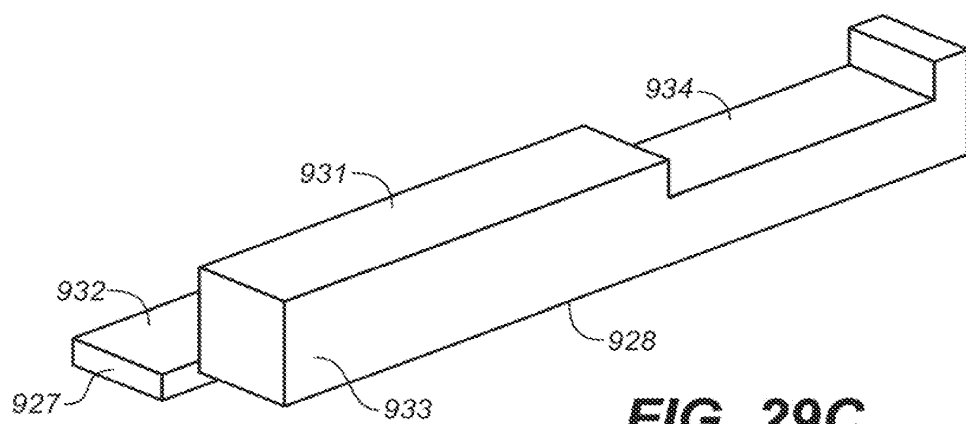
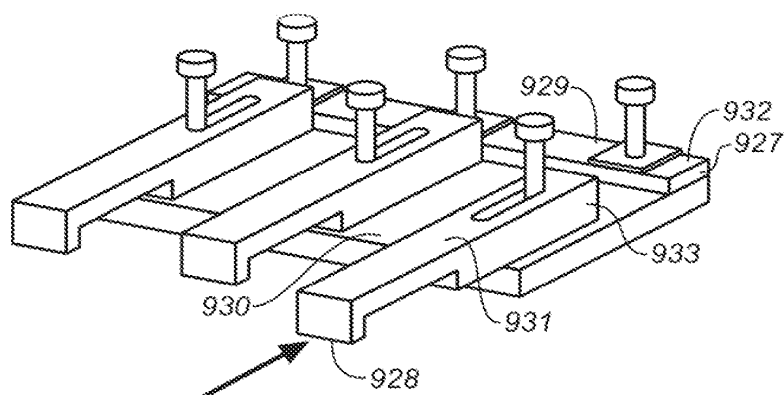


FIG. 29C

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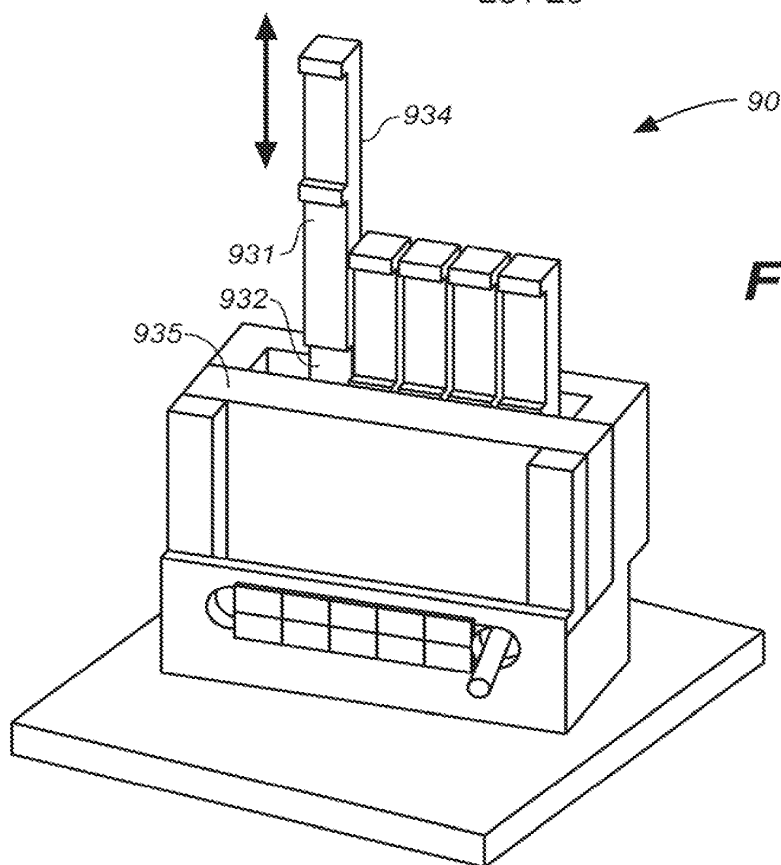
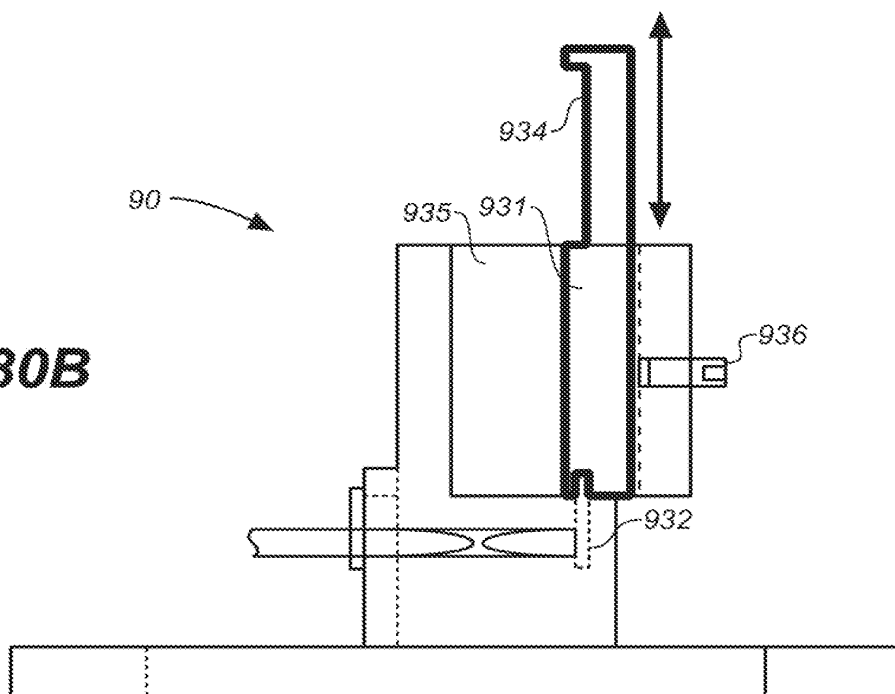


FIG. 30A

FIG. 30B



PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT/US13/43453

International Application No.

30 MAY 2013

30-05-2013

International Filing Date

PCT International Application RO/US

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum)

2294PCT

Box No. I TITLE OF INVENTION	
FLOW CYTOMETER	
Box No. II APPLICANT ☐ This person is also inventor	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)	
CYTOJENE CORP. 4097 Cortona Court San Jose, California 95135 United States of America	
Telephone No. (408) 223-7722	
Facsimile No.	
Applicant's registration No. with the Office	
E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send, notifications issued in respect of this international application to that e-mail address if those offices are willing to do so. ☐ as advance copies followed by paper notifications; or ☐ exclusively in electronic form (no paper notifications will be sent). E-mail address:	
State (that is, country) of nationality: US	
State (that is, country) of residence: US	
This person is applicant for the purposes of: ☐ all designated States ☒ the States indicated in the Supplemental Box	
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.	
Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative	
Name and address: (Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country.)	
SCHREIBER, Donald E. Post Office Box 2926 Kings Beach, California 96143-2926 United States of America	
Telephone No. (530) 546-6041	
Facsimile No. (530) 546-2341	
Agent's registration No. with the Office 29,435	
E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send, notifications issued in respect of this international application to that e-mail address if those offices are willing to do so. ☒ as advance copies followed by paper notifications; or ☐ exclusively in electronic form (no paper notifications will be sent). E-mail address: donaldschreiber0@gmail.com	
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Form PCT/RO/101 (first sheet) (16 September 2012)

See Notes to the request form

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S) <i>If none of the following sub-boxes is used, this sheet should not be included in the request.</i>	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i> CHEN, Yong Qin 4097 Cortona Court San Jose, California 95135 United States of America	This person is: ☐ applicant only ☒ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality: US	State <i>(that is, country)</i> of residence: US
This person is applicant for the purposes of: ☐ all designated States ☒ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i>	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i>	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box	
Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i>	This person is: ☐ applicant only ☐ applicant and inventor ☐ inventor only <i>(If this check-box is marked, do not fill in below.)</i> Applicant's registration No. with the Office
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box	
☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.	

Supplemental Box	If the Supplemental Box is not used, this sheet should not be included in the request.	
1. If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:	Continuation of Box no. II	
(i) if more than one person is to be indicated as applicant and/or inventor and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;	CYTOJENE CORP. All designated states except the United States	
(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;	Continuation of Box no. III CHEN, Yong Qin United States	
(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;		
(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;	Box No. VI PRIORITY CLAIM (Continuation)	
(v) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.	item (4)	
	Filing Date	19 OCT 2012 (19.10.2012)
	Number	61/715,836
	Member of WTO	US
2. If the applicant intends to make an indication of the wish that the international application be treated, in certain designated States, as an application for a patent of addition, certificate of addition, inventor's certificate of addition or utility certificate of addition: in such case, write the name or two-letter code of each designated State concerned and the indication "patent of addition," "certificate of addition," "inventor's certificate of addition" or "utility certificate of addition," the number of the parent application or parent patent or other parent grant and the date of grant of the parent patent or other parent grant or the date of filing of the parent application (Rules 4.11(a)(i) and 49bis.1(a) or (b)).	item (5)	
	Filing Date	29 APR 2013 (29.04.2013)
	Number	61/816,819
	Member of WTO	US
3. If the applicant intends to make an indication of the wish that the international application be treated, in the United States of America, as a continuation or continuation-in-part of an earlier application: in such case, write "United States of America" or "US" and the indication "continuation" or "continuation-in-part" and the number and the filing date of the parent application (Rules 4.11(a)(i) and 49bis.1(d)).		

Box No. V DESIGNATIONS				
The filing of this request constitutes under Rule 4.9(a) the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents. However, ☐ DE Germany is not designated for any kind of national protection ☐ JP Japan is not designated for any kind of national protection ☐ KR Republic of Korea is not designated for any kind of national protection <i>(The check-boxes above may only be used to exclude (irrevocably) the designations concerned (if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)</i>				
Box No. VI PRIORITY CLAIM AND DOCUMENT				
The priority of the following earlier application(s) is hereby claimed:				
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application: regional Office	international application: receiving Office
item (1) 30 MAY 2012 (30.05.2012)	61/653,245	US		
item (2) 30 MAY 2012 (30.05.2012)	61/653,328	US		
item (3) 18 OCT 2012 (18.10.2012)	61/715,819	US		
☒ Further priority claims are indicated in the Supplemental Box.				
Furnishing the priority document(s): ☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application(s) was filed with the receiving Office which, for the purposes of this international application, is the receiving Office) identified above as: ☒ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box ☐ The International Bureau is requested to obtain from a digital library a certified copy of the earlier application(s) identified above, using, where applicable, the access code(s) indicated below (if the earlier application(s) is available to it from a digital library): ☐ item (1) access code ☐ item (2) access code ☐ item (3) access code ☐ other, see Supplemental Box				
Restore the right of priority: the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (.....). (See also the Notes to Box No. VI: further information must be provided to support a request to restore the right of priority.)				
Incorporation by reference: where an element of the international application referred to in Article 11(1)(ii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(ii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.				
Box No. VII INTERNATIONAL SEARCHING AUTHORITY				
Choice of International Searching Authority (ISA) (if more than one International Searching Authority is competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used): ISA/ US				

Continuation of Box No. VII USE OF RESULTS OF EARLIER SEARCH, REFERENCE TO THAT SEARCH		
☐ The ISA indicated in Box No. VII is requested to take into account the results of the earlier search(es) indicated below (see also Notes to Box VII; use of results of more than one earlier search).		
Filing date (day/month/year)	Application Number	Country (or regional Office)
☐ Statement (Rule 4.12(ii)): this international application is the same, or substantially the same, as the application in respect of which the earlier search was carried out except, where applicable, that it is filed in a different language.		
☐ Availability of documents: the following documents are available to the ISA in a form and manner acceptable to it and therefore do not need to be submitted by the applicant to the ISA (Rule 12bis.1(f)): ☐ a copy of the results of the earlier search,* ☐ a copy of the earlier application, ☐ a translation of the earlier application into a language which is accepted by the ISA, ☐ a translation of the results of the earlier search into a language which is accepted by the ISA, ☐ a copy of any document cited in the results of the earlier search. (If known, please indicate below the document(s) available to the ISA):		
☐ Transmit copy of results of earlier search and other documents (where the earlier search was not carried out by the ISA indicated above but by the same Office as that which is acting as the receiving Office): the receiving Office is requested to prepare and transmit to the ISA (Rule 12bis.1(c)): ☐ a copy of the results of the earlier search,* ☐ a copy of the earlier application, ☐ a copy of any document cited in the results of the earlier search.		
* Where the results of the earlier search are neither available from a digital library nor transmitted by the receiving Office, the applicant is required to submit them to the receiving Office (Rule 12bis.1(a)) (See item 11. in the check-list and also Notes to Box No. VII).		
Filing date (day/month/year)	Application Number	Country (or regional Office)
☐ Statement (Rule 4.12(ii)): this international application is the same, or substantially the same, as the application in respect of which the earlier search was carried out except, where applicable, that it is filed in a different language.		
☐ Availability of documents: the following documents are available to the ISA in a form and manner acceptable to it and therefore do not need to be submitted by the applicant to the ISA (Rule 12bis.1(f)): ☐ a copy of the results of the earlier search,* ☐ a copy of the earlier application, ☐ a translation of the earlier application into a language which is accepted by the ISA, ☐ a translation of the results of the earlier search into a language which is accepted by the ISA, ☐ a copy of any document cited in the results of the earlier search. (If known, please indicate below the document(s) available to the ISA):		
☐ Transmit copy of results of earlier search and other documents (where the earlier search was not carried out by the ISA indicated above but by the same Office as that which is acting as the receiving Office): the receiving Office is requested to prepare and transmit to the ISA (Rule 12bis.1(c)): ☐ a copy of the results of the earlier search,* ☐ a copy of the earlier application, ☐ a copy of any document cited in the results of the earlier search.		
* Where the results of the earlier search are neither available from a digital library nor transmitted by the receiving Office, the applicant is required to submit them to the receiving Office (Rule 12bis.1(a)) (See item 11. in the check-list and also Notes to Box No. VII).		
☐ Further earlier searches are indicated on a continuation sheet.		
Box No. VIII DECLARATIONS		
The following declarations are contained in Boxes Nos. VIII (i) to (v) (mark the applicable check-boxes below and indicate in the right column the number of each type of declaration):		Number of declarations
☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☒ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Box No. VIII (iv) DECLARATION: INVENTORSHIP (only for the purposes of the designation of the United States of America)
The declaration must conform to the following standardized wording provided for in Section 214; see Notes to Boxes Nos. VIII, VIII (i) to (v) (in general) and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

**Declaration of inventorship (Rules 4.17(iv) and 51bis.1(a)(iv))
for the purposes of the designation of the United States of America:**

I hereby declare that I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

This declaration is directed to the international application of which it forms a part (if filing declaration with application).

This declaration is directed to international application No. PCT/..... (if furnishing declaration pursuant to Rule 26ter).

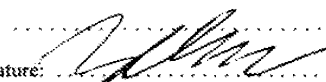
I hereby declare that the above-identified international application was made or authorized to be made by me.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Name: Yong Qin Chen

Residence: San Jose, California
(city and either US state, if applicable, or country)

Mailing Address: 4097 Cortona Court
San Jose, California 95135
United States of America

Inventor's Signature:  Date: May 24, 2013
(The signature must be that of the inventor, not that of the agent)

Name:

Residence:
(city and either US state, if applicable, or country)

Mailing Address:

Inventor's Signature: Date:
(The signature must be that of the inventor, not that of the agent)

Name:

Residence:
(city and either US state, if applicable, or country)

Mailing Address:

Inventor's Signature: Date:
(The signature must be that of the inventor, not that of the agent)

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

* RO/US

Box No. IX CHECK LIST for PAPER filings – this sheet is only to be used when filing an international application on PAPER			
This international application contains the following:		Number of sheets	This international application is accompanied by the following item(s) (mark the applicable check-boxes below and indicate in right column the number of each item):
(a) request form PCT/RO/101 (including any declarations and supplemental sheets) :	7	1. ☒ calculation sheet :	*[4]
(b) description (excluding any sequence listing part of the description, see (f), below) :	48	2. ☒ original separate power of attorney :	2
(c) claims :	30	3. ☐ original general power of attorney :	
(d) abstract :	1	4. ☐ copy of general power of attorney; reference number: :	
(e) drawings (if any) :	23	5. ☐ priority document(s) identified in Box No. VI as item(s) :	
(f) sequence listing part of the description (if any) :	0	6. ☐ Translation of international application into (language): :	
Total number of sheets :	109	7. ☐ separate indications concerning deposited microorganism or other biological material :	
		8. ☐ copy in electronic form (Annex C/ST.25 text file) on physical data carrier(s) of the sequence listing, not forming part of the international application, which is furnished only for the purposes of international search under Rule 13ter (type and number of physical data carriers) :	
		9. ☐ a statement confirming that "the information recorded in electronic form submitted under Rule 13ter is identical to the sequence listing as contained in the international application" as filed on paper. :	
		10. ☐ copy of results of earlier search(es) (Rule 12bis.1(a)) :	
		11. ☐ other (specify): :	
Figure of the drawings which should accompany the abstract:	1	Language of filing of the international application:	English
Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE			
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).			
 Donald E. Schreiber Agent for Applicants			
For receiving Office use only			
1. Date of actual receipt of the purported international application:	30 MAY 2013	30-05-2013	2. Drawings:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:			☐ received:
4. Date of timely receipt of the required corrections under PCT Article 11(2):			☐ not received:
5. International Searching Authority (if two or more are competent):	ISA / US	6. ☐ Transmittal of search copy delayed until search fee is paid	
For International Bureau use only			
Date of receipt of the record copy by the International Bureau:			

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF RECEIPT OF SEARCH COPY

(PCT Rule 25.1)

To:

DONALD E. SCHREIBER
POST OFFICE BOX 2926
KINGS BEACH, CALIFORNIA 96143-2926

Date of mailing
(day/month/year)

07 Jun 2013

Applicant's or agent's file reference
2294PCT

IMPORTANT NOTIFICATION

International application No.
PCT/US2013/043453

International filing date (day/month/year)
30 May 2013

Priority date (day/month/year)
30 May 2012

Applicant

CYTOJENE CORP.

1. Where the International Searching Authority and the receiving Office are not the same Office:

The applicant is hereby notified that the search copy of the international application was received by this International Searching Authority on the date indicated below.

Where the International Searching Authority and the receiving Office are the same Office:

The applicant is hereby notified that the search copy of the international application was received on the date indicated below.

07 Jun 2013

(date of receipt)

2. ☐ The search copy was accompanied by a nucleotide and/or amino acid sequence listing in electronic form.

3. ☐ The search copy contained a nucleotide and/or amino acid sequence listing in electronic form.

4. Time limit for establishment of international search report and written opinion of the International Searching Authority

The applicant is informed that the time limit for establishing the international search report and the written opinion of the International Searching Authority is three months from the date of receipt indicated above or nine months from the priority date, whichever time limit expires later (Rules 42.1 and 43bis.1(a)).

A copy of this Notification has been sent to the International Bureau and, where the first sentence of paragraph 1 applies, to the receiving Office.

Name and mailing address of the ISA/
Mail Stop PCT, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450
Facsimile No. 571-273-3201

Authorized officer
Nhu Thuy Tran
Telephone No. (571)272-9063

Form PCT/ISA/202 (July 2009)

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

To:

DONALD E. SCHREIBER
POST OFFICE BOX 2926
KINGS BEACH, CALIFORNIA 96143-2926

PCT

NOTIFICATION CONCERNING PAYMENT OF PRESCRIBED FEES

(PCT Rules 12*bis*.1(c), 14, 15 and 16
and Administrative Instructions,
Sections 102*bis*(c), 304, 323(b) and 707)

Date of mailing
(day/month/year)

07 Jun 2013

Applicant's or agent's file reference

2294PCT

PAYMENT DUE

see item 3 for time limits

International application No.

PCT/US2013/043453

International filing date/Date of receipt
(day/month/year)

30 May 2013

Priority date (day/month/year)

30 May 2012

Applicant

CYTOJENE CORP.

1. The applicant is hereby notified that this receiving Office has received:



the payment of all the prescribed fees, and



an overpayment, which will be refunded in due course.



no or insufficient payment of the prescribed fees and the applicant is hereby invited to pay the balance due, as summarized under item 2, within the time limit(s) indicated under item 3.

2. Fees and payment calculation:

4,896.00

4,896.00

0.00

Total fees payable

Amount paid

Balance



The details of the calculation are given in the Annex.

3. Time limit(s) for payment and amount(s) payable (Rules 14.1, 15.3 and 16.1(f)):



within ONE MONTH from the date of receipt of the international application (for the transmittal fee (if any), the search fee and the international filing fee). The amount payable for each fee is the amount applicable on the date of receipt of the international application.



within 16 MONTHS from the priority date (only for the fee for priority document). The applicant's attention is drawn to the fact that the request made by the applicant under Rule 17.1(b) will be considered not to have been made unless the fee is paid within that time limit.

4. Additional observations (if necessary):



The search copy will not be transmitted to the International Searching Authority until the search fee is paid (therefore the start of the international search will be delayed) (Rule 23.1(a) and (b)).

Name and mailing address of the receiving Office

Mail Stop PCT, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-3201

Authorized officer

Nhu Thuy Tran

Telephone No. (571)272-9063

**ANNEX TO FORM PCT/RO/102
CALCULATION OF THE PRESCRIBED FEES**

International application No.
PCT/US2013/043453

☐ T Transmittal Fee

Prescribed amount: 240.00 ☐ T
Amount paid: 240.00
Balance: = 0.00

☒ correct amount
☐ overpayment
☐ balance due

☐ S Search Fee

Prescribed amount: 2,080.00 ☐ S
Amount paid: 2,080.00
Balance: = 0.00

☒ correct amount
☐ overpayment
☐ balance due

☐ I International Filing Fee

Fixed amount for first 30 sheets: 1,312.00 ☐ i1
79 x 16.00 = 1,264.00 ☐ i2
Number of sheets Fee per sheet
in excess of 30

(excluding pages referred
to in Section 707(a-bis))

Reduction where the international application is filed
(See PCT Applicant's Guide, International Phase for
details on the availability of this reduction):

on paper together with a copy in electronic
form, in character coded format, of the request
and the abstract 0.00 ☐ r

or

in electronic form, the request not being in
character coded format 0.00 ☐ r

or

in electronic form, the request being in character
coded format 0.00 ☐ r

or

in electronic form, the request, description,
claims and abstract being in character coded
format 0.00 ☐ r

Sub-total: = 2,576.00 ☐ i1+i2-r

*Applicants from certain States are entitled to a reduction of 90% of the
international filing fee. Where the applicant is (or all applicants are) so
entitled, the total to be entered at I is 10% of the sub-total entered at
(i1+i2-r); (see Notes to the Fee Calculation Sheet as annexed to the
Request Form, PCT/RO/101, for details):* = 2,576.00 ☐ I

Amount paid: 2,576.00
Balance: = 0.00

☒ correct amount
☐ overpayment
☐ balance due

☐ P Fee for Priority Document

Prescribed amount: 0.00 ☐ P
Amount paid: 0.00
Balance: = 0.00

☒ correct amount
☐ overpayment
☐ balance due

☐ ES Fee for Earlier Search Documents

Prescribed amount: 0.00 ☐ ES
Amount paid: 0.00
Balance: = 0.00

☐ correct amount
☐ overpayment
☐ balance due

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

PCT

To:
DONALD E. SCHREIBER
POST OFFICE BOX 2926
KINGS BEACH, CALIFORNIA 96143-2926

NOTIFICATION OF THE INTERNATIONAL APPLICATION NUMBER AND OF THE INTERNATIONAL FILING DATE

(PCT Rule 20.2(c))

Confirmation No: 1081

Date of mailing
(day/month/year) 07 Jun 2013

Applicant's or agent's file reference
2294PCT

IMPORTANT NOTIFICATION

International application No.
PCT/US2013/043453

International filing date (day/month/year)
30 May 2013

Priority date (day/month/year)
30 May 2012

Applicant
CYTOJENE CORP.

Title of the invention
FLOW CYTOMETER

1. The applicant is hereby notified that the international application has been accorded the international application number and the international filing date indicated above.

2. The applicant is further notified that the record copy of the international application:

- ☒ was transmitted to the International Bureau on 07 Jun 2013 .
- ☐ has not yet been transmitted to the International Bureau for the reason indicated below and a copy of this notification has been sent to the International Bureau*:
- ☐ because the necessary national security clearance has not yet been obtained.
- ☐ because (reason to be specified):

* The International Bureau monitors the transmittal of the record copy by the receiving Office and will notify the applicant (with Form PCT/IB/301) of its receipt. Should the record copy not have been received by the expiration of 14 months from the priority date, the International Bureau will notify the applicant (Rule 22.1(c)).

3. FOREIGN TRANSMITTAL LICENSE INFORMATION

Completed by: NT

- ☐ Additional license for foreign transmittal not required. This subject matter is covered by a license already granted or the equivalent U.S. national application. Refer to that license for information concerning its scope.
- ☐ License for foreign transmittal not required. 37 CFR. 5.11(e)(1) or 37 CFR 5.11(e)(2). However, a license may be required for additional subject matter. See 37 CFR 5.15(b).
- ☒ Foreign transmittal license granted. 35 U.S.C. 184; 37 CFR 5.11 on 06 Jun 2013 :
(date)
- ☐ 37 CFR 5.15(a) ☒ 37 CFR 5.15(b)

Name and mailing address of the receiving Office
Mail Stop PCT, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450
Facsimile No. 571-273-3201

Authorized officer
Nhu Thuy Tran
Telephone No. (571)272-9063

Form PCT/RO/105 (July 2008)

PATENT COOPERATION TREATY

From the RECEIVING OFFICE

To:
DONALD E. SCHREIBER
POST OFFICE BOX 2926
KINGS BEACH, CALIFORNIA 96143-2926

PCT

INVITATION TO CORRECT DEFECTS IN THE INTERNATIONAL APPLICATION

(PCT Articles 3(4)(i) and 14(1) and Rule 26)

Applicant's or agent's file reference 2294PCT	Date of mailing <i>(day/month/year)</i> 07 Jun 2013
International application No. PCT/US2013/043453	International filing date <i>(day/month/year)</i> 30 May 2013
Applicant CYTOJENE CORP.	

1. ☒ The applicant is hereby **invited**, within the time limit indicated above, to correct, **in the international application as filed**, the defects specified on the attached:
- ☐ Annex A
 - ☒ Annex B1 *(text matter of the international application as filed)*
 - ☐ Annex C1 *(drawings of the international application as filed)*

2. ☐ The applicant is hereby **invited**, within the time limit indicated above, to correct, **in the translation of the international application** furnished under Rule 12.3 or 12.4, the defects specified on the attached:
- ☐ Annex A
 - ☐ Annex B2 *(text matter of the translation of the international application)*
 - ☐ Annex C2 *(drawings of the translation of the international application)*

Additional observations (if necessary):

HOW TO CORRECT THE DEFECTS?

Except where the defect is in the request, any correction must be submitted by filing a replacement sheet embodying the correction and a letter accompanying the replacement sheet, which shall draw attention to the difference between the replaced sheet and the replacement sheet. For a defect in the request, a correction may simply be stated in a letter if it is of such a nature that the correction can be transferred clearly onto the request record copy (Rule 26.4).

ATTENTION

Failure to correct the defects will result in the international application being considered withdrawn by this receiving Office (see Rule 26.5 for further details).

A copy of this Invitation and any attachments has been sent to the International Bureau

☒ and the International Searching Authority.

Name and mailing address of the receiving Office Mail Stop PCT, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-3201	Authorized officer Nhu Thuy Tran Telephone No. (571)272-9063
---	---

Form PCT/RO/106 (April 2007)

ANNEX A TO FORM PCT/RO/106

International application No.

PCT/US2013/043453

The receiving Office has found the following defects in the international application as filed:1. As to **signature** of the international application (Rules 4.15, 26.2bis(a) and 90.4), the request:

- a. ☐ is not signed* by the applicant or, if there is more than one applicant, by at least one of them
- b. ☐ is not accompanied by the statement referred to in the check list in Box No. IX of the request explaining the lack of the signature of an applicant for the designation of the United States of America
- c. ☐ is signed by what appears to be an agent/common representative but:
☐ the international application is not accompanied by a power of attorney appointing him
☐ the power of attorney accompanying the international application is not signed by all the applicants
- d. ☐ other (*specify*):

* Although Rule 4.15 requires that all applicants must sign the request (e.g. including all inventors/applicants for the designation of the United States of America), for the purposes of Article 14(1)(a)(i), if there is more than one applicant, it shall be sufficient that the request be signed by one of them (Rule 26.2bis(a)).

However, the applicant's attention is drawn to the fact that the national law applied by each designated Office may require, in connection with the processing of the international application in the national phase, that the applicant furnish the confirmation of the international application by the signature of any applicant for the designated State who has not signed the request (Rule 51bis.1(a)(vi)).

2. As to indications concerning the **applicant*** who is entitled, according to Rule 19.1, to file the international application with the receiving Office, the request (Rules 4.4, 4.5 and 26.2bis(b)):

- a. ☐ does not properly indicate the applicant's name (*specify*):
- b. ☐ does not indicate the applicant's address
- c. ☐ does not properly indicate the applicant's address (*specify*):
- d. ☐ does not indicate the applicant's nationality
- e. ☐ does not indicate the applicant's residence

☐ Further observations about indications concerning other applicants (if applicable):

* Although Rules 4.4 and 4.5 require indications concerning the applicant, or if there are several applicants, of each of them, for the purposes of Article 14(1)(a)(ii), if there is more than one applicant, it shall be sufficient that the indications required under Rule 4.5(a)(ii) and (iii) be provided in respect of one of them who is entitled according to Rule 19.1 to file the international application with the receiving Office (Rule 26.2bis(b)).

However, the applicant's attention is drawn to the fact that the national law applied by each designated Office may require, in connection with the processing of the international application in the national phase, that the applicant furnish any missing indication required under Rule 4.5(a)(ii) and (iii) in respect of any applicant for the designated State (Rule 51bis.1(a)(vii)).

3. As to the **language** of certain elements of the international application, other than the description and claims (Rules 12.1(c) and 26.3ter(a) and (c)):

- a. ☐ the **request** is not in a language of publication accepted by this receiving Office; (the) language(s) accepted by this receiving Office is/are:
- b. ☐ the **text matter of the drawings** is not in the language in which the international application is to be published, which is:
- c. ☐ the **abstract** is not in the language in which the international application is to be published, which is:

4. The **title** of the invention:

- a. ☐ is not indicated in Box No. I of the request (Rule 4.1(a))
- b. ☐ is not indicated at the top of the first sheet of the description (Rule 5.1(a))
- c. ☐ as appearing in Box No. I of the request is not identical with the title heading the description (Rule 5.1(a))

5. As to the **abstract** (Rules 8 and 26.1(b)):

- ☐ the international application does not contain an abstract

ANNEX B1 TO FORM PCT/RO/106

International application No.

PCT/US2013/043453

This receiving Office has found that, with regard to the presentation of the **text matter of the international application as filed**, the physical requirements are not complied with to the extent that compliance therewith is necessary for:

1. ☒ reasonably uniform international publication (Rules 11 and 26.3(a)(i)) (*defects to be specified*):

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2. ☐ satisfactory reproduction (Rules 11 and 26.3(b)(i))

Further observations (if necessary):

ALL NEW SHEETS OF DESCRIPTION, CLAIM AND ABSTRACT ARE REQUIRED

ANNEX C1 TO FORM PCT/RO/106

International application No.

PCT/US2013/043453

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Further observations (if necessary):

PATENT COOPERATION TREATY

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COMMUNICATION IN CASES FOR WHICH
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Confirmation No: 1081

Date of mailing (day/month/year)	07 Jun 2013
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Applicant's or agent's file reference
2294PCT

REPLY DUE	See paragraph 1 below
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International application No.
PCT/US2013/043453

International filing date (day/month/year)	30 May 2013
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Applicant
CYTOJENE CORP.

1. ☐ REPLY DUE within _____ months/days from the above date of mailing
- ☒ NO REPLY DUE, however, see below _____
- ☒ IMPORTANT COMMUNICATION
- ☐ INFORMATION ONLY

2. COMMUNICATION:

The attention of the applicant is directed to the indications in Box No. II and/or III of the request form in respect of the above indicated international application. In respect of " CYTOJENE CORP" in Box No. [II] of the request, the applicant has been indicated as "applicant for the purposes of all designated States except the United States of America" and in respect of " CHEN, YONG QIN" in Box No. [III] of the request, the applicant/inventor has been indicated as "applicant for the purposes of the United States of America only."The applicant is herewith informed that, due to a change in the national law of the United States of America, as from 16 September 2012, it is no longer necessary in all cases to indicate the inventor(s) as applicant(s) for the purposes of the designation of that State. If the applicant(s) indicated as "applicant for the purposes of all designated States except the United States of America" is qualified to be an applicant under U.S. law, the applicant may wish to consider submitting to the International Bureau within 30 months from the priority date a request under PCT Rule 92bis for the recording of a change, requesting that the applicant indicated in the request form as "applicant for the purposes of all designated States except the United States of America" be indicated as "applicant for the purposes of all designated States", and that the applicant/inventor indicated in the request form as "applicant for the purposes of the United States only" be indicated as "inventor only."

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Authorized officer
Nhu Thuy Tran
Telephone No. (571)272-9063

Form PCT/RO/132 (July 1992; reprint January 2004)

AP03Rec'dPCT 17 JUN 2013

**BEFORE THE UNITED STATES
RECEIVING OFFICE**

Applicant : CYTOJENE CORP. Docket no. 2294PCT
Serial No : PCT/US2013/043453
Filed : May 30, 2013
For : FLOW CYTOMETER
Authorized Officer: Nhu Thuy Tran

MAIL STOP PCT
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RESPONSE TO FORM PCT/RO/106

This communication responds to an "Invitation to Correct Defects in the International Application," Form PCT/RO/106, dated the 07 Jun 2013, for the patent application identified above. A copy of the Form PCT/RO/106 dated 07 June 2013, accompanies this correspondence.

REMARKS

Form PCT/RO/106

Annex B1 included in the Form PCT/RO/106 issued by the United States Receiving Office ("RO/US") on 07 Jun 2013, in box 1.a. requires submission of replacement Description, Claims and Abstract for the patent application identified above because the identified portions of the patent application filed May 30, 2013, "do not admit of direct reproduction."

Accompanying this correspondence are replacement copies of this patent application's Description, Claims and Abstract printed on A4 paper directly from the Adobe Acrobat PDF files that were transmitted electronically to the USPTO on May 30, 2013.

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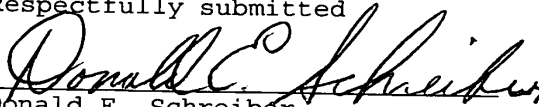
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Because the accompanying replacement portions of this patent application were printed directly from the files transmitted to the USPTO on May 30th, surely the accompanying replacement pages surely introduce no new matter into this patent application.

Respectfully submitted


Donald E. Schreiber
Reg. No. 29,435

Dated: 14 June, 2013

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Agent for Applicant(s)

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FLOW CYTOMETERTechnical Field

5 The present disclosures relate generally to the technical field of flow cytometry and, more particularly, to the structure and operation of an improved flow cytometer together with various individual subassemblies included therein.

Background Art

10 Flow cytometry is a biophysical technique employed in cell counting, sorting, biomarker detection and protein engineering. In flow cytometry, cells suspended in a stream of liquid pass through an electronic detection apparatus. Flow cytometry allows simultaneous multiparametric analysis of physical and/or
15 chemical characteristics of up to thousands of cells per second.

Flow cytometry has various applications including in the fields of molecular biology, pathology, immunology, plant biology and marine biology. Flow cytometry also has broad
20 application in medicine (especially in transplantation, hematology, tumor immunology and chemotherapy, prenatal diagnosis, genetics and sperm sorting for sex preselection). In marine biology, the autofluorescent properties of photosynthetic plankton can be exploited by flow cytometry in
25 characterizing abundance and community composition. In protein engineering, flow cytometry is used in conjunction with yeast display and bacterial display to identify cell surface-displayed protein variants with desired properties. A common variation of flow cytometry is physically sorting
30 particles based on their properties thereby purifying a population of interest.

An entire flow cytometer system includes the following main components.

- 35 1. A flow cell through which a liquid stream, usually called a sheath flow, liquid or fluid, carries and hydrodynamically aligns cells or particles so that they pass single file through the flow cell.

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2. A measuring subsystem system coupled to the flow cell that detects cells or particles passing through the flow cell and is usually either:
- a. an impedance or conductivity measuring subsystem; or
 - b. an optical illumination subsystem together with an optical sensing subsystem.
3. A conversion subsystem for converting the output signal from the measuring subsystem into computer processable data.
4. A computer for analyzing the data produced by the conversion subsystem.

The optical illumination subsystems provides a collimated and then focused beam of light, usually laser light of a single wavelength, that impinges upon the hydrodynamically-focused stream of liquid passing through the flow cell. Accordingly, the flow cytometer system may have one or more light sources that may include:

1. one or more lamps, e.g. mercury or xenon;
2. one or more high-power water-cooled lasers, e.g. argon, krypton or dye laser;
3. one or more low-power air-cooled lasers, e.g. argon (488 nm), HeNe (red-633 nm), HeNe (green) and HeCd (UV); and/or
4. one or more diode lasers (blue, green, red and violet).

The optical sensing subsystem includes one or more detectors aimed where the focused liquid stream passes through the light beam. Such detectors may include:

1. detectors in line with the light beam (Forward Scatter or FSC);
2. detectors perpendicular to it (Side Scatter or SSC); and
3. fluorescence detectors.

Each suspended particle passing through the beam scatters the light, and fluorescent material present in the particle or attached to the particle excited by the impinging light emit light at a longer wavelength than that of the impinging light.

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Detecting and analyzing brightness changes in a combination of scattered and fluorescent light at each detector (one for each fluorescent emission peak) permits deriving various types of information about the physical and chemical structure of each individual particle. FSC correlates with cell volume. Due to light being scattered off of internal components within a cell, SSC depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). Some flow cytometers omit a fluorescence detector and detect only scattered light. Other flow cytometers form images of each cell's fluorescence, scattered light, and transmitted light.

The flow cytometer system's conversion subsystem, which may include one or more amplifiers which may be either linear or logarithmic, generally includes one or more Analogue-to-Digital Converters ("ADCs") for converting the measuring subsystem's output signal into data that is then processed by the computer.

Modern flow cytometers usually include up to four (4) lasers and numerous fluorescence detectors. Increasing the number of lasers and detectors permits labeling cells with several different antibodies, and can more precisely identify a target population by their phenotypic markers. Some instruments can even capture digital images of individual cells, allowing for the analysis of a fluorescent signal location within or on the surface of cells.

Sample Illumination

In most of the instruments, particles of interest, such as blood cells or microspheres, are carried by the sheath flow using hydrodynamic focusing into a viewing zone inside a cuvette or jet stream and illuminated there by a focused laser beam. The technique provides the means to accurately identify and count particles of interest without being overwhelmed by background noise occurring outside a registration time window (Practical Flow Cytometry, Howard M. Shapiro, Wiley (2003) ISBN 0471411256). To increase detection sensitivity, the cross section of the focused laser beam is usually elliptical, with

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the minor axis along the direction of flow. In order to maintain the threshold integrity, the laser profile must have a smooth or bell shaped profile along the flow direction. One common method for producing such an beam is to elongate a
5 nearly collimated circular Gaussian beam along the direction of flow with a beam expander made of either prism or cylindrical lens pair, then focus the beam down with a spherical lens. Since the shape of the beam at the focus is the spatial Fourier transform of the beam at far field, this
10 produces a Gaussian shaped elliptical spot with minor axis along the flow.

Conventional lasers are expensive, bulky and power hungry. More recently, laser diodes ("LD") have become available. Differing from conventional lasers, the new generation of LDs
15 is cost effective, compact and power efficient, and shows great promise for new generation of compact biomedical instruments. A LD emits light having an elliptical cross-section with the ellipse's major axis, frequently called the fast axis, perpendicular to the LD's junction, and the ellipse's minor
20 axis, frequently called the slow axis, parallel to the LD's junction. Unfortunately, the beam quality of a typical LD, particularly along its fast axis, leaves much to be desired, preventing its wide acceptance in flow cytometric applications.

In principle, the quality of the LD beam can be
25 significantly improved by spatial filtering. If a small pinhole or a single mode optical fiber is positioned at the focal point of a lens, such that it only accepts the lowest order spatial mode, the beam passing through the pinhole or single mode optical fiber will be of nearly perfect Gaussian
30 shape. United States Patent no. 5,788,927 discloses that such a beam can then be collimated and expanded in the direction of flow through the cytometer, and finally focused down to an elliptical shaped Gaussian beam with minor axis along the flow direction. Unfortunately, the size of desktop instrumentation
35 limits the diameter of a pinhole to less than 5 micron. The core size of a visible wavelength single mode optical fiber also has a similar dimension. The challenge to manufacture such a precision spatial filter and maintain its long-term

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stability not only increases the cost of LD based laser system, but also reduces its reliability.

More recently, in an effort to reduce the possible side lobes due to the edge effect of limited numerical aperture of collimating lens, United States Patent no. 6,713,019 ("the '019 patent") discloses rotating the LD by ninety degrees (90°) such that its slow axis is parallel to the direction of flow. A beam diffusing section, such as a concave cylindrical lens, is then introduced to diffuse the collimated beam in the direction perpendicular to the flow, followed by a beam spot forming section, such as a spherical focusing lens, to form an elliptical spot within the cytometer's particle viewing zone. As described in detail in the '019 patent, the laser beam after the spot forming section is extremely astigmatic. In particular, the width of the beam at the viewing zone in the direction perpendicular to the flow is comparable or even wider than the width of the flow channel. This not only reduces the amount of laser energy impinging upon the particle and consequently the signal intensity, but also increases undesired background scattering from the liquid-flow cell interface. Instead of rotating the LD, United States Patent nos. 7,385,682 and 7,561,267 disclose using a large numerical aperture aspheric lens for LD collimation. Such a design, however, cannot correct the fringe effect inherent in the LD's beam profile. Consequently, there presently exists a need for a simple LD based optical system for use in flow cytometers that can reliably produce a focused elliptical beam with near Gaussian shape along its minor axis and a width along major axis.

Viewing Zone Microscope Objective

Modern flow cytometers include a spatial filter, usually either a mechanical pinhole or a large core optical fiber, located at an image location of an objective lens to prevent undesired background light from entering the cytometer's detector(s). Because particles remain in the cytometer's viewing zone for a few microseconds, microscope objectives with large numerical aperture must be used to maximize light

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collection efficiency. To support multiple spatially separated excitation laser beams in flow cytometers, as disclosed in United States Patent no.4,727,020, it is also desirable to use an objective with large field of view. In order to achieve
5 these goals, United States Patent nos. 6,5100,07 and 7,110,192 disclose an objective design using modified apochromat with a gel-coupled or epoxy bonded near hemisphere lens as the optical element closest to the sample that is followed by multiple meniscus lenses. While such microscope objectives provide both
10 a satisfactory numerical aperture and field of view, they significantly sacrificed image quality thereby:

1. limiting effective use of the spatial filter; and
2. exhibiting poor background light discrimination.

Further, such refractive microscope objectives are bulky,
15 expensive to manufacture and often exhibit severe chromatic aberration. To overcome these limitations Published Patent Cooperation Treaty ("PCT") Patent Application no. W0 01/27590 discloses an alternative objective design based on a spherical concave mirror. The design offers large numerical aperture and
20 good image quality along the optical axis. However, due to its poor off-axis characteristics, such a design is unsuitable for flow cytometers having multiple, spatially separated laser beams.

25 **Sheath Liquid Supply**

The performance of a flow cytometer depends critically on a stable liquid sheath flow. In particular, flow cytometers that have multiple spatially separated excitation laser beams or perform droplet sorting rely on a constant velocity of the
30 liquid sheath flow for timing synchronization. As disclosed in United States Patent no. 5,245,318, conventional flow cytometers provide a stable liquid sheath flow by using an air-tight fluidic system that either:

- 35 1. applies constant air pressure in a sheath liquid reservoir to push the fluid through the flow cell;
or
2. by sucking the fluid from the sheath liquid reservoir through the flow cell using a vacuum pump.

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These systems are bulky, expensive to manufacture and prone to failure. More recently, United States Patent no. 8,187,888 discloses including a sheath liquid subsystem that pumps the liquid sheath flow from the sheath liquid reservoir into the viewing zone and a waste sheath liquid pump that pumps waste sheath liquid from the viewing zone into the waste tank. Although it appears that the disclosed sheath liquid subsystem has never been used in velocity critical flow cytometers, this patent reports that the disclosed sheath liquid subsystem overcomes most of the drawbacks of conventional sheath liquid flow stabilization by:

1. damping pump pulsations by locating:
 - a. one fluidic capacitor between the sheath liquid pump and the flow cell; and
 - 15 b. another fluidic capacitor between the flow cell and the waste pump; and
2. a pump controller whose operation is responsive to a pressure sensor that measures the pressure differential between the inlet and outlet of the flow cell.

20 The disclosed sheath liquid subsystem has other limitations. For example, the pressure sensor located near the outlet of the flow cell could be a potential source of contamination.

25 **Peristaltic Pump**
Sample Liquid Supply

Peristaltic pumps are volumetric pumps in which a set of linearly or circularly moving rollers progressively compresses a compressible tube to propel the fluid through the tube. Peristaltic pumps are widely used particularly to pump clean/sterile or aggressive fluids to avoid cross contamination with exposed pump components.

30 Conventional peristaltic pump exhibits a pulsation each time a roller rolls off the tube near the pump outlet, caused by the temporary increase of tube volume when the compressed tube expands back to its original shape. The pulsation is undesirable in applications that require smooth flow. Many attempts have been made in the past to reduce the pulsation. For example, United States Patent nos. 3,726,613 and 3,826,593

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introduced a cam operated pusher which synchronously exerts an external pressure on the tube to compensate for the tube expansion. In United States Patent no. 4,834,630, a plurality of tubes mounted on segmented rollers are joined together at the pump inlet and outlet by T-shaped couplers such that pulsations from individual tubes would be reduced by averaging. United States Patent no. 7,645,127 proposed a pump tube with slightly larger inner diameter near the inlet so that the tube decompression near the pump outlet is compensated by the compression of a larger volume tube near the inlet. The various methods either significantly increased the complexity of the peristaltic pump or had limited success in reducing the pulsation effect.

15 Multicolor Fluorescence Detection

In many multicolor fluorescence detection instrumentations, such as flow cytometers, (Practical Flow Cytometry, Howard M. Shapiro, Wiley (2003) ISBN 0471411256), the fluorescence light emitted from the object of interest is:

1. collected by a microscope objective;
2. reimaged through a small pinhole or a multimode optical fiber;
3. then collimated and separated into multiple colored bands; and
4. finally detected by photo detectors such as photomultiplier tube (PMT), PIN photodiode or avalanche photodiode (APD).

A PMT is essentially a special type of electron tube. This "pre-semiconductor age" device is bulky and expensive. In addition, it has poorer quantum efficiency and less reproducible spectral response than silicon based semiconductor detectors, particularly in the biologically important red to near infrared spectral region. Despite the disadvantages, PMT has excellent noise characteristics. For example, the dark current of a typical 13mm PMT (e.g., the R9305 from Hamamatsu Corporation of Japan) is only about 1nA. In contrast, an APD's dark current would be 10 times greater even if its active area were reduced to 1/20th of that of the PMT. As a result, PMT

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has been the de-facto low-level light detector in many commercial fluorescence detection flow cytometers. Only in certain scientific applications where event rate is low and dark current may be discriminated against by expensive photon-counting techniques that the PMT has been replaced by APD detectors. (c.f., High-Throughput Flow Cytometric DNA Fragment Sizing, A.V. Orden, R.A. Keller, and W. P. Ambrose, *Anal. Chem.*, 2000, 72 (1), p 37-41). More recently, a Geiger mode APD array was also promoted as PMT replacement. (For example, the multi-pixel photon counter of Hamamatsu Photonics of Japan and the solid-state photomultiplier of SensL Inc. of Ireland.) These detectors, however, also have high dark current and are nonlinear at high event rate.

The only industry where APD has found wide acceptance is in optical communication. It is known that if the APD's active area is reduced to less than 1mm^2 , the corresponding dark current will be reduced to the same level as a PMT. In optical communication, the light is a laser beam out of single mode optical fiber. Such a beam can be easily collimated then focused down to an area much smaller than 1mm^2 . It should be noted that the color separation devices used in the fluorescence light detection instruments, as described in United States Patent no. 6,683,314 and references therein, are almost identical in function and architecture to the wavelength division multiplexers (WDM) widely used in optical communication, as described in United States Patent nos. 4,482,994, and 5,786,915. A fundamental reasons preventing the use of small area APD in fluorescence detection instrumentation is the well known theorem of etendue conservation: the fluorescence light coming through a pinhole or multimode optical fiber is an extended light source with an etendue hundreds of times greater than that of a laser beam out of a single mode optical fiber. Consequently, as illustrated in FIG. 26, it cannot be collimated over an extended distance unless the diameter of the beam is significantly expanded. Unfortunately, the larger the beam diameter, the greater the technical challenge to focus it down to a small spot. Since efficient color separation can only be accomplished

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economically with collimated light beam, small area APD has not been considered viable for multicolor fluorescence light detection applications. Clearly, a technology capable of collimating a large etendue light beam over an extended
5 distance without significantly expanding the beam diameter would be highly desirable. Such a technology would enable a WDM like device for fluorescence light detection with characteristics comparable to low noise semiconductor detectors.

10

Disclosure

The present disclosure provides an improved flow cytometer together with various improved components included therein.

It is an object of the present disclosure to provide a
15 simple and reliable diode laser based optical system capable of delivering a focused laser beam of elliptical cross section with a Gaussian like intensity distribution along its minor axis and a width along major axis optimized for flow cytometric applications.

20 An object of the present disclosure is an imaging quality microscope objective that is easy to manufacture. has long working distance, large numerical aperture. large field of view and minimal chromatic aberration.

An object of the present disclosure ia a simple fluidics
25 system for flow cytometers that is not only reliable, compact and easy to manufacture, but also capable of supporting velocity critical applications such as in instruments with multiple spatitally separated excitation laser beams or in droplet sorters.

30 A object of the present disclosure is simple design for a peristaltic pumb can provide a pulseless liquid flow is highly desirable.

An object of the present disclosure to provide a peristaltic pump with minimal pulsation.

35 A object of the present disclosure to provide a peristaltic pump that is simple to manufacture and operate.

an object of the present disclosure to provide a device capable of collimating a light beam from an extended light

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source over an extended distance without significantly expanding the said beam diameter. It is also an object of the present disclosure to provide a WDM system using the said device to separate the said light beam into multiple colored bands.

- 5 Further, it is an object of the present disclosure to provide such a WDM system that is compatible with low noise semiconductor detectors. In addition, due to the diversity of fluorescent probes, it is an object of the present disclosure to provide such a WDM system that is reconfigurable.

10 Disclosed herein is a flow cytometer that includes:

1. a LD based optical subsystem for impinging a beam of light upon particles passing through a viewing zone;
2. a composite microscope objective for gathering and
15 imaging light scattered from or fluoresced by particles passing through the viewing zone;
3. a fluidic subsystem for supplying a liquid sheath flow to the viewing zone;
4. a peristaltic pump for injecting into the liquid
20 sheath flow a liquid sample flow carrying particles that passes together with the liquid sheath flow through the viewing zone;
5. a multimode optical fiber that receives scattered and fluoresced light from the viewing zone that the composite microscope objective gathers and images;
25 and
6. a wavelength division multiplexer for optically separating into color bands light received via the optical fiber .

30 The LD based optical subsystem in accordance with the present disclosure for illuminating particles passing through the flow cytometer's viewing zone generally includes:

1. a laser diode oriented with its slow axis parallel to the direction of flow;
2. a collimating lens that converts the diverging beam
35 from the LD into a collimated beam of elliptical shape with its major axis perpendicular to the flow;

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3. a focusing lens system that reduces the laser beam at the viewing zone to an optimal width in the direction perpendicular to the flow; and
4. finally a high power cylindrical focusing element placed in the proximity of the viewing zone with its axis perpendicular to the direction of flow.

The high power cylindrical focusing element transposes the far field profile of the LD along its slow axis to its Fourier conjugate at the viewing zone along the direction of flow, while maintains the transverse beam profile, such that the laser beam profile at the viewing zone is optimal for flow cytometric applications.

The composite microscope objective in accordance with the present disclosure generally includes:

1. a concave spherical mirror;
2. a transparent aberration compensation plate with the flow cytometer's a viewing zone being located between the mirror and the plate. Scatter and fluorescence light emitted from particles in the viewing zone is collected by the mirror and reflected back toward the compensation plate. Optical aberrations originating from the mirror are significantly reduced after light passes through the compensation plate. In one embodiment of the present disclosure, the viewing zone is located inside a flow cell provided by rectangular glass cuvette with a small rectangular channel through which a particle carrying liquid flows. The concave mirror is made of an optically transparent material, such as glass or optical quality plastics, of piano-convex shape with a highly-reflective coating on the convex side for internal reflection. The piano-side of the mirror is either gel-coupled or bonded to one side surface of the cuvette. The plano-aspheric compensation plate is made of a transparent material, such as glass or optical quality plastics, with the piano side gel-coupled or bonded to the opposite side of the cuvette. The piano-convex shaped mirror and the aspheric compensation plate may also be formed integrally with the cuvette. In yet another embodiment of the present disclosure, the viewing zone is in a jet stream with both the concave

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mirror and the compensation plate being free standing from the viewing zone, and the mirror is preferably a front surface concave mirror.

5 The fluidic system in accordance with the present disclosure generally includes a sheath liquid reservoir from which a liquid pump draws sheath liquid. Sheath liquid then flows from the liquid pump to an inlet of a T-coupling. One outlet arm of the T-coupling connects to a bypass that returns a fraction of the pumped sheath liquid back to the sheath
10 liquid reservoir with the returned sheath liquid flowing into air within the sheath liquid reservoir. A second outlet arm of the T-coupling connects to a sheath route that includes a reservoir capsule followed by a particle filter and then the flow cell. The sheath liquid exiting the flow cell then goes
15 to the waste tank. The fluidic resistance along the bypass is designed to be lower than the fluidic resistance along the sheath route. Consequently, only a small fraction of the sheath liquid goes through the flow cell. Note that typical sheath flow rate in flow cytometric applications is a few tens
20 of milliliter per minute. The bypass therefore permits using higher flow rate liquid pumps that not only are much less expensive and more reliable, but also operates at higher pulsation frequency which is much easier to attenuate. Since the exit of the bypass route connects to air, it also serves
25 as a large fluidic capacitor for significantly reducing pulsation in sheath liquid flowing along the sheath route. During operation, the inlet portion of the filter cartridge is filled with air. Therefore, the filter cartridge also serves as a fluidic capacitor, for further reducing pulsation in the
30 sheath liquid at the flow cell to negligible level. Due to the large fluidic resistance at the flow cell, the air trapped near the inlet of the filter cartridge becomes compressed. If the liquid pump is turned off, the compressed air in filter cartridge being pushed back towards the sheath liquid reservoir
35 becomes stored in the reservoir capsule whose size is chosen to prevent the trapped air from reaching the T-coupling.

The peristaltic pump in accordance with the present disclosure generally includes a plurality of rollers located

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at the periphery of a rotor that moves the rollers circularly inside a housing's arcuate curved track and a compressible tube that the rollers compress against the track. In one embodiment of the present disclosure, the track of the peristaltic pump's housing has one recess so the compressible tube is progressively decompressed to full expansion then compressed to full closure every time one of the rollers moves past the recess. The location and shape of the recess maintains the total volume of liquid within the compressible tube from the recess to the pump's outlet substantially invariant. The effect of tube expansion as a roller moves past the pump's outlet is compensated by the tube compression when a different other roller immediately upstream of the pump outlet moves into the recess' compressing section. In another embodiment of the present disclosure, the track of the pump housing may contain a plurality of recesses, providing for a plurality of roller upstream of the pump outlet to progressively modify the tube compression in multiple sections along the compressible tube. The locations and shapes of the plurality of recesses are designed such that the modification of tube compression at these sections substantially compensates the effect due to the tube expansion near the pump outlet. In yet another embodiment of the present disclosure, the compressible tube is kept fully closed underneath the roller except in the inlet and exit sections. A variable speed motor is used to drive the pump. When a roller reaches the exit section, the motor's rotation programmatically speeds up to compensate for the tube's expansion.

The wavelength division multiplexer ("WDM") in accordance with the present disclosure generally includes at least two optical elements. The first optical element collimate a beam of light received from an extended light source, such as the light from a pinhole or from a multimode optical fiber. The first optical element magnifies the extended light source, for example, as defined by the pinhole, or the core of the multimode optical fiber, to an image having a size similar to the effective cross section of the first optical element thereby creating a collimated light beam between the first

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optical element and its image. A second optical element is positioned near the image, and relays the first optical element with unit magnification down the optical path. In this way the second optical element effectively doubles the collimated path length. Additional optical elements in the same 1:1 image relay configuration may also be included in the present disclosure to further extend the collimated optical path. The cascaded unit-magnification image relay architecture of the present disclosure provides a greatly extend the collimated optical path length without large beam expansion. As a result, WDM techniques well-established in the optical communication industry can be readily adapted for fluorescence light detection. In particular, multiple colored bands present in the beam of light can be separated using dichroic filters, located along the optical path with the separated light being tightly focused into small spots compatible with low noise semiconductor photodetectors.

In one embodiment of WDM, the first optical element is a lens and the second element is a concave mirror, although it is apparent to those skilled in the art that other types of refractive and/or reflective optical components may also be used to achieve the same design goal. Similar to its counterpart in optical communication, the optical path in the WDM of the present disclosure may be folded using dichroic filters. In one embodiment of the present disclosure, the light path is folded into a zig-zag configuration. Preferably, to facilitate the flow cytometer's reliable reconfiguration each dichroic filter is bonded to a mechanical holder having a reference surface that is optically parallel to the filter's reflective surface. As a result, all of the WDM's filters can be accurately positioned along the optical path by referencing the filter's holder against a common optical flat.

In another embodiment of the present disclosure, the collimated beam passing through the dichroic filter is further branched out into multiple colored bands using secondary dichroic filters. It is apparent to those skilled in the art that dichroic filters may be inserted anywhere along the long, narrow and collimated beam path afforded by the present

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disclosure's relay imaging to thereby permit delivering a tightly focused beam to photo detectors using a variety of optical configurations, such as the star configuration discussed in United States Patent no. 6,683,314, the branched configuration discussed in United States Patent no. 4,727,020 and other types of WDM optical configuration widely practiced in the optical communication industry. Instead of concave mirrors, the WDM may be replaced by curved dichroic filters to further increase the number of colored bands selected by the WDM.

These and other features, objects and advantages will be understood or apparent to those of ordinary skill in the art from the following detailed description of the preferred embodiment as illustrated in the various drawing figures.

Brief Description of Drawings

FIG. 1 is a diagram schematically illustrating a preferred embodiment of a flow cytometer in accordance with the present disclosure that includes:

- a) a LD based optical illumination subsystem;
- b) a composite microscope objective upon which light emitted from the LD based optical illumination subsystem impinges, the composite microscope objective having a fluid-passing channel formed therethrough with a particle illumination viewing zone located inside a cuvette thereof;
- c) a fluidic system for supplying a pulsation free flow of sheath liquid to the fluid-passing channel formed through the composite microscope objective;
- d) a peristaltic pump for introducing a pulsation free flow of sample liquid that carries cells or particles to be analyzed into the sheath flow of liquid supplied by the fluidic system; and
- e) a wavelength division multiplexer ("WDM") having a zig-zag configuration for separating a beam of light into several different colored bands, the WDM receiving via an optical fiber light that is scattered from cells or particles as they pass

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through the composite microscope objective's fluid-passing channel and are illuminated therein by light emitted from the LD based optical illumination subsystem.

5 FIG. 2 is a schematic view depicting a typical high power edge emitting LD that illustrates the fast and slow axes of light emitted therefrom.

FIG. 2A shows a typical far field profile for a laser beam emitted from the LD chip depicted in FIG. 2.

10 FIG. 3A depicts a 3-dimensional view of a conventional, prior art LD based optical illumination subsystem for flow cytometric instruments together with the system's flow cell.

FIG. 3B depicts a typical time dependent profile of light scattering from a cell or particle passing through the laser beam depicted in FIG. 3A at the focus thereof within the system's flow cell.

15 FIG. 4A is an elevational view of an alternative prior art LD based optical illumination subsystem configuration across the liquid flowing through the fluid-passing channel that improves the beam profile at the focus thereof in the flow cytometer system's viewing zone.

FIG. 4B is a plan view along the liquid flowing through the fluid-passing channel of the alternative prior art LD based optical illumination subsystem depicted in FIG. 4A.

25 FIG. 5A is an elevational view across liquid flowing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1 wherein the LD's slow axis oriented transversely to the liquid flow.

FIG. 5B is a plan view along liquid flowing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1 wherein the LD's slow axis oriented transversely to the liquid flow.

30 FIG. 5C depicts a typical time dependent profile of light scattering from a cell or particle passing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1.

FIG. 6 is a perspective view of an alternative embodiment of a LD based optical illumination subsystem in accordance with

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the present disclosure adapted for use in a flow cytometer system in which a jet stream of liquid passes through the viewing zone.

FIG. 6A is an enlarged perspective view of the alternative embodiment of a LD based optical illumination subsystem of depicting in greater detail the jet stream of liquid passes through the viewing zone.

FIG. 7 is a perspective view of an alternative embodiment LD based optical illumination subsystem in accordance with the present disclosure adapted for use in a flow cytometer system which orients the LD's slow axis parallel to the direction of liquid flowing through the fluid-passing channel of the composite microscope objective depicted in FIG. 1.

FIG. 8 is a perspective view of a composite microscope objective in accordance with the present disclosure adapted for use in the flow cytometer system depicted in FIG. 1, the composite microscope objective having a fluid-passing channel formed therethrough with the particle illumination viewing zone located inside a cuvette therein.

FIG. 9A is a cross-sectional elevational view of the composite microscope objective taken along the line 9A-9A in FIG. 8 that includes ray traces from three (3) spatially separated locations in the viewing zone to an image plane for the objective illustrating scatter and fluorescence emission propagation.

FIGs. 9B1-9B3 are spot diagrams near the image plane depicted in FIG. 9A for the three (3) spatially separated light emission locations depicted in FIG. 9A.

FIG. 10 is a cross-sectional elevational view similar to that of FIG. 9A for an alternative embodiment composite microscope objective in accordance with the present disclosure that includes ray traces from three (3) spatially separated locations in a viewing zone therein to an image plane for the objective illustrating scatter and fluorescence emission propagation.

FIG. 11 is a perspective view of yet another alternative embodiment a composite microscope objective in accordance with the present disclosure adapted for use in the flow cytometer

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system depicted in FIG. 1, the alternative embodiment composite microscope objective having a fluid-passing channel formed therethrough with the particle illumination viewing zone located inside a cuvette therein.

5 FIG. 12 is a perspective view of yet another alternative embodiment a composite microscope objective in accordance with the present disclosure adapted for use in the flow cytometer system depicted in FIG. 1, the alternative embodiment composite microscope objective being adapted for a viewing zone that is
10 located inside the jet stream depicted in FIGs. 6 and 6A.

FIG. 13 is a perspective view of yet another alternative embodiment a composite microscope objective in accordance with the present disclosure adapted for use wherein a viewing zone is located on the surface of a microscope slide.

15 FIG. 14 is a schematic diagram depicting a fluidic subsystem in accordance with the present disclosure for supplying stable liquid sheath flow to a flow cytometers flow cell that includes:

- 20 1. a small capsule located between a sheath liquid pump and the flow cell; and
2. a particle filter located between the small capsule and the flow cell,

both the particle filter and the small capsule providing air reservoirs for dampening pump pulsations.

25 FIG. 15 is a schematic diagram depicting an alternative embodiment fluidic subsystem similar to that illustrated in FIG 14 that replaces the small capsule with a length of tubing for providing an air reservoir.

30 FIGs. 16A and 16B are histograms that compare measured particle flight times at the flow cell when the inlet portion of the particle filter has air trapped therein (FIG. 16A), and when there is no air within the fluidic subsystem between sheath liquid pump the and the flow cell (FIG. 16B).

35 FIG. 17 is a perspective view of a 3-roller peristaltic pump in accordance with the present disclosure depicting the pump's rollers, tube and surrounding pump housing.

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FIGs. 18A through 18D depict simplified views for several states of the 3-roller peristaltic pump depicted in FIG. 17 with the rollers in different locations.

FIGs. 19 is a detailed longitudinal cross-sectional view of the peristaltic pump's tube being partially compressed by the pump's roller.

FIG. 19A and 19B are detailed cross-sectional views orthogonal to the peristaltic pump's tube's length taken along the lines 19A and 19B in FIG. 19 illustrating the tube's partial compression by the roller.

FIGs. 20A and 20B are schematic diagrams illustrating the pump's rollers and tube viewed along the pump's circular coordinates to depicting the pulseless provided by the peristaltic pump.

FIGs. 21 is graph depicting the functional relationship with respect to the roller position when it rolls off the exit section of the compressible tube of:

1. the total volume of liquid in the exit half of the pump; as well as
2. the liquid volumes in the pump's:
 - a. recess section; and
 - b. exit section.

FIGs. 22 is a simplified plan view of a 4-roller peristaltic pump in accordance with the present disclosure.

FIGs. 23 is a simplified plan view of a 6-roller peristaltic pump in accordance with the present disclosure.

FIG. 24A is a longitudinal cross-sectional illustration of rollers and a compressible tube for a pulsation minimizing 3-roller peristaltic pump in accordance with the present disclosure having a rotor with programmable speed.

FIG. 24B is simplified plan view illustrating the pulsation minimizing 3-roller peristaltic pump in accordance with the present disclosure having a rotor with programmable speed.

FIG. 24C is a graph depicting for the pulsation minimizing peristaltic pump depicted in FIG. 24B having a programmable speed rotor:

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1. negative volume change rate with respect to the roller position;
2. rotor speed; and
3. pump flow rate.

5 FIG. 25 is a diagram illustrating optical ray tracing for an exemplary 6 port wavelength division multiplexer ("WDM") using zig-zag configuration in accordance with the present disclosure.

FIG. 26 is a diagram illustrating ray tracing of prior art collimating devices showing the device's limitation in collimating an extended light source.

FIG. 27 is a perspective illustration of an embodiment of a 6 ports WDM using a combination of zig-zag and branched configurations in accordance with the present disclosure.

15 FIG. 28 is a perspective illustration of another embodiment of a WDM having a concave dichroic filters in accordance with the present disclosure.

FIGs. 29A and 29B are perspective illustrations depicting assembly process for constructing replaceable dichroic filter assembly for an reconfigurable WDM in accordance with the present disclosure.

FIGs. 29C is a perspective illustration of a replaceable dichroic filter assembly build in accordance with the illustrations of FIGs. 29A and 29B.

25 FIGs. 30A and 30B are perspective illustrations of a WDM in accordance with the present disclosure depicting installing into the WDM the replaceable dichroic filter assembly depicted in FIG. 29C and its removal therefrom.

30 **Best Mode for Carrying Out the Disclosure**

Flow Cytometer

FIG. 1 depicts a flow cytometer in accordance with the present disclosure identified by the general reference number

35 40. The flow cytometer 40 includes:

1. a LD based optical subsystem 50;
2. a composite microscope objective 60;

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3. a fluidic subsystem 70 for supplying a liquid sheath flow;
4. a peristaltic pump 80 for injecting a liquid sample flow that contains particles to be analyzed into the liquid sheath flow supplied by the fluidic subsystem 70, the liquid sample flow becoming hydrodynamically focused by the liquid sheath flow passes through a viewing zone with the composite microscope objective 60 gathering and imaging light scattered and/or fluoresced by particles in the viewing zone;
5. an optical fiber 852 that receives light scattered and/or fluoresced by particles in the viewing zone that the composite microscope objective 60 gathers and images; and
6. a wavelength division multiplexer 90 (" WDM 90") for optically processing scattered and/or fluoresced light received from the optical fiber 852.

Optical Subsystem 50

- The optical subsystem 50 includes a LD 501 that, as depicted in greater detail in FIG. 2, emits a diverging beam of light from an edge thereof. As more graphically depicted in FIGs 2 and 2A, the diverging beam of light has an elliptically shaped cross-sectional profile with both a major axis, a.k.a. the fast axis, and a minor axis, a.k.a. the slow axis. The diverging beam of light emitted from the LD 501 impinges upon a collimating lens 502 which converts the diverging beam of light emitted by LD 501 into a collimated beam of light having an elliptical cross-section. Although not essential, the optical subsystem 50 may also include an optional mirror 503 positioned to direct the collimated elliptical beam of light towards the composite microscope objective 60. A plano convex lens 504, positioned near the composite microscope objective 60, reduces the major axis of the elliptically shaped beam of light that is oriented perpendicular to the direction in which the liquid sample and the surrounding liquid sheath flow through the viewing zone

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within the composite microscope objective 60. At the viewing zone, the width of the elliptically shaped beam of light:

1. perpendicular to the direction in which the liquid sample flow passes through the viewing zone is preferably slightly less than the width of the liquid sheath flow; while
2. still sufficiently wide so particles in the sample flow pass through a nearly flat portion of the elliptically shaped beam of light at the beam's maximum intensity.

In accordance with the present disclosure, it is apparent to those skilled in the art that the plano convex lens 504 may be replaced by other types of optical elements such as an achromatic doublet lens or combination of spherical lenses, cylindrical lenses, and/or prism pairs. Alternatively, the mirror 503 and the lens 504 may also be replaced by a concave mirror. For polarization sensitive applications of the flow cytometer 40, an optional polarization conditioning element, such as a half-wave plate, may also be placed in the collimated section of the beam of light extending from the collimating lens 502 to the lens 504. Finally, before passing through the viewing zone the beam of light passes through a high power cylindrical lens 505, positioned adjacent to the viewing zone. As depicted in FIG. 1, the axis of the cylindrical lens 505 is oriented perpendicular to the direction in which the liquid sample flow passes through the viewing zone, and the focal length of the cylindrical lens 505 produces a tight focusing of the beam of light's minor axis at the viewing zone.

An advantage of the optical subsystem 50 in comparison with conventional LD based optical subsystem may be discerned more clearly in FIGs. 2 and 2A. Most commercially available laser diodes suitable for use in a flow cytometer emit a beam of light from an edge thereof. As depicted in FIG. 2, a gain section 509 of such a LD chip 510 is highly confined in the transverse direction indicated by an arrow 511. Consequently, to achieve high output power LD manufacturers often sacrifice beam quality, particularly along the transverse or fast axis direction that is oriented parallel to the arrow 511. FIG. 2A

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shows this characteristic of light emitted from a LD wherein multiple fringes 512 due to gain confinement are clearly visible at the far field in the minor axis direction of the emitted beam of light. It should be noted that the fringes 512 appearing in the illustration of FIG. 2A contain only a minute amount of total energy in the beam of light, and therefore have little impact on the conventional M-square characterization of the corresponding beam profile. However, as discussed in greater detail below, the fringes 512 do have a detrimental effect on the performance of conventional flow cytometers. Alternatively, gain confinement along the slow axis direction of an edge emitting LD that is oriented perpendicular to the arrow 511 is much more relaxed. Consequently, as shown in FIG. 2A, the far field beam profile is much smoother along the slow axis of the LD's beam of light.

FIG. 3A depicts a conventional LD based optical subsystem for a flow cytometer. Those elements depicted in FIG. 3A that are common to the optical subsystem 50 illustrated in FIG. 1 carry the same reference numeral distinguished by a prime (') designation. As depicted in FIG. 3A, the conventional optical subsystem orients the fast axis of the LD 501 parallel to the direction in which the liquid sample flow passes through the viewing zone. In its most simplified configuration, the elliptical beam profile of the LD 501' is directly transposed by the spherical focusing lens 504' into the viewing zone. In an attempt to achieve optimal aspect ratio for the focused beam of light, various different conventional LD based optical subsystems have also included beam shaping optical elements in addition to those depicted in FIG. 3A

The detrimental effect of fringes 512 along the fast axis of the LD 501' for conventional optical subsystem configurations clearly appears in the light scattering time profile depicted in FIG. 3B. Since scattering or fluorescence intensity is directly proportional to the local laser power impinging upon a particle, any fine structure in the beam of light's profile along the direction in which the liquid sample flow passes through the viewing zone will appear in the time profile of the signal produced by the flow cytometer. Such

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structures in the time profile are indistinguishable from signals generated by small particles, and will therefore cause the flow cytometer to trigger falsely and misidentify particles. In addition, the fringes 512 will also lead to
5 uncertainty in the measurement of other cytometric parameters, such as in the area and width of the pulse depicted in FIG. 3B.

FIGs. 4A and 4B depict a yet another prior art optical subsystem for LD based flow cytometric applications disclosed in the '019 patent identified previously. Those elements
10 depicted in FIGs. 4A and 4B that are common to the optical subsystem 50 illustrated either in FIG. 1 or 3A carry the same reference numeral distinguished by a double prime ("') designation. As depicted in FIGs. 4A and 4B, by orienting the
15 slow axis of the LD 501" parallel to the direction in which the liquid sample flow passes through the viewing zone the optical subsystem depicted in FIGs. 4A & 4B effectively overcomes the problem caused by the fringes 512 as described above. Unfortunately, the beam-diffusing element 513" placed before
20 the spherical focusing lens 504" in FIGs. 4A and 4B to diffuse the beam of light perpendicular to the direction in which the liquid sample flow passes through the viewing zone produces a highly astigmatic beam of light near the viewing zone. Specifically, focusing this astigmatic beam of light at the
25 viewing zone in the direction in which the liquid sample flow passes through the viewing zone increases the width of the beam of light perpendicular to the direction in which the liquid sample flow passes through the viewing zone so the beam's width become similar to or even wider than the sheath flow. Consequently, the optical subsystem depicted in FIGs. 4A and
30 4b not only diminished the amount of light energy impinging upon particles flowing through the viewing zone, the optical subsystem also increases undesirable scattering of light from the interface between the liquid sheath flow and adjacent parts of the composite microscope objective 60.

35 FIG. 5 highlights the main differences between the optical subsystem disclosed in the '019 patent and the optical subsystem 50 depicted in FIG. 1. Instead of placing an out-of-plane beam-diffusing element 513" before the spherical beam

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focusing lens 504 as shown in FIG. 4, the high power cylindrical lens 505, depicted in FIGs. 5A and 5B as a cylindrical plano-convex lens, is placed along the beam of light after the spherical beam focusing lens 504 and is preferably juxtaposed with the composite microscope objective 60. As shown in FIGs. 5A and 5B, the cylindrical lens 505 focuses the minor axis of the beam of light in the viewing zone while leaving the major axis of the beam of light essentially unchanged. Consequently, the optical subsystem 50 depicted in FIGs. 1, 5A and 5B establishes a beam of light profile at the viewing zone which is elliptical with:

1. a tightly focused minor axis that spans across the combined liquid sample and sheath flows; and
2. a smooth minor axis profile in the direction of the combined liquid sample and sheath flows that is the Fourier conjugate of the far field beam profile along the slow axis of LD 501.

Meanwhile, as shown in FIG. 5B, the out-of-plane beam width is unaffected by the cylindrical lens 505. FIG. 5C shows a measured time profile of light scattered from a micro particle using the optical subsystem 50 depicted in FIGs. 1, 5A and 5B. The LD 501 used in making the measurement presented in FIG. 5C is the same as that used in generating the measured time profile of light scattered from a micro particle appearing in FIG. 3B. As shown in FIG. 5C, the side lobes caused by the fringes 512 along the fast axis of the LD 501 no longer have any material effect on performance of the flow cytometer 40.

FIG. 6 depicts yet another alternative diode laser based optical subsystem in accordance with of the present disclosure adapted for use in a flow cytometer. Those elements depicted in FIGs. 6 and 6A that are common to the optical subsystem 50 illustrated in FIGs. 1, 5A and 5B carry the same reference numeral distinguished by a triple prime ('') designation. The optical subsystem 50''' depicted in FIGs. 6A and 6B is almost identical to that shown in FIGs. 1, 5A and 5B except that the viewing zone occurs without a composite microscope objective 60 because it occurs in a free-flowing jet stream 519 that includes both the sample and sheath flows that is emitted from

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a nozzle 518. Consequently, for the configuration of the optical subsystem 50'" depicted in FIGs. 6A and 6B the high power cylindrical lens 505 is detached from the viewing zone that is located within the jet stream 519.

5 In the exemplary embodiments of the present disclosure depicted in FIGs. 1, 5A, 5B, 6A and 6B, the minor axis, i.e. the slow axis, of the LD 501 is oriented perpendicular to the direction in which the liquid sample flow passes through the viewing zone. However, it will be apparent to those skilled
10 in the art that using an alternative optical configuration the major axis, i.e. the fast axis, of the LD 501 may be reoriented to be perpendicular to the direction in which the liquid sample flow passes through the viewing zone. FIG. 7 depicts one
15 example of such an alternative configuration of optical elements. Those elements depicted in FIG.7 that are common to the optical subsystem 50 illustrated in FIGs. 1, 5A, 5B, 6A and 6B carry the same reference numeral distinguished by a quadruple prime ("''') designation. As shown, the slow axis of the LD 501''' is oriented in the z-direction. The beam of light
20 emitted from the LD 501''' is then rotated to the in-plane y-direction by a pair of ninety degrees (90°) reflection mirrors 523a and 523b. In the illustration of FIG. 7, a normal to the first elliptically-shaped light beam reorienting mirror 523a is oriented in the x-y plane at forty-five degrees (45°)
25 to the x-axis, and a normal to the second elliptically-shaped light beam reorienting mirror 523b is oriented in the y-z plane at forty-five degrees (45°) to the z-axis.

Composite Microscope Objective 60

30 FIG. 8 depicts one embodiment in accordance with the present disclosure for the composite microscope objective 60 depicted in FIGs. 1, 5A, 5B and 7. As depicted in FIG. 8, the composite microscope objective 60 images a viewing zone that is located inside a prismatically-shaped glass cuvette 603
35 within a small flow channel 604, that preferably has a rectangular cross-sectional shape, through which passes the particle carrying combined liquid sample and sheath flows. A piano-concave back-surface mirror 601 included in the composite

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microscope objective 60 is made of an optically transparent material preferably having a refractive index similar to that of the glass cuvette 603, such as glass or optical quality plastics. To minimize optical loss, the back-surface mirror 5 601 includes a flat front surface that is optically coupled to an abutting flat surface of the prismatically-shaped cuvette 603. Optical coupling of the back-surface mirror 601 to the cuvette 603 may employ an index-matching gel, optical adhesive or direct optical bonding. Alternatively, the back-surface 10 mirror 601 may also be formed integrally with the cuvette 603.

The composite microscope objective 60 also includes a plano-ashperic corrector plate 602 that is also made of an optically transparent material preferably having a refractive index similar to that of the glass cuvette 603, such as glass 15 or optical quality plastics. To reduce optical loss, a flat surface of the corrector plate 602 may be optically coupled to an abutting flat surface of the prismatically-shaped cuvette 603 on a face thereof that is diametrically opposite to the back-surface mirror 601. Optical coupling of the corrector 20 plate 602 to the cuvette 603 may employ an index-matching gel, optical adhesive or direct optical bonding. The aspheric surface of the corrector plate 602 furthest from the corrector plate 602 may carry an anti-reflective coating to reduce optical transmission loss, although such a coating is not a 25 mandatory requirement for a composite microscope objective 60 in accordance with the present disclosure. The shape of the aspheric surface of the corrector plate 602 is similar to that in a classical Schmidt camera, (Schmidt, B., *Mitt. Hamburg Sternwart* 7 (36) 1932). As known by those skilled in the art, 30 the corrector plate of a Schmidt camera includes a circularly shaped neutral zone where the corrector plate does not deviate rays of light passing through the plate. For use in the composite microscope objective 60, outside of the neutral zone of the corrector plate 602, where the plate thickness is 35 thinnest, the corrector plate 602 has negative optical power while inside the neutral zone the corrector plate 602 has positive optical power. The exact shape of the aspheric corrector plate 602 may be readily obtained using any

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commercially available optical ray tracing tool by any person having ordinary skill in the art. Note that in the flow cytometer 40 the beam of light generated by the optical subsystem 50 depicted in FIGs. 1, 5A, 5B and 7 enters the
5 cuvette 603 perpendicularly to the flow channel 604 through one (1) of the two (2) faces of the cuvette 603 that do not abut the back-surface mirror 601 or corrector plate 602.

FIG. 9A depicts the result of ray tracing for the embodiment of composite microscope objective 60 illustrated in
10 FIG. 8. As depicted in FIG. 9A, scatter and fluorescence emission from three (3) spatially separated locations in the flow channel 604 near the center of the cuvette 603:

1. initially propagate toward back-surface mirror 601 to be internally reflected by the back-surface
15 mirror 601;
2. then pass first through the cuvette 603;
3. subsequently pass through the aspheric corrector plate 602; and
4. finally forms three (3) distinct images near an
20 image plane 605.

Note that rays traversing the composite microscope composite microscope objective 60 depicted in FIG. 9A are nearly optically-uniform and that light emitted near the center of the cuvette 603 traverses the corrector plate 602 at near normal
25 incidence. Consequently, the composite microscope objective 60 introduces very little chromatic dispersion in light emitted near the center of the cuvette 603.

Further, it is well known in the astrophysics community that Schmidt camera offers the unparalleled combination of a
30 fast focal ratio and a large field of view with near diffraction limited optical performance. The principal drawback in a conventional Schmidt camera is that the image surface lies inside the instrument. For the composite microscope objective 60, light near the center of the cuvette
35 603 propagates opposite to that of a conventional Schmidt camera and therefore the image surface lies outside the composite microscope objective 60. Consequently, the present disclosure takes full advantage of the optical performance of

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the Schmidt camera without experiencing its limitation. FIGs. 9B1 through 9B3 depict spot diagrams near the image plane 605 for three (3) emission locations in viewing zone within the flow channel 604 that are separated 150 micron from each other.

5 The diameters of all images depicted in FIGs. 9B1 through 9B3 are less than 35 microns.

Light emitted from the viewing zone within the flow channel 604 of the composite microscope objective 60 depicted in FIGs. 8 and 9A that traverses the aspheric corrector plate

10 602 suffers from a small amount of chromatic aberration. FIG. 10 depicts an alternative embodiment for the composite microscope objective 60 depicted in FIGs. 1, 5A, 5B and 7 in accordance with the present disclosure. Those elements depicted in FIG. 10 that are common to the composite microscope

15 objective 60 illustrated in FIGs. 8 and 9A carry the same reference numeral distinguished by a prime (') designation. The shapes of the back-surface mirror 601' and the aberration corrector plate 602' depicted in FIG. 10 are modified slightly to produce collimated afocal images of the emission locations

20 near the viewing zone within the flow channel 604'. In FIG. 10 the composite microscope objective 60' also includes a chromatic compensating doublet lens 609 inserted between the corrector plate 602' and the image plane 605'. In addition to focusing the light emitted from the corrector plate 602' onto

25 the image plane 605', the doublet lens 609 also serves to further reduce the residual chromatic aberration introduced by the aspheric corrector plate 602'.

It is not essential that the flat surface of the corrector plate 602 to be optically coupled to the cuvette 603. FIG. 11

30 depicts an alternative embodiment of the composite microscope objective 60 of the present disclosure. Those elements depicted in FIG. 11 that are common to the composite microscope objective 60 illustrated in FIGs. 8 and 9A carry the same reference numeral distinguished by a double prime (")

35 designation. FIG. 11 depicts the aberration corrector plate 602" optically decoupled from the cuvette 603". Although not essential for operation of the composite microscope objective 60", to improve the light transmission efficiency both surfaces

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of the corrector plate 602" and the exposed flat surface of the cuvette 603" may carry an anti-reflectively coating. It is understood that the corrector plate 602" shown in FIG. 11 is held in fixed relationship to the combined back-surface mirror 5 601 and cuvette 603 by a mechanical support not depicted in FIG. 11. Similar to the composite microscope objective 60 and 60' depicted respectively in FIGs. 9A and 10, the composite microscope objective 60" with detached corrector plate 602" may be configured to provide either finite focal length image, or 10 an afocal system which in turn is focused to a finite distance image plane by addition of the chromatic compensating doublet lens 609.

FIG. 12 depicts yet another alternative embodiment of the composite microscope objective 60. Those elements depicted in 15 FIG. 12 that are common to the composite microscope objective 60 illustrated in FIGs. 8, 9A and 11 carry the same reference numeral distinguished by a triple prime ('') designation. The composite microscope objective 60'" depicted in FIG. 12 is adapted for collecting scatter and fluorescence emission from 20 cells or other microscopic particles carried in the jet stream 519 emitted by the nozzle 518. The composite microscope objective 60'" consists of a concave, spherically shaped, front surface mirror 610 and an aberration corrector plate 612. The front surface mirror 610 may be made of glass or other types 25 of hard material with a highly reflective coating on the concave surface 611. or made of metal with polished concave surface 611. Similar to the corrector plate 602, the piano-aspheric corrector plate 612 is made of a thin piece of transparent material, such as glass or optical quality 30 plastics. The aspheric surface may be formed on either side of the corrector plate 612. Preferably, both surfaces of the corrector plate 612 are coated with an anti-reflective coating to reduce optical transmission loss, although such a coating is not a mandatory requirement for a corrector plate 612 in 35 accordance with the present disclosure. It is understood that the front surface mirror 610 and the corrector plate 612 are held in fixed relationship to each other by a mechanical support not depicted in FIG. 12. Scatter and fluorescence

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light emitted from cells or other types of microscopic particles in the viewing zone inside the jet stream 519 is reflected by the concave surface 611 of the front surface mirror 610. The aberration due to reflection from the concave surface 611 is corrected by the corrector plate 612 after light traverses through the corrector plate 612. It is understood by those having skill in the art that the composite microscope objective 60'" may be configured to provide either a finite-focused image similar to that depicted in FIG. 9A, or a collimated afocal image which is focused at finite distance from the composite microscope objective 60'" by a chromatic aberration correction doublet similar to the doublet lens 609 depicted in FIG. 10.

FIG. 13 depicts an adaptation of the composite microscope objective 60 for imaging specimens fixed to the surface of a transparent substrate such as a glass slide. Those elements depicted in FIG. 13 that are common to the composite microscope objective 60 illustrated in FIGS. 8, 9A and 11 carry the same reference numeral distinguished by a quadruple prime ("") designation. The composite microscope objective 60"" depicted in FIG. 13 includes two (2) optical elements, one a plano-concave back surface mirror 617 made of a transparent material such as glass or optical quality plastics, and an aberration corrector plate 618. As depicted in FIG. 13, the specimen to be imaged is fixed to a front surface 615 of a transparent, usually glass slide 616. The slide 616 is optically coupled, preferably using a thin layer of index matching fluid, to the flat surface of the back surface mirror 617. Scatter and fluorescence light emitted by the specimen:

1. initially propagates through the slide 616 and the back surface mirror 617;
2. is internally reflected by the back surface mirror 617 back through the slide 616;
3. then through the corrector plate 618; and
4. finally forms an image at an image plane that is located beyond the corrector plate 618.

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Fluidic Subsystem 70

FIG. 15 depicts a fluidic subsystem 70 in accordance with the present disclosure that includes a sheath liquid reservoir 702 and a liquid pump 701 that draws sheath liquid from the sheath liquid reservoir 702. The liquid pump 701 may be a diaphragm pump, or a peristaltic pump, or a piston pump or any types of continuous fluid pump. An outlet of the liquid pump 701 connects to an inlet of a T-coupling 703 that receives sheath liquid from the liquid pump 701. The T-coupling 703 has two (2) outlets a first one of which connects to a bypass conduit 710 for returning a fraction of the sheath liquid received by the T-coupling 703 from the liquid pump 701 back to the sheath liquid reservoir 702. Returning a fraction of the sheath liquid received by the T-coupling 703 from the liquid pump 701 back to the sheath liquid reservoir 702 is advantageous for two (2) reasons.

1. As depicted in FIG. 1, the bypass conduit 710 is left open to the surrounding atmosphere which effectively dampens pulsation to thereby significantly reducing the pulsation inherent in the operation of the liquid pump 701.
2. Returning a fraction of the sheath liquid received by the T-coupling 703 from the liquid pump 701 back to the sheath liquid reservoir 702 also effectively reduces the throughput of the liquid pump 701 thereby allowing the use of comparatively high flow rate, low cost pumps in the flow cytometer 40.

Denote the flow resistance of the bypass conduit 710 as "r" and the flow resistance of path from the T-coupling 703 to the flow channel 604 of the cuvette 603 as "R." The output resistance to the sheath pump is then

$$R_p = \frac{rR}{r+R} \quad (1)$$

Since $R \gg r$, the behavior of the liquid pump 701 is therefore dominated by the resistance of the bypass conduit 710 whose fluid dynamic properties are temperature insensitive. Thus, the configuration of the fluidic subsystem 70 depicted in FIG.

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15 also provides a simple mechanism for achieving a temperature insensitive sheath liquid flow to the flow channel 604.

As depicted in FIG. 15, a second outlet of the T-coupling 703 connects to the to the flow channel 604 that extends through the cuvette 603 preferably first via a small reservoir capsule 704 and then via a filter cartridge 705. As depicted in FIG. 16, a piece of tubing 704' approximately 4 ft. long may be substituted for the small reservoir capsule 704. During initialization of the fluidic subsystem 70, some air becomes trapped in the filter cartridge 705 near its inlet which as depicted in FIG. 15 is located above an outlet of the filter cartridge 705. The air trapped in the filter cartridge 705 acts as an additional fluidic capacitor effectively reducing to negligible level the pulsation in sheath liquid emitted into the flow channel 604. Due to the large fluidic resistance at the flow channel 604, the air trapped inside the filter cartridge 705 becomes compressed. When the liquid pump 701 is turned off, a trapped in the filter cartridge 705 is pushed back towards the T-coupling 703 analogous to a discharging capacitor. Without the small reservoir capsule 704, some air ejected from the filter cartridge 705 reaches the bypass conduit 710 due to its low fluidic resistance, and will be pushed out of the fluidic subsystem 70 once the liquid pump 701 is turned on again. Without additional air supply, such a scenario will repeat until most of the air becomes purged from the fluidic subsystem 70 causing the filter cartridge 705 to lose its effectiveness as a pulsation damper. The purpose of the small reservoir capsule 704 or the piece of tubing 704' is therefore to provide a reservoir for isolating the filter cartridge 705 from the bypass conduit 710 ensuring that air trapped inside the filter cartridge 705 remains within the fluidic subsystem 70 despite repeated on-off operations of the liquid pump 701.

The pulsation damping effect of the trapped air near the inlet of the filter cartridge 705 is clearly evident in the histograms depicted in FIG. 16A and 16B. FIG. 16A depicts measured particle flight times at the flow channel 604 when a pocket of air is trapped near the inlet of the filter cartridge

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705. FIG. 16B depicts measured particle flight times at the flow channel 604 when the trapped air is purged from the fluidic subsystem 70. The result depicted in the histograms of FIGs. 16A and 16B is made using two (2) knife edge shaped
5 laser beams focused near the center of the flow channel 604 that are spaced approximately 200 micrometers apart. The horizontal axis of the FIGs. 16A and 16B is the flight time a particle takes from one laser beam to the other measured by recording the peak arrival time of light scattered from the
10 particle at ninety degrees (90°) from the excitation beams. In both cases, the average flight time for particles to cross the two laser beams is the same. As shown in FIG. 16A, when the filter cartridge 705 retains some air, all particles take about the same amount of time to cross the two laser beams.
15 If the filter cartridge 705 retains no air, as shown in FIG. 16B, the distribution of flight times not only broadens, but also becomes bimodal. In other words, some particles take less time while others take longer than average amount of time to cross the two laser beams, a phenomenon that can be easily
20 attributed to sheath liquid velocity pulsation at the flow channel 604.

In the embodiments of the present disclosure discussed so far, the fluidic resistance along bypass conduit 710 as well as between the T-coupling 703 and the flow channel 604 is not
25 adjustable. As should be apparent to those ordinary skilled in the art, flow restrictors such a fixed restrictor or adjustable valves 711, 711' and 712, 712' may be advantageously inserted in the bypass conduit 710 and between the T-coupling 703 and the flow channel 604 to permit adjusting the flow rate
30 through the flow channel 604. Alternatively, the velocity of sheath liquid flowing through the flow channel 604 may also be adjusted using a liquid pump 701 that is driven by a variable speed brushless DC motor.

35 Peristaltic Pump 80

One embodiment of an peristaltic pump 80 in accordance with the present disclosure appears in FIG. 17. The pump includes a housing 809 with arcuate curved track 808, three

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rollers 810, 811 and 812 attached to a rotor 816 rotatable within the housing 809, and a compressible tube 807 sandwiched between the compressible tube 807 of the housing 809 and the rollers 810, 811 and 812. As depicted schematically in FIGs. 5 18A through 18D, the rollers 810, 811 and 812 of the peristaltic pump 80 are spaced at substantially equal angular distances, separations or spacings from each other around the perimeter of the rotor 816. For simplicity, it is assumed in the following discussions that the rotor 816 rotates 10 counterclockwise, although it is to be understood that the discussions apply equally well to a peristaltic pump with clockwise rotating rotor. The compressible tube 807 of the housing 809 may be divided into several sections:

1. an open section between point 801 and point 806 15 where the experiences no compression;
2. a pump inlet section between point 801 and point 802 where the is progressively compressed until fully closed when a roller rolls over the section;
3. two pumping sections between point 802 and point 20 803, as well as between point 804 and point 805 wherein the is fully closed by the roller;
4. a recess section between point 803 and point 804 in which the progressively expands from fully closed to fully open as a roller rolls through the 25 expansion part of the recess section from point 803 to point 813;
5. then the is progressively compressed to fully closed as a roller rolls through a compression part of the recess section from point 813 to point 804; 30 and
6. the exit section between point 805 and point 806 where the progressively expands from fully closed to fully open as a roller rolls through the section.

In other words, when a roller rolls anticlockwise over the 35 from inlet point 801 to outlet point 806, the inner gap of the :

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1. progressively decreases from fully open at point 801, to fully closed at point 802 and remain closed until point 803;
2. then progressively expands back to fully open at point 813;
3. then progressively decreases to fully closed at point 804, and remain closed until the roller reaches point 805; and
4. finally progressively expands back to fully open at point 806.

The size of the gap inside the is schematically illustrated in FIGs. 18A through 18D as the spacing between dashed circle and the solid compressible tube 807. As illustrated in FIGs. 18A through 18D, in this embodiment of the peristaltic pump 80 the angular distances, separations or spacings between points 801 and 803, points 802 and 813, point 813 and 805, as well as between point 804 and 866 are identical to the angle between adjacent rollers. As a result, when the roller 810 rolls through the pumping section from point 804 to point 805, as depicted in FIGs. 18A through 18B, its interaction with the completely determines the fluid flow rate of the peristaltic pump 80. Once the roller 810 reaches the exit section between point 805 and 806, as shown in FIG. 18C, the underneath the roller 810 starts to progressively expand and a gap starts to grow. Meanwhile, the roller 811 arrives at the compression part of the recess section and starts to progressively compress the . In the peristaltic pump 80, the shape of the compression part of the recess section between point 813 and point 804 along the compressible tube 807 is such that the volume of liquid pushed out by the compression of the underneath roller 811 in the compression part of the recess section between point 813 and point 804 substantially fills the volume created by the expansion underneath roller 810 in the exit section between point 5 and point 6. During this period, the compressible is partially open underneath both rollers 810 and 811 and completely closed underneath roller 12. Consequently, the pumping action in mainly delivered by roller 12. In particular, since by design the total volume of liquid in the

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section of the between point 13 and point 6 remains substantially constant during this period, the flow rate of the peristaltic pump 80 in the state shown in FIG. 18C remains substantially the same as that in the state shown in FIGs. 18A and 18B. Once the roller 810 passes point 806, roller 811 reaches the pumping section between point 804 and point 805. Note there is no physical difference between the rollers 810, 811 and 812, the flow rate of the peristaltic pump 80 therefore remains substantially constant throughout the entire process.

The mechanism of the pulseless peristaltic pump of the present disclosure may be understood more clearly if it is viewed along a circular coordinate following the movement of the rollers. Referring to FIG. 19, denote as V the volume of the fluid inside a compressible tube 819 from the outlet to a nearest roller 820 that closes off the compressible tube 819, i.e., the amount of fluid represented by the hatched area 818 hatched area 818 shown in FIG. 19. Clearly, V depends on the angular position, θ , of the roller 20, as well as δ , the amount of tube compression exerted by all other downstream rollers.

$$V = V(\theta, \delta_1, \delta_2, \dots) \quad (1)$$

Consequently, the flow rate, F , of a peristaltic pump is related to the time derivative of V_c by:

$$-F = \frac{dV}{dt} = \frac{\partial V}{\partial \theta} R + \sum_i \frac{\partial V}{\partial \delta_i} \frac{d\delta_i}{dt} \quad (2)$$

Here R is the rotational speed of the rotor and the subscripts are used to identify multiple downstream rollers. The first term on the right hand side of Eqn. (2) represents the contribution from the roller that closes off the tube. The partial derivative $\frac{\partial V}{\partial \theta}$ is therefore independent of θ . The summation term represents contributions from all other downstream rollers partially compressing the compressible tube 819. Now let ΔS be the cross sectional area change due to the compression of the compressible tube 819 by the , and L the length of tube where its cross sectional shape is affected by the tube compression. Then, it is obvious to a person skilled in the art that L is proportional to the tube compression δ , and ΔS proportional to its square, δ^2 . Consequently, ΔV , the

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volume of fluid lost due to the compression of the tube by the roller, follows Eqn. (3):

$$\Delta V \propto L \cdot \Delta S \propto \delta^3 = (D - G)^3 \quad (3)$$

5 where D is the inner diameter of the compressible tube and G the minimum gap indicated in FIGs. 19, 19A and 19B which is also represented in FIGs. 18A through 18D by the spacing between the dashed circle and the solid compressible tube 807 of the housing 809. Now referring to FIGs. 20A AND 20B, in the
10 circular coordinate system FIG. 20A corresponds to the state of pump shown in FIGs. 18A and 18B. During this period, there is no roller downstream of the roller 810' and the summation term in Eqn. (2) vanishes. FIG. 20B corresponds to the state of the pump shown in FIG. 18C. The is closed off by roller
15 12' and partially compressed by the rollers 810' and 811'. However, volumetric changes introduced by the two rollers 810' and 811' substantially cancel each other. Consequently, the summation term in Eqn. (2) vanishes as well. As a result, the flow rate of the peristaltic pump 80 remains substantially
20 constant regardless of roller positions.

The shape of the compressible tube 807 satisfying the above requirement can be readily derived from Eqn. (3). Referring to FIG. 18C, if the gaps of the arcuate compressible tube 807 in the compression part of the recess section between
25 point 813 and point 804, $G_{13,4}$, and in the exit section between point 805 and point 806, $G_{5,6}$, follow the equation

$$(D - G_{13,4})^3 + (D - G_{5,6})^3 = D^3 \quad (4)$$

then the total fluid volume in the two sections remains
30 substantially constant, as shown in FIG. 21. In the peristaltic pump 80, the shape of the pump housing 809 is symmetrical with respect to its center line, such that the entrance half of the pump housing 809 is the mirror image of the exit half of the housing 809, as shown in FIG. 17. The
35 peristaltic pump 80 can therefore be operated both in counterclockwise and clockwise rotation very little pulsation, although it is understood that the symmetry is not required to realize a pulseless peristaltic pump in accordance with the

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present disclosure. For example, as long as the gaps of the arcuate compressible tube 807 in the section between point 13 and point 3, $G_{13,3}$, and in the section between point 2 and point 1, $G_{2,1}$, follow Eqn. (5),

5
$$(D - G_{13,3})^3 + (D - G_{2,1})^3 = D^3 \quad (5)$$

a peristaltic pump in accordance with the present disclosure will exhibit little pulsation when the rotor 816 rotates clockwise.

10 FIG. 22 depicts an alternative embodiment of a peristaltic pump in accordance with the present disclosure. Those elements depicted in FIG. 22 that are common to the peristaltic pump 80 illustrated in FIG. 17 carry the same reference numeral distinguished by a prime (') designation. The peristaltic pump
15 80' includes a compressible tube 807' having two (2) recesses 820 and 821, and four (4) rollers 822, 823, 824 and 825. In the embodiment depicted in FIG. 22, the fluid volume loss due to the tube expansion near the outlet of the pump is compensated by the combined effect of the compression of the
20 compressible tube by rollers 822 and 823 near the two recesses 820 and 821.

FIG. 23 depicts yet another alternative embodiment of a peristaltic pump in accordance with the present disclosure. Those elements depicted in FIG. 23 that are common to the
25 peristaltic pump 80 illustrated in FIG. 17 and the peristaltic pump 80' illustrated in FIG. 22 carry the same reference numeral distinguished by a double prime (") designation. The peristaltic pump 80" includes six (6) rollers and an arcuate compressible tube 807" having two recesses 818" and 819". In
30 the peristaltic pump 80", the fluid volume loss due to the tube expansion near the outlet of the pump is compensated by the action of the roller immediately upstream of the one recess 818" or 819" near the pump outlet.

Pulsation due to the expansion of a compressed
35 compressible tube near the outlet of a peristaltic pump may also be overcome by a peristaltic pump having a programmable rotor speed. FIGs. 24A through 24C illustrate pertinent aspects of an alternative embodiment mechanism for minimizing

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peristaltic pump pulsation in accordance with the present disclosure for a 3-roller peristaltic pump. As depicted in FIG. 24B, the track 828 is substantially circular between the pump inlet and pump exit section. Consequently, as indicated by the spacing between the dashed circle 829 and the solid curve of the track 828 the compressible tube is completely closed by various ones of the pump's three (3) rollers 826, 827 and 829 between the pump inlet and pump outlet. FIG. 24A illustrates schematically in a circular coordinate system the roller positions for the peristaltic pump depicted in FIG. 24B. Since there is only one roller downstream of the one that closes off the tube, Eqn. (2) is much simplified:

$$-F = R \left(\frac{\partial v_c}{\partial \theta} + \frac{\partial v_c}{\partial \delta} \frac{d\delta}{d\theta} \right) \quad (6)$$

Here the tube compression $\delta(\theta)$ is explicitly expressed as a function of roller position θ . The terms inside the parentheses represent the change rate of fluid volume with respect to roller position. The first term is the contribution from the roller that closes off the tube, i.e., roller 827 in FIG. 24A, and the second term the contribution from the roller in the exit section. Note that by definition, the volume change rate is negative and the second term inside the parentheses vanishes when there is no roller in the exit section. The dotted curve in FIG. 24C is a representative plot of the negative volume change rate with respect to the position of the roller. The bump along the curve, due to the tube expansion when a roller rolls off the tube near the pump outlet, is the cause for pulsation in conventional peristaltic pumps having a constant rotor speed. However, for the peristaltic pump depicted in FIGs. 24A through 24C the rotor speed, R , shown as dashed curve in FIG. 24C, is set to vary in synchronism with the rotor position and inversely proportional to the change rate of the fluid volume. Consequently, the flow rate of the pump, which is the product of the rotor speed and the change rate of the fluid volume, remains constant, as indicated by the solid line at the top of FIG. 24C. Note the terms inside the parentheses of Eqn. (6) are uniquely determined by the mechanical structure of the pump. The rotor

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speed profile can therefore be readily generated from the shape of the track 828 in accordance with Eqn. (3). To those skilled in the art, there are many ways to realize a programmable rotor, for example, with stepping motor or DC servo motor.

5

WDM Device 90

FIG. 25 shows the optical ray trace for an exemplary 6 ports wavelength division multiplexer of the present disclosure using zig-zag configuration. As shown in FIG. 25, fluorescence light going through a pinhole or emitted from the facet of a multimode optical fiber, such as the optical fiber 852 depicted in FIG. 1, forms an extended object or light source at location 901, i.e. the optical input of the WDM 90. The size of the object is defined by the diameter of the pinhole or the core diameter of the multimode optical fiber. Note that the practical size of the pinhole or the core diameter of the multimode optical fiber is measured in millimeters, in contrast to the diameter of single mode optical fibers that are measured in micrometers. Consequently, the etendue of the fluorescence light source, defined as the product of beam size and its divergence angle, is hundreds times greater than its counterpart in optical communication. According to the theorem of the conservation of etendue (Julio Chaves, *Introduction to Nonimaging Optics*, CRC Press, 2008 [ISBN 978-1420054293]), light from such an extended source, similar to that from a flash light, can only be kept collimated for a very limited distance, particularly when the diameter of the collimated portion needs to be small.

As depicted in FIG. 25, a collimating optical element, in this case an achromatic doublet lens 902, captures the light from source 901, and projects a magnified image of the object near a final focusing lens 905. The size of the image near 905 is kept approximately the same as the effective size of the collimating optical element 902. Consequently, beam of light propagating between lens 902 and the lens 905 is effectively collimated. As shown in FIG. 25, so long as the magnification factor is kept small, for example, less than around 10, using a simple singlet lens 905 the collimated beam of light can

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readily focused down to a spot smaller than that of the beam of light received by the WDM 90 at location 901. The ability to focus the beam of light down to such a small size permits placing a small area semiconductor detector at a focal point

5 906 of the focusing lens 905 for efficient photo detection.

A dichroic filter 903, oriented at a slanted angle, is inserted into the optical path approximately midway between the collimating optical element 902 and the lens 905. The dichroic filter 903 passes the color band of interest and reflects the

10 remaining colors in the beam of light for further processing within the WDM 90. An optional band pass filter 904 may be inserted following the dichroic filter 903 to further improve the color isolation capability of the WDM 90.

Light reflected from the dichroic filter 903 impinges upon

15 a second optical element 907, preferably a concave mirror. The concave mirror 907 has a radius of curvature approximately equal to the distance between the collimating optical element 902 and the image near focusing lens 905. The concave mirror 907 therefore creates a second image of the collimating lens

20 902 near a second focusing lens 908. The light beam between the concave mirror 907 and the second image at the lens 908 has substantially the same diameter as the beam of light between the collimating lens 902 and the first image near the focusing lens 905. The relay imaging concave mirror 907 therefore

25 effectively doubles the collimated beam path without expanding the beam's diameter. Again, the extended yet collimated beam can be easily focused down to a spot smaller than that of the light source at 901. A second dichroic filter 909 is then inserted approximately midway between the relay imaging concave

30 mirror 790 and the second image near focusing lens 908. The second dichroic filter 909 passes another band of color in the beam of light received by the WDM 90 at location 901 and reflects the remainder of the impinging beam of light for further processing.

35 As shown in FIG. 25, additional relay collimating optical elements 910, 911, 912, 913 and dichroic filters 914, 915, 916, 917 can be cascaded in the same way to produce multiple images near focusing lenses 918, 919, 920 and 921, each of these

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images corresponding to a specific color band of light received by the WDM 90 at location 1. As shown in FIG. 25, due to the present disclosure's 1:1 imaging relay architecture, the spots of light produced by focusing lenses 906, 908, 918, 919, 920 and 921 are all smaller than the source of the beam of light, and therefore can be easily captured by small area APD's.

Although FIG 25 illustrates a 6-port wavelength division multiplexer for a beam of light from the extended light source, it is readily apparent to those skilled in the art that WDM's having different numbers of ports can be easily built in accordance with the present disclosure. It is also apparent to those skilled in the art that although the WDM 90 preferably uses achromatic doublets as the first collimating optical element, singlet lens can also be used since the images created before the focusing lenses 906, 908, 918, 919, 920 and 921 are all nearly monochromatic. Instead of using concave mirrors for relaying the beam of light reflected from the dichroic filters, one may also use refractive optics as relay element to extend the path of the collimated beam of light. A clear advantage of the zig-zag architecture used in the WDM 90, however, is the possibility for using array detectors, which would lead to a more compact WDM suitable for portable instrumentation.

FIG. 26 illustrates an optical ray trace for a prior art collimating device. The technique depicted in FIG. 26 is extensively used in conventional multicolor fluorescence instruments, for example, in United States Patent no. 6,683,314. As shown in FIG. 26, the beam of light diverges rapidly beyond the image 924 created by the collimating optical element 923. Consequently, the only option for constructing a multi-color device is to insert dichroic filters in between the collimating element 923 and its image 924.

Due to the constraint of etendue conservation, the diameter of the collimated beam must be significantly expanded to accept multiple dichroic filters in the section. The expanded beam creates serious challenge to refocusing the collimated beam down to small spots suitable for small area semiconductor detectors. To overcome these difficulties, some instrument manufacturers have chosen to use Mt exclusively for

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fluorescence detection such as in the main stream flow cytometers manufactured by Becton-Dickinson, Becman Coulter and Partec 's and the MegaBACE series of DNA sequencers by GE Amersham. Other instruments, such as the Luminex multiplexed
5 bead analyzers, have selected certain color bands with known bright fluorescence, and uses large area APD for detecting light in the selected color bands.

FIG. 27 illustrates a perspective view of an alternative embodiment for a 6-port WDM 90 using a combination of zig-zag and branched configurations. The design is a modification of
10 zig-zag configuration depicted in FIG. 25. In the alternative embodiment depicted in FIG. 27, band pass filter 904 of FIG. 25 is replaced by a dichroic filter 904'. The filter 904' is positioned to let one color pass through and reflects other
15 colors at ninety degrees (90°). The optical path length of the beam of light passing through the dichroic filter 904' and that being reflected from the 904' are substantially the same, such that one arm is focused by lenses 905 and the other by lens
20 905' to small spots compatible with small area semiconductor detectors placed at focal location 906 and 906'. As shown in FIG. 25, the remaining color of the light reflected by dichroic filter 903 is relay imaged by a concave mirror 907 and the configuration including optical elements 903, 904', 905 and 905' is cascaded two (2) more times to form a 6 port-WDM.

FIG. 28 illustrates a perspective view of an alternative embodiment for a 8-port WDM 90. By replacing the concave relay imaging mirrors 907 and 910 in FIG. 27 with concave shaped dichroic filters 907' and 910', the WDM depicted in FIG. 28 provides 2 more color bands in comparison with the WDMs
30 depicted in FIGs. 25 and 27.

Numerous fluorescence probes for use in flow cytometry have been developed over the years. More recently, multiple fluorescence proteins have also become an important tool in biomedical studies. To accommodate different types of
35 fluorescence probe, various techniques have been developed to enable user selection of dichroic filters suitable for their particular needs. A significant challenge for replaceable dichroic filters is avoiding direct contact of the coated

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filter surface with any hard flow cytometer reference frame. Repeated direct contact between the coated filter surface and any hard reference frame may damage a replaceable dichroic filter. Presently, most conventional solutions addressing this problem use precision-machined mechanical spacers for holding replaceable dichroic filters in place. One example of such a solution appears in United States Patent no. 6,683,314. However, such a solution becomes unreliable if the detector's active area is smaller than 1.0 mm².

FIGs. 29A and 29B depict fabricating a replaceable dichroic filter assembly 934 illustrated in FIG. 29C suitable for small area detectors. Assembly of the replaceable dichroic filter assembly 934 begins in FIG. 29A which depicts constructing a reference template for its fabrication. The reference template is a staircase made of two (2) optically parallel glass plates 925 and 926. Bonding the two (2) glass plates 925 and 926 together in optical contact ensures that a surface 929 of the glass plate 925 becomes optically parallel to a surface 930 of the glass plate 926. A front surface 932 of a replaceable dichroic filter 927 is then pressed against the surface 929 of the template. A filter holder 928, which loosely fits the dichroic filter 927, includes a reference surface 931 and a filter slot 933. During assembly of the replaceable dichroic filter, the filter slot 933 is partially filled with epoxy adhesive and the reference surface 931 of the filter holder 928 is pressed against the surface 930 of the template while filter holder 928 slides toward the dichroic filter 927. While the epoxy adhesive sets, part of the dichroic filter 927 remains seated within the filter slot 933 while pressure is applied against the dichroic filter 927 and filter holder 928. It should be apparent to those skilled in the art that the epoxy adhesive may be either UV or thermally curable, or made by blending together components of an A/B mixture. FIG. 29C depicts the E934~ fabricated as depicted in FIGs. 29A and 29B and described above. The assembly process depicted in FIGs. 29A and 29B and described above ensures that front surface 932 of the replaceable dichroic filter assembly 934 will be optically parallel to the reference surface 931,

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and indented with respect to the latter at a spacing accurately determined by the thickness of the glass plate 925.

FIGs. 30A and 30B depict an embodiment of the present disclosure where the fore-mentioned replaceable dichroic filter assembly 934 is used in the WDM 90 for optically processing a beam of light from an extended light source. A notable feature of the WDM 90 is a glass reference block 935 having an optically flat surface. As will be apparent to those skilled in the art, the glass reference block 935 may be made of other materials. As shown in FIG 30B, when installing a dichroic filter 927 the reference surface 931 of the replaceable dichroic filter assembly 934 slides against the flat surface of the glass reference block 935 and is kept in contact therewith by a spring loaded screw 936. Consequently, the coated front surface 932 of the replaceable dichroic filter assembly 934 remains optically parallel to the optical flat and accurately located. In the meantime, the indentation of front surface 932 with respect to the reference reference surface 931 protects it from in physical contact with any object during filter replacement.

It is apparent to those skilled in the art that many modifications and variations of the described embodiments of the replaceable dichroic filter assembly 934 are possible. For example, an alternative embodiment of the present disclosure is a pedestal assembled using a first and a second round optical flat. When assembling the replaceable dichroic filter assembly 934, the reference surface of a filter holder rests against a surface of the first optical flat and the coated surface of the dichroic filter rests against the flat surface of the second optical flat. Epoxy bonding then holds the coated surface of the dichroic filter optically parallel to the reference surface of a filter holder, yet indented at a distance accurately determined by the thickness of the second optical flat.

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Industrial Applicability

Although an embodiment of the present disclosure of an LD based optical system for flow cytometric application has been

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described in some detail, and equally advantageous embodiments have also been described for a stream based flow cytometric instrument, it will be apparent to those of ordinary skill in the art that many modifications and variations of the described
5 embodiment are possible in the light of the above teachings without departing from the principles and concepts of the disclosure as set forth in the claims.

Although an embodiment of the present disclosure of wavelength division multiplexing device for separating light
10 beam from an extended light source into multiple color bands has been described in some detail, and several other equally advantageous embodiments have also been described, it will be apparent to those ordinary skilled in the art that many modifications and variations of the described embodiments are
15 possible in the light of the above teachings without departing from the principles and concepts of the disclosure as set forth in the claims.

Although the present invention has been described in terms of the presently preferred embodiment, it is to be understood
20 that such disclosure is purely illustrative and is not to be interpreted as limiting. Consequently, without departing from the spirit and scope of the disclosure, various alterations, modifications, and/or alternative applications of the disclosure will, no doubt, be suggested to those skilled in the
25 art after having read the preceding disclosure. Accordingly, it is intended that the following claims be interpreted as encompassing all alterations, modifications, or alternative applications as fall within the true spirit and scope of the disclosure.

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The Claims

What is claimed is:

1. A flow cytometer (40) comprising:
 - a) a laser diode ("LD") based optical subsystem (50) for directing a beam of light into a viewing zone of said flow cytometer (40) through which a sample liquid carrying particles flows, the sample liquid being hydrodynamically focused within the viewing zone by a liquid sheath flow that also flows through the viewing zone, the optical subsystem (50) including:
 - i. a LD for emitting a diverging beam of light from an edge thereof, the diverging beam of light having an elliptically shaped cross-sectional profile with both a major axis and a minor axis;
 - ii. a collimating lens for converting the diverging beam of light emitted from said LD into a collimated elliptical beam of light, wherein the minor axis of said collimated elliptical beam of light is oriented parallel to a direction in which particles pass through the viewing zone;
 - iii. a beam compressing optical element for reducing the size of said elliptical beam of light at the viewing zone whereby a width of said major axis of said elliptical beam of light oriented perpendicular to the direction in which particles pass through the viewing zone is less than a width of said liquid sheath flow; and
 - v. a cylindrical focusing element positioned adjacent to the viewing zone with an axis of said cylindrical focusing element being oriented perpendicular to the direction in which particles pass through the viewing zone whereby:

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- 35 1) said minor axis of said beam of light
 becomes focused at the viewing zone; and
- 2) the size of said major axis of said
 elliptical beam of light at the viewing
 zone remains essentially unchanged;
- 40 b) a composite microscope objective (60) for imaging
 light scattered from and fluoresced by a particle
 present within the viewing zone, the composite
 microscope objective (60) including:
- i. a concave mirror upon which scattered and
45 fluoresced light impinges;
- ii. an aberration corrector plate made of optically
 transparent material wherein said aberration
 corrector plate is an aspheric lens that has:
- 1) a first zone of said aberration corrector
50 plate outside a neutral zone thereof where
 said aberration corrector plate:
- A. is thinnest; and
- B. has negative optical power; and
- 2) a second zone of said aberration corrector
55 plate inside the neutral zone has positive
 optical power
- light reflected from the composite microscope
 objective (60) passing through said aberration
 corrector plate,
- 60 the viewing zone of said flow cytometer (40) being
 located between said concave mirror and said
 aberration corrector plate;
- c) a fluidic subsystem (70) for supplying the liquid
 sheath flow to the viewing zone, the liquid sheath
65 flow lacking pulsations, the fluidic subsystem (70)
 including:
1. a liquid pump for supplying liquid drawn from
 a reservoir;
- ii. a T-coupling having at least one (1) inlet and
70 two (2) outlets,
- 1) the inlet of said T-coupling receiving
 liquid from said liquid pump;

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- 75 2) a first fraction of the liquid received by
 the inlet flowing via a first one of the
 outlets and via a bypass conduit back to
 the reservoir; and
- 80 3) a second fraction of the liquid received
 by the inlet flowing via a second one of
 the outlets and via a particle filter to
 the viewing zone of said flow cytometer
 (40);
- 85 d) a peristaltic pump 80 for supplying the sample
 liquid carrying the particles, the sample liquid
 being hydrodynamically focused within the viewing
 zone by the liquid sheath flow, the peristaltic pump
 80 comprising:
- 90 i. a pump housing having a arcuate curved track
 formed therein that extends between a pump
 inlet and a pump outlet;
- 95 ii. a plurality of rollers that are attached to a
 rotor, the rollers having a substantially equal
 angular spacing between each pair of
 immediately adjacent rollers, the rotor being
 rotatable together with the rollers attached
 thereto inside said pump housing; and
- 100 ii a compressible tube sandwiched between said
 rollers and the arcuate curved track of said
 pump housing, the arcuate curved track
 including:
- 105 1) an exit section wherein, as a roller rolls
 through the exit section, said
 compressible tube adjacent to said roller
 progressively expands from fully closed at
 a beginning of said exit section to fully
 open at the pump outlet where said roller
 breaks contact with said compressible
 tube; and
- 110 2) at least one pumping section along the
 arcuate curved track between the pump
 inlet and the pump outlet wherein said

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compressible tube is compressed to fully
closed by at least one of said rollers;
and

- 115 e) a wavelength division multiplexer 90 ("WDM (90)")
for separating into multiple colored bands a beam of
light emitted initially from the viewing zone and
imaged by the composite microscope objective (60)
into an optical fiber for transmission to the WDM
(90), the WDM (90) comprising:
- 120 i. a collimating optical element that magnifies an
to produce an image of substantially the same
size as the effective size of said collimating
optical element;
- 125 ii. at least one dichroic filter located between
said collimating optical element and said
image, wherein said dichroic filter separates
the collimated beam of light into two (2)
branches of distinctive colors;
- 130 iii. a focusing optical element located in one of
said branches, wherein the beam of light in
said branch is focused to a spot having a
diameter of less than 1.0 mm by said focusing
optical element; and
- 135 iv. an image relay optical element located near the
image produced by said collimating optical
element in the other branch, wherein said image
relay optical element produces an image of said
collimating optical element at substantially
unit magnification.

2. The flow cytometer (40) of claim 1 wherein said
cuvette has a rectangularly-shaped , and wherein the viewing
zone of the flow cytometer (40) is located within a channel
having a rectangularly-shaped cross-section that is located
within said cuvette.

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3. The flow cytometer (40) of claim 1 wherein said cuvette has a tubularly-shaped cross-section, and wherein the viewing zone of the flow cytometer (40) is located within a channel having a circularly-shaped cross-section that is located within said cuvette.

4. The flow cytometer (40) of claim 1 wherein the sample liquid and the liquid sheath flow form a jet stream in which the viewing zone of the flow cytometer (40) is located.

5. The flow cytometer (40) of claim 2 wherein said cylindrical focusing element is in optical contact with an entrance face of said rectangularly-shaped cuvette.

6. The flow cytometer (40) of claim 2 wherein said cylindrical focusing element is separated from said rectangularly-shaped cuvette.

7. The flow cytometer (40) of claim 3 wherein said cylindrical focusing element is separated from said tubularly-shaped cuvette.

8. The flow cytometer (40) of claim 4 wherein said cylindrical focusing element is separated from said jet stream.

9. The flow cytometer (40) of claim 1 further comprising a polarization conditioning element through which said collimated elliptical beam of light passes.

10. The flow cytometer (40) of claim 1 wherein an optical image of the viewing zone is formed outside the composite microscope objective (60).

11. The flow cytometer (40) of claim 10 wherein the viewing zone is located within a flow channel included in a rectangularly-shaped cuvette made of optically transparent material.

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12. The flow cytometer (40) of claim 11 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

13. The flow cytometer (40) of claim 12 wherein the piano-surface of said piano-concave back surface mirror is optically coupled to a flat surface of said cuvette.

14. The flow cytometer (40) of claim 13 wherein an optical adhesive material accomplishes the optical coupling.

15. The flow cytometer (40) of claim 13 wherein an index matching gel accomplishes the optical coupling.

16. The flow cytometer (40) of claim 13 wherein an index matching fluid accomplishes the optical coupling.

17. The flow cytometer (40) of claim 13 wherein optical contact bonding accomplishes the optical coupling.

18. The flow cytometer (40) of claim 13 wherein the piano-concave back surface mirror formed integrally with said cuvette means.

19. The flow cytometer (40) of claim 11 wherein said aberration corrector plate is a piano-aspherical lens.

20. The flow cytometer (40) of claim 19 wherein a piano-surface of said aberration corrector plate is optically coupled to a flat surface of said cuvette opposite of said piano-concave back surface mirror.

21. The flow cytometer (40) of claim 20 wherein an index matching gel accomplishes the optical coupling.

22. The flow cytometer (40) of claim 20 wherein an index matching fluid accomplishes the optical coupling.

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23. The flow cytometer (40) of claim 20 wherein optical contact bonding accomplishes the optical coupling.

24. The flow cytometer (40) of claim 20 wherein the piano-aspherical lens is formed integrally with said cuvette.

25. The flow cytometer (40) of claim 19 wherein said aberration corrector plate is detached from said cuvette.

26. The flow cytometer (40) of claim 10 wherein the viewing zone is inside a jet stream.

27. The flow cytometer (40) of claim 25 wherein said concave mirror is a front surface mirror.

28. The flow cytometer (40) of claim 10 wherein the viewing zone is located on a surface of a flat, transparent substrate.

29. The flow cytometer (40) of claim 28 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

30. The flow cytometer (40) of claim 29 wherein the piano-surface of said piano-concave back surface mirror is optically coupled to said flat, transparent substrate.

31. The flow cytometer (40) of claim 30 wherein an optical adhesive material accomplishes the optical coupling.

32. The flow cytometer (40) of claim 30 wherein an index matching gel accomplishes the optical coupling.

33. The flow cytometer (40) of claim 30 wherein an index matching fluid accomplishes the optical coupling.

34. The flow cytometer (40) of claim 30 wherein optical contact bonding accomplishes the optical coupling.

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35. The flow cytometer (40) of claim 29 wherein said piano-concave back surface mirror is formed integrally with said flat, transparent substrate.

36. The flow cytometer (40) of claim 28 wherein said aberration corrector plate is detached from said flat, transparent substrate.

37. The flow cytometer (40) of claim 1 wherein the particle filter has inlet thereto for receiving liquid from the T-coupling, the inlet of the particle filter being disposed so that air becomes trapped within the particle filter at the inlet thereto.

38. The flow cytometer (40) of claim 37 wherein when said liquid pump is turned off air cannot enter into the bypass conduit.

39. The flow cytometer (40) of claim 38 further comprising a small capsule disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

40. The flow cytometer (40) of claim 38 further comprising a length of tubing disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

41. The flow cytometer (40) of claim 38 further comprising an adjustable valve located in the bypass conduit between the first one of the outlets of the T-coupling and the reservoir for restricting liquid flow therebetween.

42. The flow cytometer (40) of claim 38 further comprising an adjustable valve located between the second one

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of the outlets of the T-coupling and the viewing zone for restricting liquid flow therebetween.

43. The flow cytometer (40) of claim 38 wherein the throughput of the liquid pump is adjustable.

44. The flow cytometer (40) of claim 1 wherein the arcuate curved track of said pump housing includes at least two (2) pumping sections, the arcuate curved track further including at least one recess section located between said
5 pumping sections along the arcuate curved track, and wherein said compressible tube at said recess section becomes decompressed to full expansion then compressed to fully closed when one (1) of said rollers rolls through said recess section.

45. The flow cytometer (40) of claim 44 includes a plurality of recess sections along said arcuate curved track upstream of the pump outlet, the angular spacing between the compression part of said recess section adjacent to the pump
5 outlet and said exit section of said arcuate curved track being substantially the same as the angular spacing between each pair of immediately adjacent rollers.

46. The flow cytometer (40) of claim 44 wherein said compression part of said recess section adjacent to the pump outlet has a shape complementing a shape of said exit section of said arcuate curved track to maintain the total fluid volume
5 inside a section of said compressible tube extending from said recess section to the pump outlet substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

47. The flow cytometer (40) of claim 44 having a plurality of recess sections respectively interspersed between immediately adjacent pairs of a plurality of pumping sections.

48. The flow cytometer (40) of claim 46 wherein both:

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- a) angular spacing between adjacent pairs of recess sections; and
- b) angular spacing between said exit section of said arcuate curved track and an adjacent recess section to said exit section
- 5 are substantially the same as the angular spacing between each pair of immediately adjacent rollers.

49. The flow cytometer (40) of claim 47 wherein shapes of a plurality of recess sections of said arcuate curved track complement a shape of said exit section of said arcuate curved track to maintain a fluid volume in sections of said compressible tube at the plurality of recess sections and said exit section substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

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50. The flow cytometer (40) of claim 1 wherein a speed of said rotor is programmably controlled to vary substantially in inverse proportion to the fluid volume change rate in said compressible tube due to its changing compression near the exit section of said arcuate curved track.

51. The flow cytometer (40) of claim 1 wherein at least one additional dichroic filter is located between said image relay optical element and the image produced by said image relay optical element, wherein said dichroic filter produces two (2) branches of the beam of light having distinctive colors.

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52. The flow cytometer (40) of claim 51 wherein another focusing optical element is located in one of said branches and focuses the beam of light in the branch into a spot having a diameter of less than 1.0 mm.

53. The flow cytometer (40) of claim 52 wherein successive combinations of said image relay optical element, dichroic filter and focusing optical element are cascaded to

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produce additional focused spots having a diameter of less than 1.0 mm for multiple colored bands of said beam of light.

54. The flow cytometer (40) of claim 52 wherein the dichroic filter is assembled using a template that include two (2) optically flat glass plates bonded together in optical contact, and wherein the dichroic filter is bonded to a filter holder using the template such that a coated filter surface of the dichroic filter is indented and optically parallel to a reference surface of the filter holder.

55. The flow cytometer (40) of claim 54 wherein the reference surface of the filter holder rests against an optically flat surface of an reference block included in the WDM (90) thereby providing consistent optical alignment when installing the dichroic filter into the WDM (90).

56. A LD based optical subsystem (50) for impinging a beam of light into a viewing zone in which a particle is present, the optical subsystem (50) comprising:

- 5 a) a LD for emitting a diverging beam of light from an edge thereof, the diverging beam of light having an elliptically shaped cross-sectional profile with both a major axis and a minor axis;
- 10 b) a collimating lens for converting the diverging beam of light emitted from said LD into a collimated elliptical beam of light, wherein the minor axis of said collimated elliptical beam of light is oriented parallel to a direction in which particles pass through the viewing zone;
- 15 c) a beam compressing optical element for reducing the size of said elliptical beam of light at the viewing zone whereby a width of said major axis of said elliptical beam of light oriented perpendicular to the direction in which particles pass through the viewing zone is less than a width of said liquid sheath flow; and
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- 25 d) a cylindrical focusing element positioned adjacent to the viewing zone with an axis of said cylindrical focusing element being oriented perpendicular to the direction in which particles pass through the viewing zone whereby:
- i. said minor axis of said beam of light becomes focused at the viewing zone; and
 - ii. the size of said major axis of said elliptical beam of light at the viewing zone remains essentially unchanged.

57. The optical subsystem (50) of claim 56 further comprising a cuvette having a rectangularly-shaped cross-section, and wherein the viewing zone is located within a channel having a rectangularly-shaped cross-section that is located within said cuvette.

58. The optical subsystem (50) of claim 56 further comprising a cuvette having a tubularly-shaped cross-section, and wherein the viewing zone is located within a channel having a circularly-shaped cross-section that is located within said cuvette.

59. The optical subsystem (50) of claim 56 wherein the sample liquid and the liquid sheath flow form a jet stream in which the viewing zone is located.

60. The optical subsystem (50) of claim 57 wherein said cylindrical focusing element is in optical contact with an entrance face of said rectangularly-shaped cuvette.

61. The optical subsystem (50) of claim 57 wherein said cylindrical focusing element is separated from said rectangularly-shaped cuvette.

62. The optical subsystem (50) of claim 58 wherein said cylindrical focusing element is separated from said tubularly-shaped cuvette.

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63. The optical subsystem (50) of claim 59 wherein said cylindrical focusing element is separated from said jet stream.

64. The optical subsystem (50) of claim 56 further comprising a polarization conditioning element through which said collimated elliptical beam of light passes.

65. A method for delivering an elliptically shaped beam of light using a LD based optical subsystem (50), the beam of light having a smooth profile at a focus of a minor axis thereof that is located at a viewing zone through which a
5 sample liquid flows, the sample liquid being hydrodynamically focused within the viewing zone by a liquid sheath flow that also flows through the viewing zone, the method comprising the steps of:

- 10 a) providing a LD that emits a diverging beam of light from an edge thereof, the diverging beam of light having an elliptically shaped cross-sectional profile with both a major axis and a minor axis;
- 15 b) impinging the diverging beam of light emitted by the LD upon a collimating lens for converting the diverging beam of light emitted therefrom into a collimated elliptical beam of light wherein the minor axis of said collimated elliptical beam of light is oriented parallel to a direction in which sample liquid passes through the viewing zone;
- 20 c) after passing through said collimating lens, impinging the collimated elliptical beam of light upon an beam compressing optical element for reducing the size of said elliptical beam of light at the viewing zone whereby a width of said major axis of said elliptical beam of light oriented
25 perpendicular to the direction in which sample liquid passes through the viewing zone becomes less than a width of said liquid sheath flow; and
- 30 d) after passing through said beam compressing optical element, impinging the beam of light upon a

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cylindrical focusing element positioned adjacent to the viewing zone with an axis of said cylindrical focusing element being oriented perpendicular to the direction in which sample liquid passes through the viewing zone whereby:

- i. said minor axis of said beam of light becomes focused at the viewing zone; and
- ii. the size of said major axis of said elliptical beam of light at the viewing zone remains essentially unchanged.

66. The method of claim 65 wherein the viewing zone is located within a channel having a rectangularly-shaped cross-section that is located within a cuvette.

67. The method of claim 65 wherein the viewing zone is located within a channel having a circularly-shaped cross-section that is located within a cuvette.

68. The method of claim 65 wherein the viewing zone is located within a jet stream.

69. The method of claim 66 further comprising a step of establishing an optical contact between said cylindrical focusing element and an entrance face of said cuvette.

70. The method of claim 66 further comprising a step of establishing a spacing between said cylindrical focusing element and said cuvette.

71. The method of claim 67 further comprising a step of establishing a spacing between said cylindrical focusing element and said cuvette.

72. The method of claim 68 further comprising a step of establishing a spacing between said cylindrical focusing element and said jet stream.

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73. The method of claim 65 further comprising a step of inserting a polarization conditioning element between the collimating lens and the beam compressing optical element whereby the collimated elliptical beam of light passes through the polarization conditioning element.

74. A composite microscope objective (60) adapted for imaging light scattered from and fluoresced by a particle present within a viewing zone comprising:

- a) a concave mirror upon which scattered and fluoresced light impinges;
- b) an aberration corrector plate made of optically transparent material wherein said aberration corrector plate is an aspheric lens that has:
 - i. a first zone of said aberration corrector plate outside a neutral zone thereof where said aberration corrector plate:
 - 1) is thinnest; and
 - 2) has negative optical power; and
 - ii. a second zone of said aberration corrector plate inside the neutral zone has positive optical power,light reflected from the composite microscope objective (60) passing through said aberration corrector plate,

the viewing zone being located between said concave mirror and said aberration corrector plate.

75. The composite microscope objective (60) of claim 74 wherein an optical image of the viewing zone is formed outside the composite microscope objective (60).

76. The composite microscope objective (60) of claim 75 wherein the viewing zone is located within a flow channel included in a rectangularly-shaped cuvette made of optically transparent material.

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77. The composite microscope objective (60) of claim 76 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

78. The composite microscope objective (60) of claim 77 wherein a piano-surface of said piano-concave back surface mirror is optically coupled to a flat surface of said cuvette.

79. The composite microscope objective (60) of claim 78 wherein an optical adhesive material accomplishes the optical coupling.

80. The composite microscope objective (60) of claim 78 wherein an index matching gel accomplishes the optical coupling.

81. The composite microscope objective (60) of claim 78 wherein an index matching fluid accomplishes the optical coupling.

82. The composite microscope objective (60) of claim 78 wherein optical contact bonding accomplishes the optical coupling.

83. The composite microscope objective (60) of claim 78 wherein the piano-concave back surface mirror formed integrally with said cuvette means.

84. The composite microscope objective (60) of claim 76 wherein said aberration corrector plate is a piano-aspherical lens.

85. The composite microscope objective (60) of claim 84 wherein a piano-surface of said aberration corrector plate is optically coupled to a flat surface of said cuvette opposite of said piano-concave back surface mirror.

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86. The composite microscope objective (60) of claim 85 wherein an index matching gel accomplishes the optical coupling.

87. The composite microscope objective (60) of claim 85 wherein an index matching fluid accomplishes the optical coupling.

88. The composite microscope objective (60) of claim 85 wherein optical contact bonding accomplishes the optical coupling.

89. The composite microscope objective (60) of claim 85 wherein the piano-aspherical lens is formed integrally with said cuvette.

90. The composite microscope objective (60) of claim 84 wherein said aberration corrector plate is detached from said cuvette.

91. The composite microscope objective (60) of claim 75 wherein the viewing zone is inside a jet stream.

92. The composite microscope objective (60) of claim 90 wherein said concave mirror is a front surface mirror.

93. The composite microscope objective (60) of claim 75 wherein the viewing zone is located on a surface of a flat, transparent substrate.

94. The composite microscope objective (60) of claim 93 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

95. The composite microscope objective (60) of claim 94 wherein a piano-surface of said piano-concave back surface mirror is optically coupled to said flat, transparent substrate.

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96. The composite microscope objective (60) of claim 95 wherein an optical adhesive material accomplishes the optical coupling.

97. The composite microscope objective (60) of claim 95 wherein an index matching gel accomplishes the optical coupling.

98. The composite microscope objective (60) of claim 95 wherein an index matching fluid accomplishes the optical coupling.

99. The composite microscope objective (60) of claim 95 wherein optical contact bonding accomplishes the optical coupling.

100. The composite microscope objective (60) of claim 94 wherein said piano-concave back surface mirror is formed integrally with said flat, transparent substrate.

101. The composite microscope objective (60) of claim 93 wherein said aberration corrector plate is detached from said flat, transparent substrate.

102. A method for characterizing microscopic species using a microscope objective device comprising:

- a) A concave mirror;
- b) An aberration corrector plate made of optically transparent material wherein said aberration corrector plate is an aspheric lens with the zone of said plate means outside of the neutral zone wherein the thickness of said plate means is thinnest, having negative optical power, and the zone inside the neutral zone having positive optical power
- c) A viewing zone located in between said concave mirror and said aberration corrector plate.

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103. The method of claim 102 wherein an optical image of the viewing zone is formed outside the device.

104. The method of claim 103 wherein the viewing zone is located within a flow channel contained in a rectangularly-shaped cuvette means made of optically transparent material.

105. The method of claim 104 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

106. The method of claim 105 wherein a piano-surface of said piano-concave back surface mirror means is optically coupled to a flat surface of said cuvette means.

107. The method of claim 106 wherein an optical adhesive material accomplishes the optical coupling.

108. The method of claim 106 wherein an index matching gel accomplishes the optical coupling.

109. The method of claim 106 wherein an index matching fluid accomplishes the optical coupling.

110. The method of claim 106 wherein optical contact bonding accomplishes the optical coupling.

111. The method of claim 106 wherein the piano-concave back surface mirror is formed integrally with said cuvette.

112. The method of claim 104 wherein said aberration corrector plate is a plano-aspherical lens.

113. The method of claim 112 wherein a piano-surface of said aberration corrector plate is optically coupled to a flat surface of said cuvette means opposite of said concave mirror.

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114. The method of claim 113 wherein an index matching gel accomplishes the optical coupling.

115. The method of claim 113 wherein an index matching fluid accomplishes the optical coupling.

116. The method of claim 113 wherein optical contact bonding accomplishes the optical coupling.

117. The method of claim 113 wherein the piano-aspherical lens is formed integrally with said cuvette.

118. The method of claim 112 wherein said aberration corrector plate is detached from said cuvette.

119. The method of claim 102 wherein the viewing zone is inside a jet stream.

120. The method of claim 119 wherein said concave mirror is a front surface mirror.

121. The method of claim 102 wherein the viewing zone is located on a surface of a flat, transparent substrate.

122. The method of claim 121 wherein said concave mirror is a piano-concave back surface mirror made from an optically transparent material.

123. The method of claim 122 wherein a piano-surface of said piano-concave back surface mirror means is optically coupled to said flat, transparent substrate.

124. The method of claim 123 wherein an optical adhesive material accomplishes the optical coupling.

125. The method of claim 123 wherein an index matching gel accomplishes the optical coupling.

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126. The method of claim 123 wherein an index matching fluid accomplishes the optical coupling.

127. The method of claim 123 wherein optical contact bonding accomplishes the optical coupling.

128. The method of claim 122 wherein said piano-concave back surface mirror is formed integrally with said flat, transparent substrate.

129. The method of claim 128 wherein said aberration corrector plate is detached from said flat, transparent substrate.

130. A fluidic subsystem (70) for supplying a liquid flow pulsation free to an outlet of the fluidic subsystem (70), the fluidic subsystem (70) comprising:

- 5 a) a liquid pump for supplying liquid drawn from a reservoir;
- b) a T-coupling having at least one (1) inlet and two (2) outlets,
 - 10 i. the inlet of said T-coupling receiving liquid from said liquid pump;
 - ii. a first fraction of the liquid received by the inlet flowing via a first one of the outlets and via a bypass conduit back to the reservoir; and
 - 15 iii. a second fraction of the liquid received by the inlet flowing via a second one of the outlets and via a particle filter to the outlet of the fluidic subsystem (70).

131. The fluidic subsystem (70) of claim 130 wherein the particle filter has inlet thereto for receiving liquid from the T-coupling, the inlet of the particle filter being disposed so that air becomes trapped within the particle filter at the inlet thereto.

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132. The fluidic subsystem (70) of claim 131 wherein when said liquid pump is turned off air cannot enter into the bypass conduit.

133. The fluidic subsystem (70) of claim 132 further comprising a small capsule disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

134. The fluidic subsystem (70) of claim 132 further comprising a length of tubing disposed between the second one of the outlets of said T-coupling and the particle filter for storing air ejected from the particle filter when the liquid pump is turned off.

135. The fluidic subsystem (70) of claim 132 further comprising an adjustable valve located in the bypass conduit between the first one of the outlets of the T-coupling and the reservoir for restricting liquid flow therebetween.

136. The fluidic subsystem (70) of claim 132 further comprising an adjustable valve located between the second one of the outlets of the T-coupling and the outlet of the fluidic subsystem (70) for restricting liquid flow therebetween.

137. The fluidic subsystem (70) of claim 132 wherein the throughput of the liquid pump is adjustable.

138. A method for supplying a liquid flow pulsation free to an outlet of the fluidic subsystem (70), the method comprising:

- 5 a) a liquid pump for supplying liquid drawn from a reservoir;
- b) a T-coupling having at least one (1) inlet and two (2) outlets,
 - i. the inlet of said T-coupling receiving liquid from said liquid pump;

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- 10 ii. a first fraction of the liquid received by the
 inlet flowing via a first one of the outlets
 and via a bypass conduit back to the reservoir;
 and
- 15 iii. a second fraction of the liquid received by the
 inlet flowing via a second one of the outlets
 and via a particle filter to the outlet of the
 fluidic subsystem (70).

139. The method of claim 138 wherein during normal operation certain amount of air is trapped near the inlet portion of said filter cartridge means.

140. The method of claim 139 wherein said reservoir means holds sufficient amount of liquid such that when said pump means is turned off, portion of the tubing between said Tee means and said reservoir means is still filled with liquid,
5 preventing said trapped air from leaking into said bypass means.

141. The method of claim 140 wherein said reservoir means is a capsule.

142. The method of claim 140 wherein said reservoir means is a piece of tubing.

143. The method of claim 140 wherein an adjustable flow restrictor means is placed in the bypass route.

144. The method of claim 140 wherein an adjustable flow restrictor means is placed in the sheath route.

145. The method of claim 140 wherein the throughput of the sheath pump is adjustable.

146. A peristaltic pump 80 comprising:

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- a) a pump housing having a arcuate curved track formed therein that extends between a pump inlet and a pump outlet;
- 5 b) a plurality of rollers that are attached to a rotor, the rollers having a substantially equal angular spacing between each pair of immediately adjacent rollers, the rotor being rotatable together with the rollers attached thereto inside said pump housing;
- 10 and
- c) a compressible tube sandwiched between said rollers and the arcuate curved track of said pump housing, the arcuate curved track including:
- 15 i. an exit section wherein, as a roller rolls through the exit section, said compressible tube adjacent to said roller progressively expands from fully closed at a beginning of said exit section to fully open at the pump outlet where said roller breaks contact with
- 20 said compressible tube; and
- ii. at least one pumping section along the arcuate curved track between the pump inlet and the pump outlet wherein said compressible tube is compressed to fully closed by at least one of said rollers.

147. The peristaltic pump 80 of claim 146 wherein the arcuate curved track of said pump housing includes at least two (2) pumping sections, the arcuate curved track further including at least one recess section located between said

5 pumping sections along the arcuate curved track, and wherein said compressible tube at said recess section becomes decompressed to full expansion then compressed to fully closed when one (1) of said rollers rolls through said recess section.

148. The peristaltic pump 80 of claim 147 includes a plurality of recess sections along said arcuate curved track upstream of the pump outlet, the angular spacing between the compression part of said recess section adjacent to the pump

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- 5 outlet and said exit section of said arcuate curved track being substantially the same as the angular spacing between each pair of immediately adjacent rollers.

149. The peristaltic pump 80 of claim 147 wherein said compression part of said recess section adjacent to the pump outlet has a shape complementing a shape of said exit section of said arcuate curved track to maintain the total fluid volume
5 inside a section of said compressible tube extending from said recess section to the pump outlet substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

150. The peristaltic pump 80 of claim 147 having a plurality of recess sections respectively interspersed between immediately adjacent pairs of a plurality of pumping sections.

151. The peristaltic pump 80 of claim 149 wherein both:
a) angular spacing between adjacent pairs of recess sections; and
b) angular spacing between said exit section of said
5 arcuate curved track and an adjacent recess section to said exit section
are substantially the same as the angular spacing between each pair of immediately adjacent rollers.

152. The peristaltic pump 80 of claim 150 wherein shapes of a plurality of recess sections of said arcuate curved track complement a shape of said exit section of said arcuate curved track to maintain a fluid volume in sections of said
5 compressible tube at the plurality of recess sections and said exit section substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

153. The peristaltic pump 80 of claim 146 wherein a speed of said rotor is programmably controlled to vary substantially in inverse proportion to the fluid volume change rate in said

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compressible tube due to its changing compression near the exit section of said arcuate curved track.

154. A method for delivering liquid using a peristaltic pump 80 comprising:

- a) a pump housing with arcuate curved track;
- b) A plurality of rollers attached to a rotor means rotatable inside said pump housing;
- c) Said plurality of rollers spaced at substantially equal angular spacing from each other;
- d) A compressible tube sandwiched between said rollers and the arcuate curved track of said pump housing;
- e) Said arcuate curved track of said pump housing including an exit section, wherein said compressible tube being progressively decompressed to full expansion when one of said rollers rolls off the compressible tube at the pump outlet;
- f) At least one pumping section along the arcuate curved track of said pump housing between the pump inlet and pump outlet wherein said compressible tube is compressed to full closure by one of said rollers.

155. The method of claim 154 wherein the arcuate curved track of said pump housing includes at least two (2) pumping sections, the arcuate curved track further including at least one recess section located between said pumping sections along the arcuate curved track, and wherein said compressible tube at said recess section becomes decompressed to full expansion then compressed to fully closed when one (1) of said rollers rolls through said recess section.

156. The method of claim 155 wherein the peristaltic pump 80 includes a plurality of recess sections along said arcuate curved track upstream of the pump outlet; The angular spacing between the compression part of said recess section adjacent to the pump outlet and said exit section of said arcuate curved

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track being substantially the same as the angular spacing between each pair of immediately adjacent rollers.

157. The method of claim 155 wherein said compression part of said recess section adjacent to the pump outlet has a shape complementing a shape of said exit section of said arcuate curved track to maintain the total fluid volume inside a section of said compressible tube extending from said recess section to the pump outlet substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

158. The method of claim 155 having a plurality of recess sections respectively interspersed between immediately adjacent pairs of a plurality of pumping sections.

159. The method of claim 157 wherein both:

- a) angular spacing between adjacent pairs of recess sections; and
- b) angular spacing between said exit section of said arcuate curved track and an adjacent recess section to said exit section

are substantially the same as the angular spacing between each pair of immediately adjacent rollers.

160. The method of claim 158 wherein shapes of a plurality of recess sections of said arcuate curved track complement a shape of said exit section of said arcuate curved track to maintain a fluid volume in sections of said compressible tube at the plurality of recess sections and said exit section substantially invariant when one of said rollers progressively rolls off said exit section of the arcuate curved track.

161. The method of claim 154 wherein a speed of said rotor of the peristaltic pump 80 is programmably controlled to vary substantially in inverse proportion to the fluid volume change rate in said compressible tube due to its changing compression near the exit section of said arcuate curved track.

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162. A WDM (90) for separating a beam of light emitted from an into multiple colored bands, the WDM (90) comprising:

- 5 a) a collimating optical element that magnifies an to produce an image of substantially the same size as the effective size of said collimating optical element;
- 10 b) at least one dichroic filter located between said collimating optical element and said image, wherein said dichroic filter separates the collimated beam of light into two (2) branches of distinctive colors;
- 15 c) a focusing optical element located in one of said branches, wherein the beam of light in said branch is focused to a spot having a diameter of less than 1.0 mm by said focusing optical element; and
- 20 d) an image relay optical element located near the image produced by said collimating optical element in the other branch, wherein said image relay optical element produces an image of said collimating optical element at substantially unit magnification.

163. The WDM (90) of claim 162 wherein at least one additional dichroic filter is located between said image relay optical element and the image produced by said image relay optical element, wherein said dichroic filter produces two (2) branches of the beam of light having distinctive colors.

164. The WDM (90) of claim 163 wherein another focusing optical element is located in one of said branches and focuses the beam of light in the branch into a spot having a diameter of less than 1.0 mm.

165. The WDM (90) of claim 164 wherein successive combinations of said image relay optical element, dichroic filter and focusing optical element are cascaded to produce

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additional focused spots having a diameter of less than 1.0 mm for multiple colored bands of said beam of light.

166. The WDM (90) of claim 164 wherein the dichroic filter is assembled using a template that include two (2) optically flat glass plates bonded together in optical contact, and wherein the dichroic filter is bonded to a filter holder using the template such that a coated filter surface of the dichroic filter is indented and optically parallel to a reference surface of the filter holder.

167. The WDM (90) of claim 166 wherein the reference surface of the filter holder rests against an optically flat surface of an reference block included in the WDM (90) thereby providing consistent optical alignment when installing the dichroic filter into the WDM (90).

168. A method for separating beam of light emitted from an into colored bands using a WDM (90) comprising

- a) a collimating optical element that magnifies an to produce an image of substantially the same size as the effective size of said collimating optical element;
- b) at least one dichroic filter located between said collimating optical element and said image, wherein said dichroic filter separates the collimated beam of light into two (2) branches of distinctive colors;
- c) a focusing optical element located in one of said branches, wherein the beam of light of said branch is focused to a spot having a diameter of less than 1.0 mm by said focusing optical element; and
- d) an image relay optical element located near the image produced by said collimating optical element in the other branch, wherein said image relay optical element produces an image of said collimating optical element at substantially unit magnification.

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169. The method of claim 168 wherein at least one additional dichroic filter is located between said image relay optical element and the image produced by said image relay optical element, wherein said dichroic filter produces two (2) branches of beam of light having distinctive colors.

170. The method of claim 169 wherein another focusing optical element is located in one of said branches and focuses the beam of light in the branch into a spot having a diameter of less than 1.0 mm.

171. The method of claim 170 wherein successive combinations of said image relay optical element, dichroic filter and focusing optical element are cascaded to produce additional focused spots having a diameter of less than 1.0 mm for multiple colored bands of said beam of light.

172. The method of claim 170 wherein the dichroic filter is assembled using a template that include two (2) optically flat glass plates bonded together in optical contact, and wherein the dichroic filter is bonded to a filter holder using
5 the template such that a coated filter surface of the dichroic filter is indented and optically parallel to a reference surface of the filter holder.

173. The method of claim 172 wherein the reference surface of the filter holder rests against an optically flat surface of an reference block included in the WDM (90) thereby providing consistent optical alignment when installing the dichroic filter into the WDM (90).

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ABSTRACT

The disclosed flow cytometer (40) includes:

1. a laser diode ("LD") based optical subsystem (50) for impinging a beam of light upon particles passing through a viewing zone;
2. a composite microscope objective (60) for gathering and imaging light scattered from or fluoresced by particles passing through the viewing zone;
3. a fluidic subsystem (70) for supplying a liquid sheath flow to the viewing zone;
4. a peristaltic pump (80) for injecting into the liquid sheath flow a liquid sample flow carrying particles that passes together with the liquid sheath flow through the viewing zone;
5. a multimode optical fiber (852) that receives scattered and fluoresced light from the viewing zone that the composite microscope objective (60) gathers and images; and
6. a wavelength division multiplexer (90) for optically separating into color bands light received via the optical fiber (852).

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

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PCT

INVITATION TO PAY ADDITIONAL FEES
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(PCT Article 17(3)(a) and Rules 40.1 and 40.2(e))

Date of mailing (day/month/year)		1 0 OCT 2013
Applicant's or agent's file reference 2294PCT	PAYMENT DUE within ONE MONTH from the above date of mailing	
International application No. PCT/US2013/043453	International filing date (day/month/year) 30 May 2013	
Applicant CYTOJENE CORP.		

1. This International Searching Authority

(i) considers that there are 5 (number of) inventions claimed in the international application covered by the claims indicated below/on an extra sheet:
See Extra Sheet

(ii) therefore considers that **the international application does not comply with the requirement of unity of invention** (Rules 13.1, 13.2 and 13.3) for the reasons indicated below/on an extra sheet:
See Extra Sheet

(iii) ☐ has carried out a partial international search (see Annex) ☒ will establish the international search report on those parts of the international application which relate to the invention first mentioned in claims Nos.:
1-73

(iv) will establish the international search report on the other parts of the international application only if, and to the extent to which, additional fees are paid.

2. Consequently, the applicant is hereby **invited to pay**, within the time limit indicated above, additional fees in the amount indicated below:

\$2,080	x	4	=	\$8,320
Fee per additional invention		number of additional inventions		total amount of additional fees/currency

3. The applicant is informed that, according to Rule 40.2(c), **the payment of any additional fees may be made under protest**, that is, a reasoned statement to the effect that the international application complies with the requirement of unity of invention or that the amount of the required additional fees is excessive, where applicable, subject to the payment of a protest fee.

Where the applicant pays additional fees under protest, the applicant is hereby invited, within the time limit indicated above, to pay a protest fee (Rule 40.2(e)) in the amount of _____ (amount/currency)

Where the applicant has not, within the time limit indicated above, paid the required protest fee, the protest will be considered not to have been made and the International Searching Authority will so declare.

4. ☐ Claim(s) Nos. _____ have been found to be unsearchable under Article 17(2)(b) because of defects under Article 17(2)(a) and therefore have not been included with any invention.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774
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Form PCT/ISA/206 (April 2005)

INVITATION TO PAY ADDITIONAL FEES
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International application No.
PCT/US2013/043453

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Species I) a laser diode based optical subsystem, figures 2, 2A, 3A, 3B, 4A, 4B

Species II) a composite microscope objective, figures 5A, 5B, 5C, 8, 9A

Species III) a fluidic subsystem, figures 14-15

Species IV) a peristaltic pump, figures 17-24

Species V) a wavelength division multiplexer, figures 25-30B

The claims are deemed to correspond to the species listed above in the following manner:

Species I) figures 2, 2A, 3A, 3B, 4A, 4B; claims 56-73

Species II) figures 5A, 5B, 5C, 8, 9A; claims 74-129

Species III) figures 14-15; claims 130-145

Species IV) figures 17-24; claims 146-161

Species V) figures 25-30B; claims 162-173

The following claims are generic: claims 1-55.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

Species I) figures 2, 2A, 3A, 3B, 4A, 4B; have the special technical feature of a laser diode based optical subsystem. This special technical feature is not present in species II, III, IV or V.

Species II) figures 5A, 5B, 5C, 8, 9A; have the special technical feature of a composite microscope objective. This special technical feature is not present in species I, III, IV or V.

Species III) figures 14-15; have the special technical feature of a fluidic subsystem. This special technical feature is not present in species I, II, IV or V.

Species IV) figures 17-24; have the special technical feature of a peristaltic pump. This special technical feature is not present in species I, II, III or V.

Species V) figures 25-30B; have the special technical feature of a wavelength division multiplexer. This special technical feature is not present in species I, II, III or IV.

Species I, II, III, IV and V lack unity of invention because even though the inventions of these species require the technical feature of an optical system having liquid flow, directing light, and having a microscope, this technical feature is not a special technical feature as it does not make a contribution over the prior art in view of WO 2005/033654 A2 (TUNG et al) 14 April 2005 (14.04.2005) page 18, lines 21-29; page 19, line 1, 15-16.

Since none of the special technical features of the Species I, II, III, IV and V inventions is found in more than one of the inventions, unity of invention is lacking.

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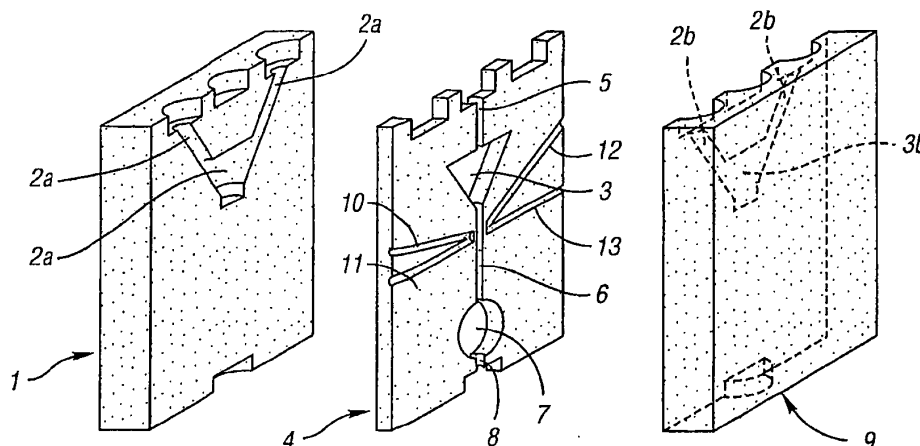
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[Continued on next page]

(54) Title: MICRO FLOW CYTOMETER WITH MULTIANGULAR WAVEGUIDE DETECTORS HAVING SPECTRAL CAPABILITIES



(57) Abstract: A micro flow cytometer having the ability to distinguish light emanating from a particle in the interrogation zone of the cytometer contains a plurality of optical waveguides for receiving emanating light, the optical waveguides positioned at different angles from the axis of the interrogation zone and in the same plane. Photodetectors measuring emanated light provide an electrical signal which is processable to reveal an angular dependence of wavelength with respect to the interrogation channel.

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MICRO FLOW CYTOMETER WITH MULTIANGULAR WAVEGUIDE
DETECTORS HAVING SPECTRAL CAPABILITIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application
5 *Serial No. 60/498,453 filed August 28, 2003., herein incorporated by reference.*

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention pertains to micro flow cytometers having spectral capabilities.

10 2. Background Art

Micro flow cytometers are known, for example as described in PCT/US02/22903, incorporated herein by reference. Such flow cytometers are distinguished from conventional cytometers by their small size and inexpensive construction. The small channel sizes of such devices encourage laminar fluid flow,
15 and their small volume requires correspondingly small amounts of analyte and focusing fluids, when the latter are used. In PCT/US02/22903, a unique detection system employing PIN diodes as detectors has been described. By modulating the incident light and amplifying the detector signal synchronously with the frequency of modulation, a detector output having lower noise than conventional
20 photomultiplier tubes is achieved. PCT/US02/22903 also describes the use of multiple wavelengths of light with suitably filtered detectors, so as to detect fluorescence from more than one species.

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In PCT published application WO 85/05680 is disclosed a conventional flow cytometer of macroscopic size in which the interrogation channel is interrupted by an orifice plate transverse to the axis of the interrogation channel. The orifice plate contains a plurality of optical waveguides which are perpendicular to the channel. However, WO 85/05680 does not disclose any variation of spectral response at the different waveguides, which rather are for the purpose of gathering a plurality of differing kinds of information based on interaction of particles in the channel with an exciting light source which flows through one of the waveguides.

It would be desirable to provide new modes of detection in micro flow cytometers, particularly enhanced ability to gather wavelength-dependent spectral data. Gathering such data may provide means to further increase detection characteristics such as signal to noise ratio, and to provide information on particles being detected which is not currently available.

SUMMARY OF THE INVENTION

It has now been surprisingly discovered that interrogation waveguides having different angular relationships with the interrogation channel of a micro flow cytometer can provide increased signal to noise ratio in particle detection, as well as allowing for investigating spectral response characteristics. In such devices, a plurality of detectors for a single color, color band, etc. may be used, but at different angles to the flow channel. The detector response exhibits an angular dependence on wavelength.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates one embodiment of a micro flow cytometer having microgrooves adapted to receive optical waveguides.

FIGURE 2 illustrates a test setup used to investigate detector response in a micro flow cytometer employing red and blue light sources.

FIGURE 3 illustrates a block diagram for detection and signal amplification of fluorescence emitted by a particle in an interrogation zone of a MFC.

FIGURES 4a and 4b represent typical traces from a MFC using a PMT detector and PIN detector with lock-in, respectively.

FIGURE 5 illustrates one relationship between signal to noise ration (S/N) at various detector angles for various colored microspheres.

FIGURE 6 illustrates the dependency of S/N ratio on wavelength at various detector angles.

FIGURE 7 illustrates actual and theoretical S/N variation with different sample column widths.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The micro flow cytometers ("MFC") of the subject invention contain at least one optical waveguide which supplies incident light to an interrogation zone of a MFC, and a plurality of collecting optical waveguides to gather light from the interrogation zone, the collecting optical waveguides positioned at different included angles with respect to the axis of the interrogation zone, and in the plane of the axis of the interrogation zone. The incident and collecting optical waveguides are preferably optical fibers.

The flow cytometer itself may be made of any convenient material, including but not limited to micromachined glass, quartz, silicon, stainless steel, ceramic, etc., injection molded plastics, and the like. However, the material of construction is preferably a cast silicone elastomer, preferably a transparent elastomer. Such MFCs can be readily cast from a micromachined master. They may also be fabricated as multiple layer structures which are subsequently bonded together. One embodiment of such a device is shown in Figure 1, where a three

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layer construction is used. In leftmost layer 1, the inner face contains gas channels 2a which communicate with leftmost portion of focusing zone 3a. A middle layer 4 contains the bulk of the focusing zone 3 and a sample fluid passage 5 in communication therewith. The focusing zone communicates with the interrogation zone or "channel" 6 which communicates with the outlet reservoir 7 and outlet passage 8. The rightmost layer 9 is, in this case, a mirror image of leftmost layer 1, and contains gas passages 2b and, rightmost focusing zone portion 3b.

The terminations of the various channels external to the device are advantageously adapted to be single plug-in connectors as are common for supply of fluids, or in the case of the connectors for the light supply and detector passageways, simple snap fittings which allow suitable coupling to external light emitters (lasers, laser diodes, etc.) and detectors (photomultiplier tubes, PIN diodes, etc). In preferred devices, these latter components may be incorporated into the body of the cell, such that only electrical connections (electrical contacts, jacks, coaxial cable), etc., may be required. The central layer 4 is also configured to contain microgrooves for receiving minimally one incident waveguide and two collector waveguides. The device shown contains microgrooves for two incident waveguides 10, 11 and two collector waveguides, 12 and 13.

As indicated previously, the micro flow cytometer ("MFC") devices may be constructed of various materials. For example, the entire device may be constructed of a hydrophobic polymer such as polydimethylsiloxane (PDMS), or may be constructed of alternative materials, including conventional thermosetting polymers, thermoplastics, glass, ceramic, metal, and the like, provided that the portions of the internal passageways of the device preceding and including the interrogation zone where both liquid and optionally gas are simultaneously present are transparent to the wavelengths of interest in an area between the light source and the detector within the interrogation zone, so that light scattered by particles passing through the interrogation zone can be analyzed appropriately as in conventional flow cytometers. The walls of the internal passages are preferably of hydrophobic material, or are coated or otherwise treated to render them hydrophobic.

A convenient method of constructing the focusing chamber, interrogation zone, and further channels and reservoirs is the use of soft lithography as disclosed by Y.N. Xia et al., SCIENCE, V. 273 p.347 ff (1996). The use of this technique also allows a simple lens or even a grating to be fabricated in the device.

- 5 Y.N. Xia et al., "SOFT LITHOGRAPHY" Annu. Rev. Matter SC1 V. 28, pp 153 - 184, (1998). The lens and grating may form part of the wall of the interrogation zone, for example, for light focusing and/or collimation or spectral dispersion.

- 10 The devices may be unfocused, *i.e.* configured such that the only fluid flowing through the interrogation zone of the device is the fluid being analyzed. This type of configuration is particularly useful at low flow rates in channels of very small dimension. However, in general, it is preferable that the devices be focused, either by means of a surrounding sheath of liquid, or of gas. As is well known, focused devices maintain the fluid being analyzed in a narrower and more well defined flow through the interrogation channel.

- 15 The optical waveguides used are preferably optical fibers of glass, and may be cast in place in the device or positioned by any other suitable means. Most preferably, MFC devices are constructed of layers of materials, with one or more layers, preferably a layer also containing the interrogation channel or a portion thereof, containing grooves or channels sized to receive and emplace the optical
20 waveguides. Providing of such channels simplifies the construction process and renders the devices uniform from device to device. Since the MFCs are preferably designed to be low cost and disposable, uniformity is particularly desirable. Traditional large scale flow cytometers are both expensive as well as specifically designed for multiple use. The concepts expressed herein can be used with
25 conventional flow cytometers as well.

- The geometry of the focusing chamber, interrogation zone, etc., is such that the sample stream is focused, either two dimensionally or multiply dimensionally, without substantial breakup of the sample stream when used as a flow cytometer. Numerous geometries are possible, and the various channel
30 chambers and zones may take numerous cross-sections. A suitable geometry is

provided in Figure 7 of PCT/US02/22903, which should not be construed as limiting. Dimensions are in mm. Focusing of the sample fluid is illustrated in Figure 8 of PCT/US02/22903, wherein sample fluid stream 41 enters focusing chamber 42 together with air sheaths or "focusing streams" 43a and 43b. As the focusing chamber narrows, the sample stream is altered in shape and constricted 44 to a narrow and coherent stream 45 within the interrogation zone. The geometry may be determined by standard commercially available fluid dynamics software.

In a typical device, the diameter of the liquid sample channel may be from 1 - 1000 μm , preferably 10-300 μm , and most preferably about 20 μm at the downstream end where it communicates with the focusing chamber. The gas channels at this point are preferably somewhat larger in dimension, for example 200-600 μm , preferably 250-500 μm , and more preferably 300-400 μm , and converge with the fluid sample channel at an acute included angle, preferably from 15-45°, more preferably 20-40°, and most preferably ca. 30°. The walls of the focusing chamber are defined by the outer walls of the gas inlet channels at its "upper" end, for example 1000 μm to 2000 μm , preferably about 1500 μm , and at the "lower" end, by the diameter of the interrogation zone into which the focusing chamber transitions, *i.e.* from 100 μm to 500 μm , preferably 200 μm to 400 μm , and most preferably about 300 μm . The transition of the upper end of the focusing chamber to the lower end, the beginning of the interrogation zone, is roughly V-shaped in plan, and the side walls may be slightly concave, as described in the figures, the actual geometry being straight, circular, elliptical, hyperbolic, parabolic, or other shape which suitably minimizes breakup of the sample stream.

The liquid sample and focusing gas flow rates (when used) are selected so as to form a focused but coherent liquid sample stream within the interrogation zone of the desired diameter. It should be noted that when gas focusing is used, the fluid sample is not necessarily completely enveloped by gas, although complete envelopment is preferred. The diameter of the sample stream in the interrogation zone will be a function of the gas flow rate, the liquid sample flow rate, and the vacuum (if any) applied at the outlet. Liquid focusing in the same manner may also be used. The lower end of the interrogation zone, for example the

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outlet reservoir, is preferably maintained at less than atmospheric pressure, for example from 10 mm Hg to 700 mm Hg. A pressure of 45 mm Hg has been found quite suitable. Alternatively, the inlet reservoirs could be maintained at positive pressure without requiring application of vacuum at the outlet. The physical geometry of the device may also affect sample stream diameter.

For example, when gas is used for focusing, the gas flow rate may range from 40 mL/hr to 15,000 mL/hr, preferably ca. 800 mL/hr. At these gas flow rates, with a 45 mm Hg vacuum, and in a channel with a 100 μ m height, the diameter of the focused liquid sample stream in the interrogation zone may be varied from as little as 15 μ m at a liquid sample flow rate of 6 mL/hr to about 100 μ m at a flow rate of 20 mL/hr. Calculations based on fluid dynamics indicate that in a device with a 300 μ m wide interrogation zone, a gas flow rate of about 700-900 mL, and a vacuum at the outlet of 45 mm Hg, the maximum focused liquid stream flow rate is approximately 50 mL/hr, at which point the liquid will contact the walls of the interrogation chamber. These same calculations indicate a minimum sample flow rate of 6.6 mL/hr without losing stream coherency. A stable 6 mL/hr stream has been observed. However, lowering the flow rate to 5 mL/hr caused the stream to be discontinuous. The geometry and dimensions of the channel can be tailored to achieve stability at different flow rates. For further discussion of the necessary calculations, reference may be made to Huh, et al., "Use of Air-Liquid Two-Phase Flow in Hydrophobic Microfluidic Channels for Disposable Flow Cytometers," BIOMEDICAL MICRODEVICES, 4 (2), 141-49 (2002), based on the work of the present inventors.

The light source and light impingement system may comprise a conventional laser, numerous types of which are commercially available. With such lasers, optical systems are generally necessary to properly direct the laser beam through the interrogation zone. In this type of light supply, conventional auxiliary components can be used. "Auxiliary" components include conventional optics including but not limited to lenses, lens systems, filters, gratings, polarizers, quarter wave plates, beam splitters, etc. These auxiliary components comprise the light impingement system when standard light sources are used. The light source may

emit a range of light, may be monochromatic, or may be multichromatic. In one embodiment, a plurality of light sources of different spectral characteristics are employed. These, for example, may emit light in the UV, visible, or IR portions of the spectrum. The wavelengths are often chosen such that they maximize
5 fluorescing modes of particles to be detected. For purposes of detection, it is also desirable that the light sources be of a wavelength other than that to be detected.

Similarly, conventional light collection systems and light detection systems can be used. These include, generally, a photomultiplier tube or equivalent device as part of the detector system, generally also associated with auxiliary
10 components such as lenses or lens systems, beam splitters, filters, gratings, etc., which also comprise the light collection system. The output of the photomultiplier and of photodiodes, when used, are generally connected to standard data acquisition devices and processed using analog or digital circuitry to maximize the signal to noise ratio. These techniques are easily implemented by those skilled in the art
15 based on the concepts presented herein. For example, a noise baseline may be established, and only peaks projecting above this baseline may be reported.

When lenses or lens systems are employed, these may be external to the flow cell, or may be cast or inserted into the flow cell to minimize the number of external auxiliary components. The impingement and collection pathways
20 terminate internally such that light impinges upon the collection zone, and is detectable following passage through the collection zone by the detector. The pathways for conventional impingement and collection pathways generally terminate "proximate" to the interrogation zone. For transparent devices, for example of acrylic resin or polyorganosiloxane, the path termini may be at an outside wall of
25 the device, or may be located in close proximity to the interrogation zone, with an optically transparent passage, for example a hole, transparent rod or window, etc., whose innermost closed end is close to the interrogation zone. The actual construction of the impingement and collection zones is not critical so long as their function is achieved. It should be noted that the material of construction, if optically
30 transparent at the desired wavelengths, may be structured in the form of a lens element or grating. While light transmission from the interrogation zone will be

conveyed by optical waveguides, and while it is preferably that the impinging light also be so conveyed, the lenses, gratings, etc. heretofore described may precede or follow the optical waveguides *per se*. Most preferably, the optical waveguides communicate directly with the interrogation channel or an optically transparent wall thereof, as described more fully below.

To reduce size and cost, it is particularly preferred that the conventional light source be supplanted by one or more solid state "laser" diodes, which are preferably located within or on the flow cell or a housing thereof. Use of such devices is made practical by a light impingement system which employs optical waveguides, preferably fiber optics. In this embodiment, the light from a laser diode light source is transmitted to the proximity of the interrogation zone by fiber optics. The optical fiber(s) terminate as close as possible to the interrogation zone to minimize scattering effects, for example terminating from 10 to 100 μm , preferably 30-80 μm from the interrogation zone. Due to the nature of the signal carrying capabilities of the optical fibers, lens systems which are commonly used in conventional systems may be eliminated, thus providing significant savings in size and cost, to the degree that in a preferred embodiment, the laser diode light source(s) may be incorporated within the body of the flow cell itself or intimately associated therewith, for example in a compact case, such that the entire device is portable, handheldable, and/or disposable. In addition to focusing fluid and sample fluid connections, only standard electrical connections need be provided, which can constitute metal contacts, jacks, etc.

The light impingement system can also be used with a remotely located light source, either of the conventional laser type or a laser diode type. In such cases, the optical fibers embedded within the body of the flow cell are preferably terminated at the exterior of the cell body, for example by conventional fiber optics coupling devices.

In like manner, the light collection system preferably comprises fiber optics as well, and preferably does so for purposes of miniaturization and disposability. As in the case of the light impingement systems, the optical fibers

may be connected to an external detector, for example a photomultiplier tube and auxiliary components desired to maximize the signal to noise ratio. However, the fiber optics may also be connected to a detector system "on board," which allows for an integrated transportable and even handheld device. The waveguide
5 configuration is also self-locating and fixtured, which minimizes the potential for interfering vibrations during measurements with a miniaturized device.

The detector system of the present invention is used in conjunction with an optical waveguide or fiber optic collection system, and preferably comprises a semiconductor detector, preferably a PIN diode. Such diodes are commercially
10 available, are capable of being biased by relatively low voltages, and are of relatively small size and cost. The PIN diode preferably includes one or more filters grown directly on the PIN structure or attached thereto. Such a filter is useful for filtering out extraneous light. For example, the filter may allow light of a fluorescence wavelength to pass while filtering out the wavelength used to excite the
15 fluorescent species, for example a dye marker. Alternatively, an optional filter may be contained in the collection pathway as a component of a collimating lens system.

The PDMS observation channel illustrated in Figure 1 was designed to have a cross-sectional area of $100 \times 300 \mu\text{m}^2$ and three fluid entry compartments. Design parameters for the channel such as lengths, angles, and volumes were
20 determined by developing a microfluidic model within the computational fluid dynamics (CFD) software Fluent 5.5 (Fluent Inc., NH). A three-dimensional computational model was utilized to simulate the hydrodynamic focusing behavior of the sample at the interrogation point of the laser under different scenarios. Specifically, models which could predict the sample focusing effectiveness of sheath
25 fluid injected at different impingement angles (20° - 60°) were constructed to find an optimal configuration for the impingement angle of the sheath fluid channels on the sample channel. Similarly, various ratios of sample flow rate to sheath fluid flow rate (1:1-1:10) were simulated to observe the effectiveness of sample fluid focusing. The final dimensions and operating parameters for the channel were
30 selected such that the sample fluid is well focused with a constant velocity in the center of the channel well before the interrogation point. The design was also set

such that the sample fluid in entire thickness direction (100 μ m) would be uniformly illuminated as necessary to minimize device performance variation.

The optical sub-system of the micro flow cytometry system must serve 1) to deliver light, preferably laser light, to a well-defined observation zone, and 2) to collect fluorescent light from the observation zone and deliver it to an optical filter for separation from scattered laser light prior to electronic detection. In contrast to conventional flow cytometers, the present invention uses integrated optical fibers. The fiberoptic system has a more compact volume, yet is still capable of transmitting excitation and fluorescence light with high efficiency. In addition, by embossing grooves that serve as fiber receptacles directly into the PDMS observation cell as shown in Figure 1, the optical fibers can be fit tightly in the system without separate alignment steps necessary during assembly. Figure 1 also illustrates that multiple light impingement and collection angles are also easily possible (45° and 90° shown), which is not the case in conventional flow cytometers. The detection system can therefore be configured to collect information from any or all angles simultaneously as desired in a given application.

In addition to the consideration of microfluidics and materials of construction, much effort has been directed to develop micro-optical components that could potentially be utilized within a flow cytometer microsystem. In a flow cytometer, the optical system usually consists of a laser, photodetectors, and optical components such as lenses, mirrors, and filters. In conventional flow cytometers photomultiple-tubes (PMTs), and occasionally avalanche photodiodes (APDs), are used for fluorescence detection in the wavelength range of 400 to 1000nm. These detectors are utilized in flow cytometry and other applications due to their high sensitivity, high internal gain ($> 10^6$), and fast response (10^{-7} - 10^{-9} s). However, PMTs are bulky, fragile, consume large amounts of power, and are generally sensitive to environmental conditions. APDs are also somewhat bulky and require high-power, resulting from their need for active thermal management to maintain high S/N. Although both PMT and APD photodetectors yield a large S/N due to their internal gain, optical systems incorporating these devices into flow cytometers are costly and difficult to miniaturize. For this reason, PIN-based photodetectors

which are 1) low-cost, 2) low power, and 3) tightly integratable with other optical components are preferably used.

Since the fluorescent signals generated from the detection of biological cells only generate a few nA of current in PIN photodiodes, a lock-in
5 amplification technique is applied to increase the S/N of the micro flow cytometer. While common in other technical applications, the lock-in amplification approach, which is also described in PCT/US02/22903, has not been previously discussed in the literature for flow cytometry. The lock-in technique is effective because it acts
10 as a narrow band-pass filter which removes much of the unwanted noise while allowing the signal which is to be measured to pass through. To achieve this, the laser excitation is modulated at a reference frequency which induces fluorescence emission from the interrogated sample at the same frequency. The modulated signals detected at the photodiode are then monitored with high sensitivity using the
15 lock-in circuit. For reliable performance, the modulation frequency is set to be two orders of magnitude higher than the bandwidth required for detecting the fluorescence emission from moving sample particles. The lock-in circuit parameters such as the time constant and sensitivity are selected to maximize S/N at the given modulation frequency.

The spectral characteristics can best be understood by consideration
20 of an MFC having two monochromatic light sources of different peak wavelengths, for example blue and red. The term "monochromatic" is used in its ordinary sense, and is meant to include light sources which emit the majority of their energy over a relatively narrow wavelength band. Examples of such monochromatic light sources include, but are not limited to, LEDs, lasers, etc. Spectral emission lamps
25 such as mercury vapor lamps, which typically emit a line spectrum, may also be used, preferably with a filter to isolate one or more wavelengths of interest.

The two light sources of the present illustration may be applied to the interrogation channel through a single optical waveguide, for example through a combining prism, rotating mirror, etc, or may be applied through two different
30 optical waveguides. When two or more optical waveguides are used for the incident

light, the waveguides are preferably positioned with their ends which are proximate the interrogation channel as close as possible to each other, and may be angled slightly with respect to each other to try to obtain a condition where a single particle is illuminated by both incident wavelengths in the same position in the channel, as shown in Figure 1. Otherwise, the output signals will be staggered from each other in time.

At least two output optical waveguides are employed in this illustration, one, for example, at an included angle of 90° to the axis of the interrogation channel, and one at an included angle of 45° to the axis of the interrogation channel. Typically, the two output optical waveguides will not receive the same light energy at any given wavelength. Rather, due to refractive, diffractive, and reflective effects, the signal strengths will differ. Moreover, the difference in signal strength will be wavelength dependent. Therefore, by measuring the difference in signal strengths at a first wavelength and the difference in signal strengths at a second wavelength, by comparing the relative differences of these "comparison" signals at the two different wavelengths, a plot of wavelength versus difference in comparison signal can be made. By measuring a difference in comparison signal strength during operation, each difference will correspond to a different wavelength. Thus, the wavelength emitted by a fluorescing particle may be measured by comparing the difference in output signal strength between two output waveguides at different included angles to the interrogation channel.

For example, once the output signal and wavelength relationship has been established, signals may be selected with regard to their comparison output to distinguish between multiple particles having different spectral signatures, *i.e.*, different fluorescent wavelengths. Thus, in a sample fluid containing two different microorganisms, each receiving a stain which will cause fluorescence at a different wavelength, the detectors, for example one or more photodiodes or PMTs may be filtered to remove the incident light, and the outputs sampled at two different angles with the interrogation channel. The signal strength at any given output waveguide will not yield any information which can distinguish between the different microorganisms. Rather, a signal merely indicates that a particle is pressing through

the interrogation zone. However, if at this instant, the difference between the two differently angled output waveguides is measured, the difference will indicate which type of particle is traversing the zone. Thus, a low difference in signal strength will correspond to one wavelength of fluorescence, attributable to one type of particle, while a higher difference will correspond to a second wavelength and hence a second type of particle. By synchronizing particle detection with output signal strength difference between the two output waveguides, a count of each type of particle can be made. In a like manner, the S/N ratio may be used in lieu of a difference or "comparison" signal. As indicated in the Example and as illustrated in Figure 6, the S/N ratio is also wavelength dependent when expressed as a ratio between variously angled detectors.

The means for measuring signal strength, amplifying signals, measuring signal strength differences, etc., are all accomplished by means well known in the electronic arts. For example, signal strengths may be amplified by conventional operational amplifiers which are readily available, or amplifiers constructed of discrete components. Differences in signal strength may be made by means of comparators or differential amplifiers. The signals may be exclusively analog, or may be digitized by analog-to-digital converters. Once digitized, the signals, their difference (comparison signals), etc., may be processed by suitable software. An output may be presented as a trace on a CRT as shown in Figures 4a, 4b, or in tabular form, etc.

The micro flow cytometers must have an inlet for supplying test fluid, and an outlet. However, these need not be made in such a fashion that fluid necessarily needs to enter or exit the device during measurement. For example, the inlet may comprise a well or reservoir holding fluid until fluid flow is desired to be established. In like fashion, the outlet may continually remove fluid from the device, or again may comprise a well or reservoir to receive fluid from the interrogation channel. In disposable MFCs, outlet wells or reservoirs may be highly desirable, to avoid the necessity of disposing of microorganism containing fluids.

In preferred devices, photodiodes are employed as the detector, for reasons advanced earlier. Due to the lack of internal amplification and noise associated with such devices, it is preferable also as earlier indicated, to modulate the incident light and to amplify detected output light synchronously. For example, a function generator may be used to modulate the incident light source such as an LED, and also to provide a signal to the amplifier for synchronous amplification. The function generator may produce any type of output, for example sinusoidal, square wave, sawtooth waves, etc. When two or more incident wavelengths are desired to be used, they are most preferably driven substantially out-of-phase with respect to each other. For two sources, for example, they may be 180° out-of-phase. Some overlap of the light intensities at multiple wavelengths may be tolerated. However, the greater the overlap, the less will be the signal-to-noise ratio. A function generator may be easily arranged to provide signals which would produce no overlap. The term "substantially out-of-phase" implies that the peak intensities of the various wavelengths can be distinguished from each other with respect to time.

Figure 3 illustrates a schematic of a synchronous ("lock in") detection system which can be assembled with conventional components. A 1 MHZ oscillator 20 is chosen to provide signal to both the laser driver 21 and synchronous detector 22. The laser driver provides a suitable driving signal for laser diode or LED 23, causing it to emit pulses of light at the 1 MHZ signal rate. The laser light, upon contacting a particle 24 in the interrogation zone causes a fluorescent output which is detected by PIN photodiode 25. The resulting electrical signal is amplified by a low noise differential amplifier 26, and optionally further amplified by additional amplifier(s) 27. The amplified signal is routed to a high Q (e.g. Q=100) bandpass filter 28, which may, for example, limit the output signal to the range of 995 KHz to 1.005 MHZ, only a 10 KHz bandwidth. This output signal from the bandpass filter is routed to the synchronous detector 22, the output of which may be digitized by analog to digital (A/D) converter 29, and the data subsequently processed and/or collected by microcontroller or microcomputer 30.

The term "emanating" is used to describe light from a particle having been illuminated by incident light. Light emanating from such a particle may have the same wavelength as the incident light, for example by the incident light having been reflected from or scattered by the particle. In most cases, however, the
5 particles will fluoresce, *i.e.*, emit one or more wavelengths different from the incident light wavelength. Fluorescence at a different wavelength provides the opportunity to eliminate the rather strong incident light from the output, resulting in a significant increase in the signal-to-noise (S/N) ratio which may be obtained. The term "comparator" means a device or plurality of devices which are able to
10 compare relative signal strengths. A comparator need not be a discrete device, but may, for example, include detectors, amplifiers, analog to digital converters, and a microprocessor. The output of the comparator may be processed further to provide any desired type of signal.

Thus, in one aspect, the subject invention pertains to a spectral micro
15 flow cytometer device with a micro flow cytometer having an inlet, an outlet, and an interrogation channel in fluid communication with the inlet and outlet, the interrogation channel defining an interrogation plane in which the channel is positioned; minimally three optical waveguides terminating proximate to the channel and in the interrogation plane, each optical waveguide directed toward the channel
20 at a different angle with respect to the axis of the channel, the inlet direction of the channel being 0°; minimally one incident light source coupled to an optical waveguide and illuminating the channel; and a comparator which compares a light intensity or a signal to noise ratio at one optical waveguide positioned at a first included angle, with a light intensity or a signal to noise ratio at a further optical
25 waveguide positioned at another included angle, the difference in intensity or in signal to noise ratio being proportional to the wavelength of light emanating from a particle passing through the channel.

In another aspect, the micro flow cytometer contains a modulated,
first monochromatic light source which directs light into an incident optical
30 waveguide such that modulated monochromatic light impinges upon particles within an interrogation channel; optionally, a modulated, second monochromatic light

source of a different color whose modulation is substantially out-of-phase with the modulation of the first monochromatic light source, and which directs light to the interrogation channel through a second incident light optical waveguide, which may be the same as or different from the first incident light waveguide; a first photodiode
5 detector sensitive to light from the first monochromatic light source, the second monochromatic light source, or both, or sensitive to a fluorescent wavelength emitted by a particle in the channel as a result of the particle being illuminated by one or more light sources, the photodiode detector communicating with the channel through a first output waveguide in a plane with the first incident waveguide and the
10 channel, and positioned at a first included angle with respect to the channel; a second photodiode detector communicating with the channel through a second output waveguide in a plane with the interrogation channel and the first incident waveguide, but at a different included angle to the channel than the first output optical waveguide; at least one amplifier which amplifies an output from the photodiode
15 detector(s); a means for selectively sampling an amplified output from the amplifier synchronously with a respective modulation frequency of the first or second monochromatic light sources to provide an output proportional to incident light fluorescent light impinging on the detector, and a second output proportional to light of a different incident color or fluorescent color; a means for comparing the signal
20 strength obtained from the first detector with the second detector at at least two different wavelengths to obtain an angularly dependent comparison signal or signal to noise ratio for each wavelength; and a means for comparing the angularly dependent comparison signals or signal to noise ratios to each other to provide spectral differentiation of light collected by the respective output waveguides.

25 A yet further aspect of the invention pertains to a method for providing spectral information for particles in an interrogation channel of a micro flow cytometer, by illuminating the particles with incident light directed to the channel through an incident optical waveguide; transmitting reflected, scattered, or fluorescent light from a particle in the channel through separate output optical
30 waveguides positioned in a plane which includes the interrogation channel, the output optical waveguides positioned at different included angles with respect to the axis of the channel; measuring light intensity or signal to noise ratio from each

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output optical waveguide at two different wavelengths, and providing comparison output signals proportional to the difference between the intensity of each respective wavelength from the output optical waveguides, thus providing a comparison output signal for a first wavelength and a second comparison output signal for a second wavelength; and comparing the comparison output signals or signal to noise ratios, by means of which spectral information relating to the particles being observed can be collected, and if desired, further processed.

Example

The PDMS observation cell with a microfluidic channel and optical fiber microgrooves is fabricated using replica molding. The process begins with the patterning of a negative tone SU-8 photoresist (SU-8 2050, Microchem Co., MA) mold on a silicon wafer using lithography. This is followed by silanization of the wafer surface using 3-(trimethoxysilyl)propyl methacrylate (Sigma-Aldrich Co., MO), which facilitates the parting of a PDMS layer during the next process step. A PDMS precursor liquid is then well mixed with a curing agent (Sylgard 184 Silicone Elastomer Base, Dow Corning Co., MI) and poured onto the SU-8 micromold on the silicon wafer. After curing for 24 hours at room temperature, the PDMS slab with embossed observation channel features is peeled off. The final size of the fabricated flow cell is $4 \times 2 \text{ cm}^2$. The microfluidic channel has a length of 1 cm and cross-sectional area of $100 \times 300 \mu\text{m}^2$.

The flow profile of the hydrodynamically-focused sample liquid is observed using optical and confocal microscopy. These methods permit characterization of the hydrodynamic focusing performance of the PDMS microfluidic channel and provide cross-sectional images of the sample fluid. For the confocal microscopy study, sample water containing fluorescein (Sigma-Aldrich, MO) is utilized as the characterized fluid. The sample fluid is excited by a blue laser contained within the confocal microscope (LSM-510, Zeiss, Germany).

Figure 2 illustrates the experimental testbed used to evaluate the performance of the microfluidic and optical design of the PDMS micro flow

cytometer previously described. FluoSpheres® polystyrene microspheres (Molecular Probes, OR) are used as a reference sample to illustrate the performance of the micro flow cytometer, using scarlet fluorescent (F-8843) microspheres with 15.5µm average diameter, and green fluorescent (F-21010) microspheres with 15 µm average diameter, as listed in Table 1. Significant variation in the absorption and emission wavelengths and intensities is observed experimentally.

TABLE 1

<i>Sample</i>	<i>Maximum Absorption Wavelength (nm)</i>	<i>Maximum Fluorescence Emission Wavelength (nm)</i>
FluoSpheres® polystyrene microspheres, F-21010 (green fluorescent, 15.0 µm)	645	680
FluoSpheres® polystyrene microspheres, F-8843 (scarlet fluorescent, 15.5 µm)	450	480

The semiconductor laser diodes used to excite the reference fluorescent microspheres include 1) a 6mW red iode laser (SCFC2-0635-0006-T-25, Optical Fiber System Inc., MA) with a peak wavelength of approximately 635nm, and 2) a 5mW blue diode laser (NDHB500APAE1 Engineering Sample, Nichia Co., Japan) with a peak wavelength of approximately 440nm. The blue and red laser excitations are modulated at 100 kHz with differing phase angles (*e.g.*, signals 180 degrees apart in phase). Multimode optical fibers (F-MCB-T, Newport Co., CA) with 100µm core diameter and 110µm cladding diameter are utilized to transmit energy to and from the interrogation zone as shown in Figure 2. The optical filters are selected according to the transmission spectral characteristics listed in Table 2 in order to separate the high-intensity excitation light from the relatively weak fluorescent light emitted by the above-described microspheres. The photocurrent generated at the PIN photodiode (S7329-01, Hamamatsu Photonics Co., NJ) is amplified using an OP-amplifier-based circuit (10⁴X, OPA655, Texas Instruments Inc., TX). The circuit converts the photocurrent signal to an amplified voltage signal. The voltage signal is processed using a lock-in amplifier (SR-840, Stanford

Research, Inc., CA) and extracted from the background noise. A block diagram is illustrated in Figure 3.

TABLE 2

<i>Sample</i>	<i>Optical Filter</i>
645/680 Polystyrene Bead	695 Band Pass (FWHM: 11.5 nm)
450/480 Polystyrene Bead	480 Long Pass (50% transmission at 480 nm)

Two independent experiments are conducted to fully validate the performance of the PIN-based signal detection process. First, fluorescence detection with and without the lock-in amplifier was performed. To achieve this, the signal detected at the PIN photodiode is guided to a pre-amplifier circuit and then identically divided into two cables. One of the divided signals is directly monitored and the other was processed by the lock-in amplifier prior to monitoring. The signals are then compared with each other.

Second, signals simultaneously obtained from a PMT (P30CWAD5-01, Electron Tubes Inc., NJ) and PIN photodiode are compared with each other to validate the performance of the signal detection system using the PIN photodiode and the lock-in amplifier. The PMT was selected as a reference detector, as it is widely used in cytometry and known to perform well in this application. The PMT is powered by a 900-1200V voltage source to obtain maximum S/N. For this experiment, one excitation and two detection optical fibers are embedded into the observation cell at the same angle. The detection fibers, one of which is connected to the PIN photodiode and the other connected to the PMT, simultaneously capture the fluorescent signals and send them to the photodetectors. The signals from the PIN photodiode with lock-in amplifier and the PMT (without signal processing) are then compared.

To better understand the influence of experimental conditions on the PDMS micro flow cytometer, scarlet/green inorganic and biological particles are

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tested under variable sample/sheath flow rate ratios, and multiple optical detection angles. Three major parameters are investigated: 1) S/N, 2) S/N variation, and 3) recovery rate. The recovery rate is estimated by injecting a sample fluid with polystyrene microspheres of known concentration ($10^6/30$ beads/ml) into the
5 microchannel. The final microsphere concentration is achieved by a 1:30 dilution in buffer solution (Tris Buffered Saline with bovine serum albumin (BSA), T6789, Sigma-Aldrich Co., MO).

To demonstrate two-color excitation and detection using the developed micro flow cytometer, two pairs of optical fibers are arranged at an angle
10 of 135° between the excitation and detection fibers as shown in Figure 2. A mixed sample consisting of scarlet and green microspheres (each diluted 1:30 with buffer solution) was injected into the microfluid channel and hydrodynamically focused. The sample was excited at a single interrogation point using two lasers with 440nm and 635nm wavelengths. In order to prevent the coupling of the two excitation light
15 sources, the red and blue laser diodes were driven 180° out-of-phase (100kHz).

To demonstrate the feasibility of using the micro flow cytometer to detect fluorescent labeled biological samples, an experiment with the fungus *Saccharomyces cerevisiae* is performed. To prepare the fungus sample, a powder sample of dried cells is mixed into warm water and maintained at 38°C for about 20
20 minutes to revive the culture. After full activation of the cells, a $2\mu\text{l}$ yeast solution is stained using Syto[®] 62 (or Syto[®] 44) (Molecular Probes, OR) and incubated for 20 minutes at room temperature. The yeast cells, with fluorescently labeled nucleic acid, are injected into the flow cell at a sample flow rate of 5ml/hr with total flow rate of 15ml/hr and excited using the 635nm red (or 440nm blue) laser diode. The
25 optical signals are separated from the excitation radiation using the 695nm band-pass (or 480 long-pass) filter.

When examined by optical and confocal microscopy, the sample fluid is well confined in the center of the channel in the horizontal direction. It is found, as predicted, that the width of the focused sample fluid can be controlled by
30 changing the ratio of sample/sheath flow rate. It is observed that by tuning the ratio

of flow rates between the sample fluid and the sheath fluid (maintaining fixed total flow rate of 15ml/hr), experimental results conferred with CFD simulation results to within 10% error.

Figure 4a demonstrates the simultaneous detection by the PMT (without advanced signal processing) and the PIN photodiode detection (4b) with lock-in technique. It is observed that all particles detected with PMT are observed with the PIN photodiode with equal or higher S/N.

The micro flow cytometer performance was evaluated for the case of fluorescent microsphere detection. Three parameters were used as performance metrics: S/N, S/N variation, and recovery rate. These performance parameters are discussed in the following paragraphs.

S/N: Figure 5 shows the average S/N corresponding to various angles between the excitation and detection fibers. The S/N values obtained from the experiments show that the device can successfully detect the fluorescence emission in different angles for 15 μ m polystyrene microspheres. The detection angle resulting in maximum S/N (~ 9.5) is 180°. For the scarlet polystyrene microspheres, the S/N is slightly reduced when measuring at 45° and 135° off the excitation direction. In contrast, the S/N at 45° and 135° for green polystyrene microspheres measurement is reduced to about 35% of that in 180°. These results have useful and significant ramifications for estimating fluorescence color that will be discussed later.

The S/N variation (expressed as the coefficient of variation percentage) varies at different angles for numerous ratios of sample/sheath flow rates. The minimum variation occurs at 180° detection and at the lowest ratio of sample/sheath flow rate. The S/N variation for the 180° detection is observed to be approximately 25% and 35% for the scarlet and green polystyrene microspheres respectively over most of the flow rate ratios investigated. The variation over this range is believed to be in part due to the variation in the dimensions and intensity of the microspheres, and in part due to the variation in the vertical position of the

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microspheres in the microfluidic channel. Furthermore, the S/N variation is observed to increase as the ratio of sample/sheath flow rate became higher. This variation increase is more obvious at 45° and 135° detection than it was at 180°. As will be discussed later, this is likely due to the wider column of sample fluid at higher at higher sample/sheath flow rate ratios, and the increased possibility of multiple particles in the interrogation zone at the same time under this condition.

The recovery rate also varies for different angles at different ratios of sample/sheath flow rate. The maximum recovery rate (75%) is obtained at 180° detection. Higher recovery rates generally are observed with increasing sample/sheath flow rate ratio. Deviations from 100% recovery are discussed later.

Experiments with simultaneous two-color excitation/detection demonstrates the feasibility of the compact optical arrangement as shown in Figure 2. The high S/N ratio (6.4 and 2.1 for scarlet and green microspheres, respectively) of the detected signal shows that the system is capable of simultaneous two-color excitation/detection with a single interrogation zone. Moreover, the recovery rates of the two-color excitation/detection system are observed to be only slightly lower than those of the single-color results. The differences are approximately 10-20%, which may be due to experimental variation, or correctable interference between the detection systems of the two colors.

In observing the electronic signal corresponding to the detection of *Saccharomyces cerevisiae* labeled with Syto 62®, the concentration of the yeast cells calculated from the experimental results is about 1.90×10^4 cells/ml, with a S/N of approximately 2.8. The results show that the micro flow cytometer is effective in detecting yeast cells, with high potential for application to other biological cells.

S/N: The data in Figure 5 indicate that the average S/N is almost invariant regardless of the detection angles for the scarlet fluorescent microspheres, whereas it is highly influenced by angle for the green fluorescent microspheres. Moreover, it is observed that the signal intensity is reduced at the 45° and 135°

angles compared to that at the 180° angle. The reduced off-angle signal intensities are likely due to numerous opportunities for scattering losses at the off angles. For instance, when the detection optical fiber is not aligned normal to the channel, the optical path can be significantly bent due to the difference in index of refraction at the interfaces between the PDMS channel wall and the fluid, and between the PDMS flow cell and the air gap in front of the optical fiber. In addition to optical path bending, the distance between the end of the detector fiber and the center of the interrogation zone, d_c , highly influences the magnitude of the detected light intensity. In the current flow cell design, the 180° detection arrangement has a shorter distance between these two points ($d_c \sim 200 \mu\text{m}$) than the 45° and 135° detection arrangements ($d_c \sim 330 \mu\text{m}$). The longer distance leads to weaker detected signals at the 45° and 135° angles, as would be predicted if the sample particles were assumed to be point light sources positioned at the center of the interrogation zone. In this case, the intensity of the light collected by the optical fiber is proportional to d_c^{-2} , and it would be predicted that the signal intensity at the 45° and 135° angles should be about 37% as that at 180°. From the experimental results, the average signal intensities at 45° and 135° were 36-42% of that at 180°, which is in reasonable agreement with the prediction.

Moreover, experimental results show that the relative S/N between various detection angles varies for sample particles with different spectra. Consequently, by taking advantage of simultaneous multiple-angle detection, a micro flow cytometer with spectrometer characteristics is possible. To test this hypothesis, both scarlet and green fluorescent microspheres (F-2101 and F-8843) and *Saccharomyces cerevisiae* labeled by Syto® 62 and Syto® 44 are examined, and the ratio of average S/N at 180° and 45° detection is calculated for both green and scarlet particle/cell detection. As shown in Figure 6, both microsphere and biological detection yield a distinct ratio of 180° to 45° S/N depending on the fluorescent color of the interrogation target. The difference of the ratio is consistent with color. Thus, spectral flow cytometers for specialized biological assays targeted at simultaneous multi-species detection are possible with the devices of the subject invention.

To determine whether particles smaller than 15 μm can be detected using this micro flow cytometer design, scarlet fluorescent polystyrene microspheres with 6 μm average diameter are also tested in the micro flow cytometer using the 180° optical fiber arrangement. The S/N (~ 1.5) is low but detectable, suggesting that the signal will be prohibitively weak if the particle size becomes smaller than 6 μm . Ideally, the intensity of fluorescence emission is proportional to the surface area of the fluorescence particles, and therefore the fluorescence intensity of the 15 μm microspheres is expected to be about 6.25 times larger than that of the 6 μm microspheres. The experimentally observed results are in close agreement with this prediction, showing a S/N difference of 6.3 (9.5/1.5).

S/N Variation: The variation of the S/N at a given detection angle can be correlated with the shape and area of the interrogation zone, Ω_i , that is defined by the intersection of the sample fluid column, the optical excitation zone, and the optical detection zone as shown in Figure 12. To account for the S/N variation observed experimentally, it is assumed that:

- The positional variation of the particle passing through Ω_i is negligible in the vertical direction.
- The particle can pass through any point in Ω_i at the same velocity, with the same probability.
- The optical excitation in Ω_i is spatially uniform.
- The detected particle is a point light source.
- The optical path bending can be ignored at any interfaces.
- The optical attenuation loss in the optical fiber is negligible.

Based on these assumptions, the intensity of the fluorescence signal I detected from the sample particle at an arbitrary point $r = (x, y)$ in Ω_i is given by.

$$I(r) = \frac{I_0}{|r - r_d|^2} \quad (1)$$

- 5 where I_0 is the original total intensity of the excited fluorescent microsphere, and r_d represents the position of the end of the detection optical fiber in the x - y coordinate system in Figure 12. The maximum and minimum signal intensities are given by the maximum and minimum of the maximum intensity calculated at each x position within the interrogation zone, Ω_i , which can be expressed as

$$\Omega_i = \left\{ (x, y) \mid |x| \leq \frac{w_c}{2}, |y| \leq \frac{d_f}{2}, f_1(x) \leq y \leq f_2(x) \right\} \quad (2)$$

$$f_1(x) = \frac{d_f}{\cos \theta} + \frac{x}{\cos \theta} \quad (3)$$

$$f_2(x) = \frac{d_f}{\cos \theta} - \frac{x}{\cos \theta} \quad (4)$$

- 15 where d_f is the excitation and detection optical fiber diameter, w_c is the width of sample fluid column, and θ is the angle between excitation and detection direction. In this case, the S/N variation $\Delta S/N$ is estimated as

$$\Delta S / N = \frac{(I_{\max} - I_{\min}) / 2}{\langle I \rangle} \quad (5)$$

where $\langle I \rangle$ is the averaged signal intensity given by

$$\langle I \rangle = \frac{I_{\max} + I_{\min}}{2}$$

(6)

Figure 7 shows the calculated S/N variations at varying sample column width and detection angle. Due to the geometrical symmetry, the calculations for 45° and 135° detection angles yield the same predictions.

As can be seen from Figure 7, the estimated S/N variation of 180° detection is close to the observed experimental result obtained from green fluorescence detection, with the difference within 10%. In contrast, the predicted S/N variation of 45° or 135° detection is much lower than that observed experimentally. This contradiction is a likely result of ignoring optical path bending in the model, which makes 180° detection less sensitive to the position of the sample particles in the microfluidic channel when compared with 45° and 135° detection.

Recovery Rate: At a low sample flow rate relative to the sheath flow rate (~3:12), the sample stream becomes unstable due to the large pressure difference between the sample and sheath flows near the channel inlets, resulting in discontinuous fluid motion. The discontinuous sample flow causes particles to become trapped in the inlet reservoir, which is one of the major reasons for the low detection recovery rate at a low sample flow rate (10-30%). As the sample flow rate increases to nearly 50% of the sheath flow rate, the sample stream can establish a stable liquid column by overcoming the pressure difference at the channel inlets. The stable liquid column is more effective at centering the sample particles in the center of the interrogation zone in a consistent manner. The stable flow of the sample particles leads to a higher recovery rate (55-75%) at the higher sample/sheath flow rate ratio.

On the other hand, larger liquid column width resulting from a higher sample flow rate is likely to prohibit a single-file sample flow, allowing multiple particles to simultaneously enter into the interrogation zone. Fluorescence signal intensities detected from the scarlet polystyrene microspheres at sample/sheath flow

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rate ratio of 6:9 and 7:8, respectively (total flow rate of 15ml/hr) show that the signal intensity distribution is approximately divided into two subgroups at a flow rate ratio increases to 7:8. The middle intensity of each subgroup relates to the others in a whole number ratio. For instance, the middle intensities in Figure 14(b) are approximately in the ratio of 1:2:3 (0.45:0.90:1.35). Assuming that the signal intensity is proportional to the number of the simultaneously detected beads, these results appear to indicate that multiple fluorescent particles are detected in the interrogation zone at the same time. To test this hypothesis, the detection algorithm is modified to account for the possibility of multiple beads passing through the interrogation zone. With this modification to the detection algorithm, the recovery rate increases by about 25-30% (for flow rate ratio 6:9 and 7:8), leading to about 95% recovery for the fluorescence detection in the micro flow cytometer.

Even with the intensity correction, 100% recovery is not observed due to some loss of microspheres in the microfluidic interconnects. To minimize such losses, a buffer fluid containing BSA is utilized. BSA is found to be effective in coating the PDMS channel wall surface to prevent particle adhesion. The BSA buffer doubles recovery rates relative to experiments with other buffers such as PBS (phosphate buffered saline) or de-ionized water, both of which have little effect on changing the surface chemistry of the channel wall.

Lower recovery rates are also observed at the 45° and 135° detection angles. This suggests that the fluorescence signal may become weak enough to be indistinguishable from the background noise when the sample particles take a fluidic path far away from the detection optical fiber. The observation is likely due to the fact that optical path bending can shift the detection zone from the center of excitation zone defined by the laser diodes. This shifting can generate a partial or total blind spot for the 45° or 135° detector fibers, resulting in a low recovery rate. This is readily correctable by modifying the optical pathways in the system through the use of micro optical components in a next generation design of the system.

Single-Color vs. Two-Color Excitation/Detection: For two-color excitation/detection setup, the S/N ratios are about 16% (for $\lambda = 635\text{nm}$) and 50%

(for λ -440nm) lower than that observed for the single-color excitation/detection setup. For the most part, this is because the background noise level in the two-color system is approximately 30% and 100% larger for scarlet and green fluorescence detection respectively. The background noise increase for the 2-color system is likely caused by the excitation light of one color leaking into the detection optical fiber. The leakage of excitation light and imperfect filtering can be solved by use of customized optical filters based upon the specific wavelength spectra of the excitation and detection targets. This will yield a two-color excitation/detection microcytometer possessing nearly the same S/N as the single-color system discussed previously.

While the inventive microsystem is highly successful to demonstrate the feasibility of producing a two-color micro-flow cytometer with PIN photodiodes and color differentiation ability, a number of design modifications would be achieved by designing the flow cell to have a channel width more comparable to the sample particle diameter. This channel design would result in a larger ratio of the sample flow rate to the sheath flow rate at a fixed combined total flow rate. In this case, a stable sample liquid column could be established while keeping its width narrow enough to confine the particle in the center of the interrogation zone with high precision. The modified sample liquid column width would improve the recovery rate and reduce the S/N variation, and promote a single-file flow in the microfluidic channel.

With respect to the optics design, micro optical components such as micro lenses and apertures could be integrated into the flow cell to prevent the optical bending and reduce background noise. This would also help increase the amount of collected light and better define the shape of the excitation area, leading to a higher S/N. These optical components would be designed to allow optical excitation to be more spatially uniform within a smaller area in the channel, which would reduce the S/N variation. Moreover, the blind spot that appears for the off-angle detection can be eliminated with a new optics design, which will naturally lead to an improved recovery rate at the off-angles. In addition, a new optics system could incorporate a larger number of detection optical fibers embedded into the

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observation cell. These optical fibers can simultaneously collect the fluorescence emission from a single particle within the interrogation zone. The signals collected at each optical fiber can be combined to amplify the signal and to cancel the random components of the background noise. It appears that practical and affordable
5 particle detection below 6 microns will be readily achievable with this design approach.

While embodiments of the invention have been illustrated and described, it is not intended that these embodiments illustrate and describe all possible forms of the invention. Rather, the words used in the specification are
10 words of description rather than limitation, and it is understood that various changes may be made without departing from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A spectral micro flow cytometer device, comprising:
 - a) a micro flow cytometer having an inlet, an outlet, and an interrogation channel in fluid communication with said inlet and said outlet, said interrogation channel defining an interrogation plane in which said interrogation channel is positioned;
 - b) a plurality of minimally three optical waveguides terminating proximate said interrogation channel and positioned in said interrogation plane, each optical waveguide of said plurality of waveguides directed toward said interrogation channel at a different angle with respect to the direction of said interrogation channel, with the inlet direction of said interrogation channel being 0°;
 - c) minimally one incident light source coupled to one of said optical waveguides and illuminating said interrogation channel;
 - d) a comparator which compares a light intensity or a signal to noise ratio at one optical waveguide at a first included angle, with a light intensity or a signal to noise ratio at a further optical waveguide at a further included angle, the difference in intensity or in signal to noise ratio being proportional to the wavelength of light emanating from a particle passing through said interrogation channel.
2. The device of claim 1, wherein a first included angle of a first optical waveguide with the interrogation channel and a further included angle of a further optical waveguide with the interrogation channel are such that the change in intensity of a first wavelength of light measured at said first optical waveguide and said further optical waveguide is different from the change in relative intensity of a second wavelength of light measured at said first optical waveguide and said further optical waveguide.

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3. The device of claim 1, wherein said optical waveguides comprise optical fibers.
4. The device of claim 1, wherein two or more incident light sources of differing spectral characteristics are employed.
- 5 5. The device of claim 4, wherein at least two incident light sources are supplied to two different optical waveguides.
6. The device of claim 1, wherein at least one incident light source is monochromatic.
- 10 7. The device of claim 1, wherein at least two monochromatic incident light sources are employed.
8. The device of claim 7, wherein said two incident monochromatic light sources are pulsed light sources out-of-phase with respect to each other.
- 15 9. The device of claim 1, comprising first and second output optical waveguides positioned at different included angles with respect to the axis of the interrogation zone, and means for calculating the signal to noise ratio of the electrical signal from photodetectors receiving light from the respective output optical waveguides.
- 20 10. The micro flow cytometer of claim 1, comprising:
a modulated, first monochromatic light source having a first peak wavelength directing light into a first incident optical waveguide such that modulated monochromatic light impinges upon particles within said interrogation channel;
optionally, a modulated, second monochromatic light source having a second peak wavelength different from said first peak wavelength, whose
25 modulation is substantially out-of-phase with the modulation of said first monochromatic light source, said second monochromatic light source directing light

to said interrogation channel through a second incident light optical waveguide, said first and second incident waveguides being the same or different;

5 a first photodiode detector sensitive to light from said first monochromatic light source, said second monochromatic light source, or both said first and said second monochromatic light sources or to a wavelength corresponding to a fluorescence emitted by a particle in said interrogation channel as a result of said particle being illuminated by one or more of said first and second light sources, said at least one photodiode detector communicating with said interrogation channel through a first output optical waveguide in a plane with said first incident waveguide and said interrogation channel, and having a first included angle with said
10 interrogation channel;

at least a second photodiode detector communicating with said interrogation channel through a second output optical waveguide in a plane with said interrogation channel and said first incident waveguide, but at a different included angle to said interrogation channel than said first output optical waveguide;
15

at least one amplifier which amplifies an output from said photodiode detector(s);

means for selectively sampling an amplified output from said at least one amplifier synchronously with a respective modulation frequency of said first and said second monochromatic light sources to provide a first output proportional to light of an incident wavelength or a fluorescent wavelength impinging on said detector, and a second output proportional to light of a different incident wavelength or a different fluorescent wavelength;
20

means for comparing the signal strength obtained from said first photodiode detector with said second photodiode detector at at least two different wavelengths to obtain an angularly dependent comparison signal for each wavelength, and/or means for calculating a signal to noise ratio from said first photodiode detector and said second photodiode detector;
25

means for comparing the angularly dependent comparison signals or the signal to noise ratios to each other to provide spectral differentiation of light collected by said first output waveguide and said second output waveguide.
30

11. A method for providing spectral information for particles in an interrogation channel of a micro flow cytometer of claim 1, comprising:

illuminating said particles with incident light from at least one incident optical waveguide;

5 transmitting reflected, scattered, or fluorescent light from a particle in said interrogation channel through at least first and second output optical waveguides, all of said optical waveguides in a plane including said interrogation channel, said first and second output optical waveguides at different included angles with respect to the axis of said interrogation channel;

10 measuring light intensity or signal to noise ratio from each output optical waveguide at each of at least two different wavelengths and providing comparison output signals proportional to the difference between the intensity of each respective wavelength from the first and second output optical waveguides, thus providing a first comparison output signal for a first wavelength and a second
15 comparison output signal for a second wavelength;

comparing said first and said second comparison output signals or signal to noise ratios, a difference being spectrally dependent.

12. The process of claim 11, wherein an output signal is amplified by a photomultiplier tube.

20 13. The process of claim 11, wherein incident light is modulated, and output signals are amplified in phase with modulated incident light.

14. The process of claim 11, wherein said incident light comprises two or more modulated, monochromatic light sources of different peak wavelengths, each of said monochromatic light sources modulated out-of-phase with all other
25 monochromatic light sources.

15. A method for spectrally distinguishing output signals from a device of claim 1, comprising

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illuminating particles in said interrogation channel with an incident light wavelength which causes said particles to fluoresce with a fluorescent wavelength different from the incident light wavelength;

5 measuring the signal to noise ratio of a photodetector signal from a first output optical waveguide and a photodetector signal from a second output optical waveguide, said first and second output optical waveguides positioned at different angles with respect to said interrogation channel;

10 identifying the fluorescent wavelength emitted by a particle by the ratio of signal to noise ratio at one photodetector to the signal to noise ratio at the other photodetector.

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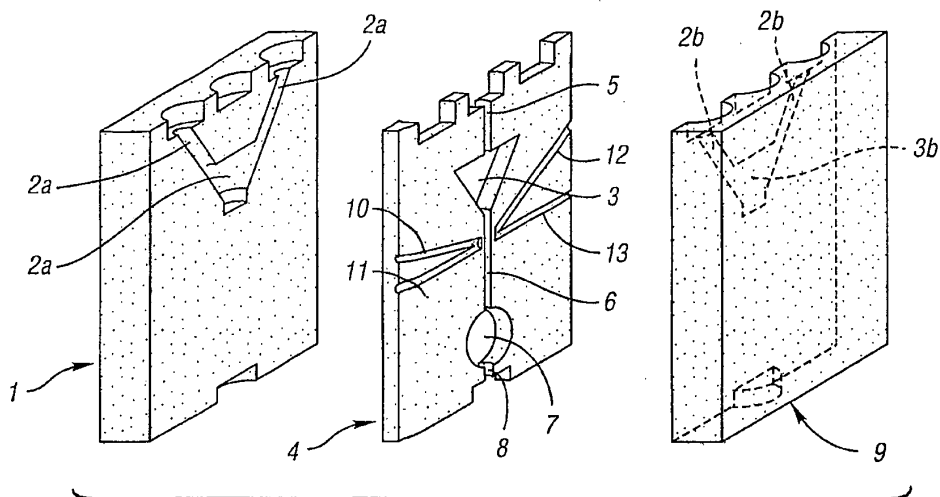


Fig. 1

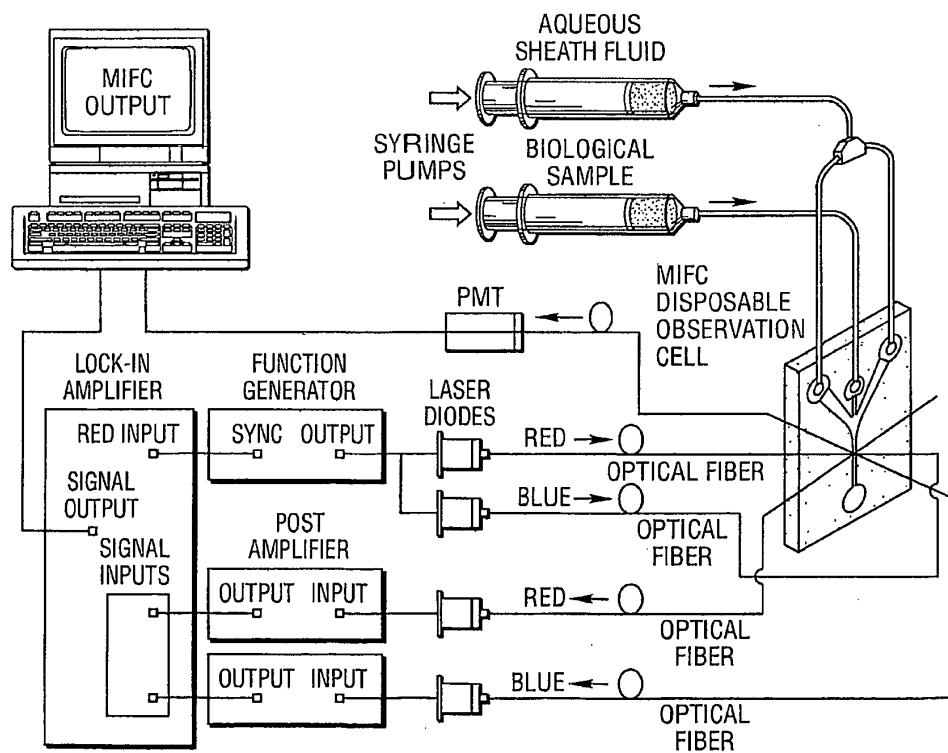


Fig. 2

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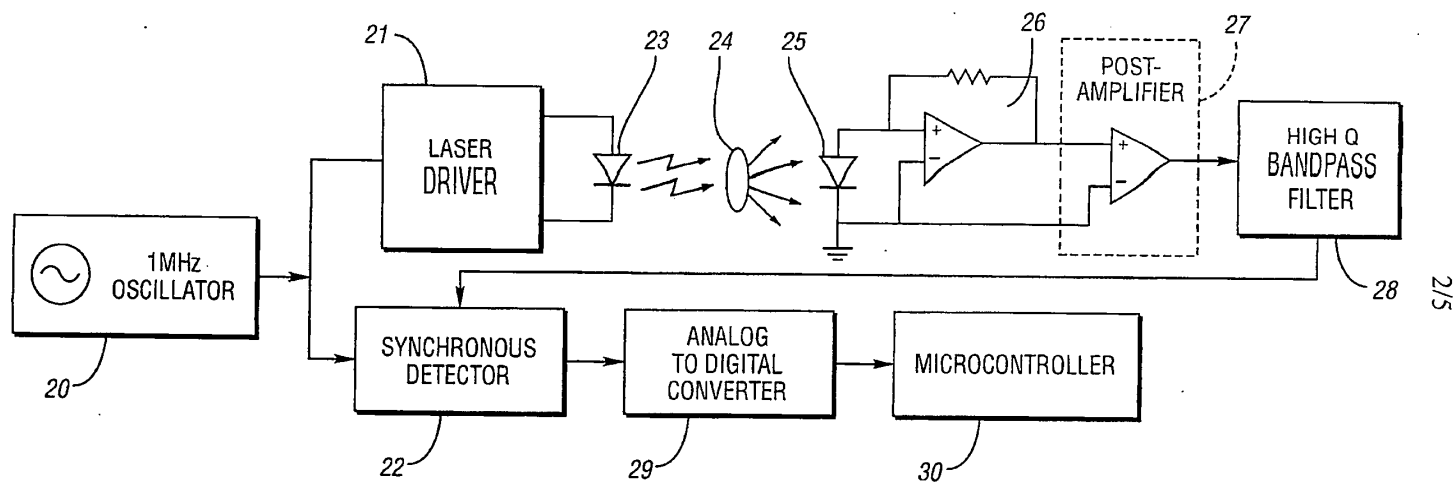
*Fig. 3*

Fig. 4a

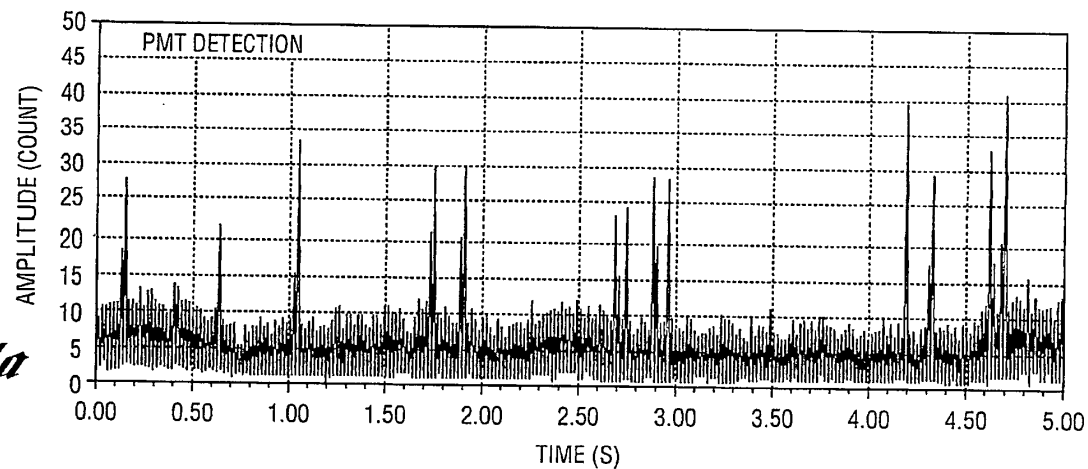
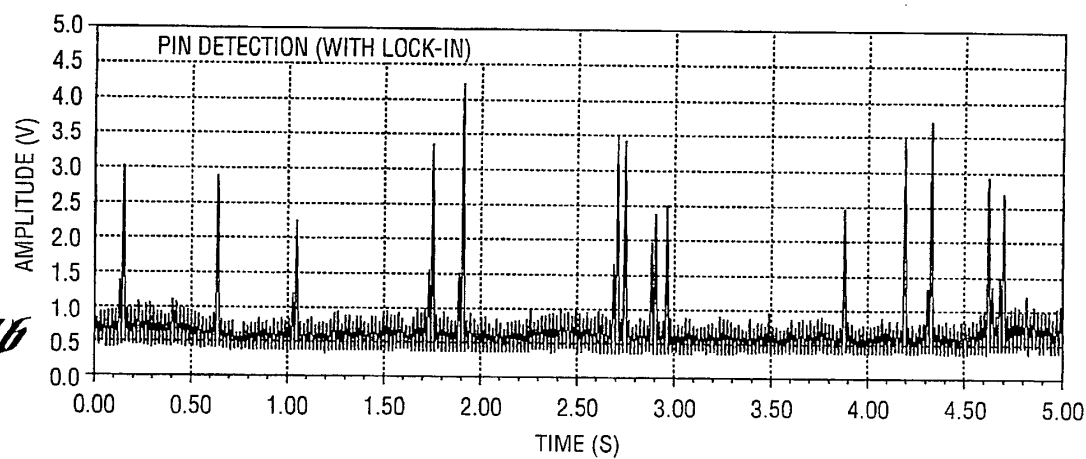


Fig. 4b



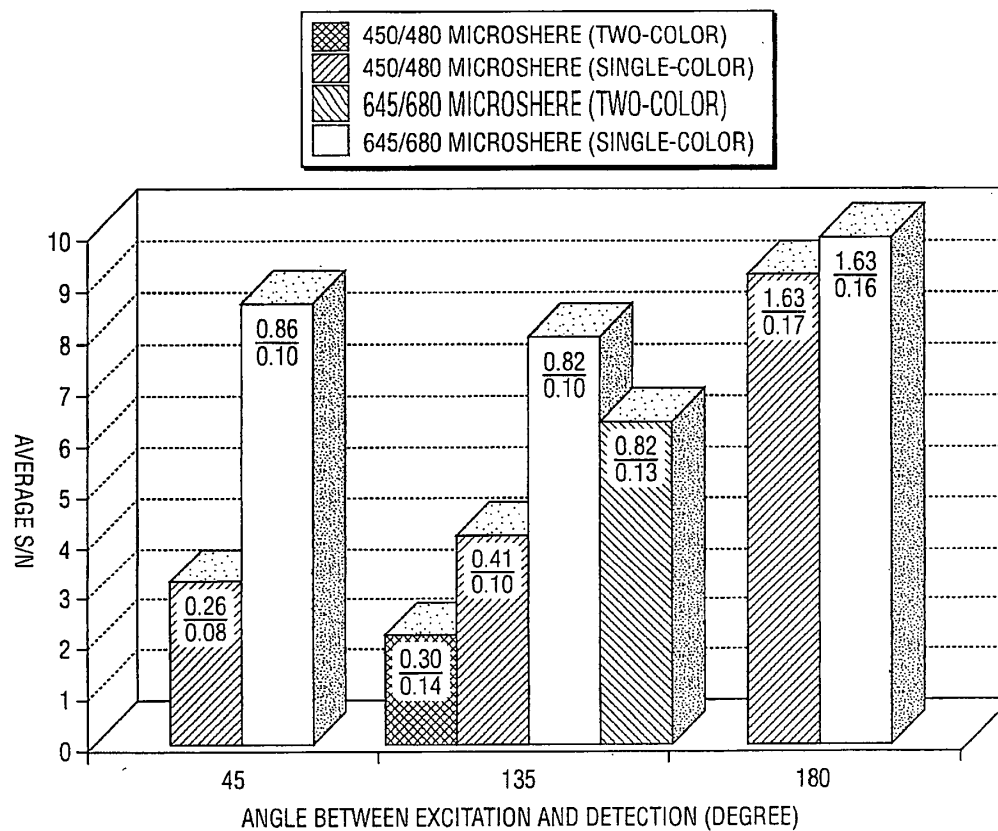


Fig. 5

WO 2005/033654

4/5

PCT/US2004/028175

PCT/US2013/043453 10.10.2013

WO 2005/033654

PCT/US2004/028175

5/5

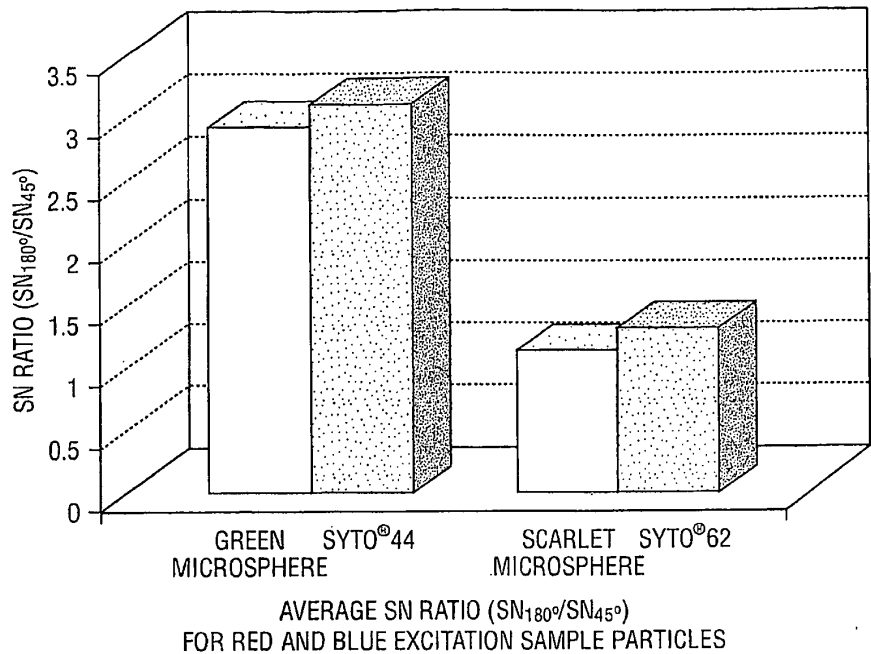


Fig. 6

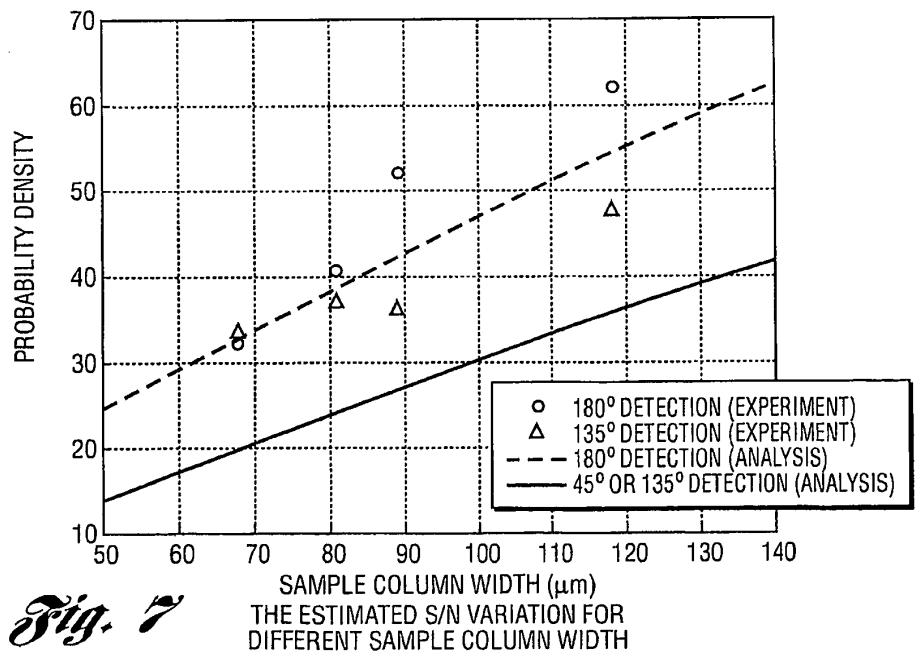


Fig. 7

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: DONALD E. SCHREIBER
POST OFFICE BOX 2926
KINGS BEACH, CA 96143-2926

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Date of mailing (day/month/year)	20 DEC 2013
Applicant's or agent's file reference 2294PCT	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US2013/043453	International filing date (day/month/year) 30 May 2013
Applicant CYTOJENE CORP.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. Following the expiration of 30 months from the priority date, these comments will also be made available to the public.

Shortly after the expiration of **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90bis.1 and 90bis.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774
---	--

Form PCT/ISA/220 (July 2010)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 2294PCT	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US2013/043453	International filing date (day/month/year) 30 May 2013	(Earliest) Priority Date (day/month/year) 30 May 2012
Applicant CYTOJENE CORP.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed.
☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☐ Certain claims were found unsearchable (see Box No. II).

3. ☒ Unity of invention is lacking (see Box No. III).

4. With regard to the title,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- ☐ the text is approved as submitted by the applicant.
☒ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. 1
☒ as suggested by the applicant.
☐ as selected by this Authority, because the applicant failed to suggest a figure.
☐ as selected by this Authority, because this figure better characterizes the invention.
- b. ☐ none of the figures is to be published with the abstract.

Form PCT/ISA/210 (first sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/043453

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-73

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/043453

Box No. IV Text of the abstract (Continuation of item 5 of the first sheet)

A flow cytometer including a laser diode based optical subsystem for impinging a beam of light upon particles passing through a viewing zone; a composite microscope objective for gathering and imaging light scattered from or fluoresced by particles passing through the viewing zone; a fluidic subsystem for supplying a liquid sheath flow to the viewing zone; a peristaltic pump for injecting into the liquid sheath flow a liquid sample flow carrying particles that passes together with the liquid sheath flow through the viewing zone; a multimode optical fiber that receives scattered and fluoresced light from the viewing zone that the composite microscope objective gathers and images; and a wavelength division multiplexer for optically separating into color bands light received via the optical fiber.

Form PCT/ISA/210 (continuation of first sheet (3)) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/043453

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - G01N 15/14 (2013.01) USPC - 356/337 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) IPC(8) - G01N 15/08, 10, 14, 21/01, 03, 05, 47, 53, 62, 64 (2013.01) USPC - 356/311, 317, 318, 337; 435/288.7; 436/164, 171, 172 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched CPC - G01N 15/08, 10, 14, 1434, 21/01, 03, 05, 47, 53, 62, 64 (2013.01) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatBase, Google Patents, Google Scholar		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0091746 A1 (FUKUDA et al) 09 April 2009 (09.04.2009) entire document	56-73
Y	WO 2001/40764 A2 (DUBELAAR) 07 June 2001 (07.06.2001) entire document	56-73
Y	US 4,515,274 A (HOLLINGER et al) 07 May 1985 (07.05.1985) entire document	59, 63, 68, 72
Y	US 2006/0221325 A1 (WELLS) 05 October 2006 (05.10.2006) entire document	64, 73
A	US 2002/0141902 A1 (OZASA et al) 03 October 2002 (03.10.2002) entire document	1-73
A	US 2008/0024758 A1 (TABATA) 31 January 2008 (31.01.2008) entire document	1-73
A	WO 2005/033654 A2 (TUNG et al) 14 April 2005 (14.04.2005) page 18, lines 21-29; page 19, line 1, 15-16	1-73
☐ Further documents are listed in the continuation of Box C. ☐		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 04 December 2013		Date of mailing of the international search report 20 DEC 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201		Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/043453

Continuation of Box III.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Species I) a laser diode based optical subsystem, figures 2, 2A, 3A, 3B, 4A, 4B

Species II) a composite microscope objective, figures 5A, 5B, 5C, 8, 9A

Species III) a fluidic subsystem, figures 14-15

Species IV) a peristaltic pump, figures 17-24

Species V) a wavelength division multiplexer, figures 25-30B

The claims are deemed to correspond to the species listed above in the following manner:

Species I) figures 2, 2A, 3A, 3B, 4A, 4B; claims 56-73

Species II) figures 5A, 5B, 5C, 8, 9A; claims 74-129

Species III) figures 14-15; claims 130-145

Species IV) figures 17-24; claims 146-161

Species V) figures 25-30B; claims 162-173

The following claims are generic: claims 1-55.

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

Species I) figures 2, 2A, 3A, 3B, 4A, 4B; have the special technical feature of a laser diode based optical subsystem. This special technical feature is not present in species II, III, IV or V.

Species II) figures 5A, 5B, 5C, 8, 9A; have the special technical feature of a composite microscope objective. This special technical feature is not present in species I, III, IV or V.

Species III) figures 14-15; have the special technical feature of a fluidic subsystem. This special technical feature is not present in species I, II, IV or V.

Species IV) figures 17-24; have the special technical feature of a peristaltic pump. This special technical feature is not present in species I, II, III or V.

Species V) figures 25-30B; have the special technical feature of a wavelength division multiplexer. This special technical feature is not present in species I, II, III or IV.

Species I, II, III, IV and V lack unity of invention because even though the inventions of these species require the technical feature of an optical system having liquid flow, directing light, and having a microscope, this technical feature is not a special technical feature as it does not make a contribution over the prior art in view of WO 2005/033654 A2 (TUNG et al) 14 April 2005 (14.04.2005) page 18, lines 21-29; page 19, line 1, 15-16.

Since none of the special technical features of the Species I, II, III, IV and V inventions is found in more than one of the inventions, unity of invention is lacking.

Search History:

Limited Classification Search

The Patent Analyst performed a limited classification search within the following US, IPC, ECLA, or F-Term classification areas:

U.S. Class/Subclass(es):

356/311, 317, 318, 337
435/288.7
436/164, 171, 172

IPC (8) Class/Subclass(es):

G01N 15/08, 10, 14, 21/01, 03, 05, 47, 53, 62, 64 (2013.01)

CPC Class Subclass(es):

G01N 15/08, 10, 14, 1434, 21/01, 03, 05, 47, 53, 62, 64 (2013.01)

See Global Search Results.

Global Patent Literature Text Search

The Patent Analyst performed the following global text search, which was not limited by classification but may or may not have been limited by other criteria:

PATBASE

1) pd<20120530 (100000)
2) PA=(cytojene) (0)
3) INV=(chen yong qin) (12)
4) PN=(WO05033654 OR US5504337 OR US2010035235 OR US8159670 OR US5360739 OR US8400632 OR US8432541) (6)
5) uc=356/311 (271)
6) uc=356/317 (784)
7) uc=356/318 (1087)
8) uc=356/337 (494)
9) uc=435/288.7 (2035)
10) uc=436/164 (3185)
11) uc=436/171 (564)
12) uc=436/172 (2777)
13) ic=g01n15/08 (6095)
14) ic=g01n15/10 (2235)

15) ic=g01n15/14 (5584)
16) ic=g01n21/01 (8932)
17) ic=g01n21/03 (6199)
18) ic=g01n21/05 (2858)
19) ic=g01n21/47 (11615)
20) ic=g01n21/53 (4373)
21) ic=g01n21/62 (2941)
22) ic=g01n21/64 (22270)
23) cpc=g01n15/08 (462)
24) cpc=g01n15/10 (153)
25) cpc=g01n15/14 (404)
26) cpc=g01n15/1434 (338)
27) cpc=g01n21/01 (356)
28) cpc=g01n21/03 (949)
29) cpc=g01n21/05 (1410)
30) cpc=g01n21/47 (742)
31) cpc=g01n21/53 (959)
32) cpc=g01n21/62 (109)
33) cpc=g01n21/64 (1468)
34) 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 (8979)
35) 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 (61142)
36) 23 or 24 or 25 or 26 or 27 or 28 or 29 or 30 or 31 or 32 or 33 (6935)
37) flow cytomet* (44427)
38) laser diode# (84760)
39) (ellip* w3 (shape# or beam# or spot#)) (100000)
40) (major w3 axis) and (minor w3 axis) (26738)
41) sheath# (100000)
42) 37 and 38 (716)
43) 39 and 40 (7353)
44) 41 and 42 (173)
45) 43 and 44 (5)
46) PN=(US5788927 OR US6713019 OR US7385682 OR US7561267) (4)
47) 43 and 42 (6)

48) 38 and (39 or 40) (3644)
49) 48 and 34 (60)
50) 48 and 35 (243)
51) 48 and 36 (85)
52) (49 or 50 or 51) and 1 (250)
53) 52 and 41 (26)
54) pn=us20090091746 (1)
55) 41 and (39 or 40) and 1 (5275)
56) 55 and 37 (164)
57) 55 and (34 or 35 or 36) (164)
58) 56 and 57 (113)
59) jet (100000)
60) cuvette (57326)
61) 41 and 59 and 60 (208)
62) 61 and 37 and 1 (67)
63) (composite#) w3 (microscop* or objective#) (22710)
64) concave# (100000)
65) aberration# w3 (correct# or fix# or mitigate#) (23551)
66) t-coupling# (1551)
67) peristaltic# (27652)
68) rollers (100000)
69) rotor# (100000)
70) (wavelength division multiplexer) or (wdm) (21363)
71) 37 and 63 (60)
72) 71 and 38 (1)
73) 63 and 64 and 65 (70)
74) 73 and 66 (0)
75) 73 and 67 (0)
76) 37 and 66 (1)
77) 37 and 67 (967)
78) 70 and 77 (0)
79) 77 and 38 (91)
80) 79 and (39 or 40) (5)

81) 68 and 69 and 37 (21)
82) 81 and 1 (21)
83) 37 and 70 (23)
84) 83 and (39 or 40) (2)
85) 83 and 64 (0)

GOOGLE PATENTS http://www.google.com/advanced_patent_search

No. of Hits	Text String
1240	flow cytometer optical subsystem elliptical beam
0	flow cytometer optical subsystem elliptical beam "beam compressing"
47	flow cytometer optical subsystem elliptical beam cylindrical lens
19	flow cytometer optical subsystem elliptical beam minor major axis
26	optical subsystem laser diode flow cell sheath flow elliptical beam
7300	optical subsystem laser diode flow cell major minor axis
44	optical subsystem laser diode flow cell major minor axis collimate focus
54	optical subsystem laser diode flow cell major minor axis composite microscope
4270	optical subsystem laser diode flow cell major minor axis cylindrical lens
9	optical subsystem laser diode flow cell major minor axis cylindrical lens microscope
34	optical subsystem laser diode flow cell major minor axis aberration correction
3	optical subsystem laser diode flow cell major minor axis sheath
54	optical subsystem laser diode flow cell major minor axis t-coupling
18	optical subsystem laser diode flow cell peristaltic pump
1010	optical subsystem laser diode flow cell wavelength division multiplexer
23	optical subsystem laser diode flow cell "wavelength division multiplexer"
6200	flow cytometer laser peristaltic pump
12	flow cytometer laser peristaltic pump wavelength division multiplexer
13	flow cytometer peristaltic pump wavelength division multiplexer
35	flow cytometer elliptical beam wavelength division multiplexer

Computer Accessed Text Databases Searched

The Patent Analyst searched the following computer accessed text databases:

Database: Google Scholar <http://scholar.google.com/>

No. of Hits	Text String
4890	"flow cytometer" laser diode
298	"flow cytometer" laser diode elliptical beam
65	"flow cytometer" laser diode elliptical beam major minor axis
8600	"flow cytometer" sheath
1	"flow cytometer" sheath t-coupling
1810	"flow cytometer" peristaltic pump
34	"flow cytometer" peristaltic pump rollers rotor tube

1380	"flow cytometer" wavelength division multiplexer
20	"flow cytometer" wavelength division multiplexer collimating focusing image relay optical
11	"flow cytometer" wavelength division multiplexer collimating focusing image relay optical dichroic filter

Date search was completed: 04 December 2013

CC/DJ

PATENT COOPERATION TREATY

ADVANCE E-MAIL

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

To:

WONG, Charles, C.
Beckman Coulter, Inc.
250 S. Kraemer Blvd, E1.SE.02
Brea, CA 92821
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 09 October 2014 (09.10.2014)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 12US00.40-WO	
International application No. PCT/US2013/043453	International filing date (day/month/year) 30 May 2013 (30.05.2013)

1. The following indications appeared on record concerning:									
☐ the applicant	☐ the inventor ☒ the agent ☐ the common representative								
Name and Address SCHREIBER, Donald, E. Post Office Box 2926 Kings Beach, CA 96143-2926 United States of America	<table border="1"> <tr> <td>State of Nationality</td> <td>State of Residence</td> </tr> <tr> <td colspan="2">Telephone No. (530) 546-6041</td> </tr> <tr> <td colspan="2">Facsimile No. (530) 546-2341</td> </tr> <tr> <td colspan="2">E-mail address donaldschreiber0@gmail.com</td> </tr> </table>	State of Nationality	State of Residence	Telephone No. (530) 546-6041		Facsimile No. (530) 546-2341		E-mail address donaldschreiber0@gmail.com	
State of Nationality	State of Residence								
Telephone No. (530) 546-6041									
Facsimile No. (530) 546-2341									
E-mail address donaldschreiber0@gmail.com									
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:									
☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence									
Name and Address WONG, Charles, C. Beckman Coulter, Inc. 250 S. Kraemer Blvd, E1.SE.02 Brea, CA 92821 United States of America	<table border="1"> <tr> <td>State of Nationality</td> <td>State of Residence</td> </tr> <tr> <td colspan="2">Telephone No.</td> </tr> <tr> <td colspan="2">Facsimile No.</td> </tr> <tr> <td colspan="2">E-mail address ☐ Notifications by e-mail authorized</td> </tr> </table>	State of Nationality	State of Residence	Telephone No.		Facsimile No.		E-mail address ☐ Notifications by e-mail authorized	
State of Nationality	State of Residence								
Telephone No.									
Facsimile No.									
E-mail address ☐ Notifications by e-mail authorized									
3. Further observations, if necessary: The new agent's file reference is: 12US00 40-WO.									
4. A copy of this notification has been sent to:									
☒ the receiving Office ☐ the International Searching Authority ☐ the Authority(ies) specified for supplementary search	☐ the International Preliminary Examining Authority ☐ the designated Offices concerned ☒ the elected Offices concerned ☒ other: SCHREIBER, Donald, E.								

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. +41 22 338 90 90	Authorized officer Sason Elisabeth e-mail pt07.pct@wipo.int Telephone No. +41 22 338 74 07
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Copy for (RO-US) 37
PATENT COOPERATION TREATY

PCT/US2013/043453

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING
OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

To:

WONG, Charles, C.
Beckman Coulter, Inc.
250 S. Kraemer Blvd, E1.SE.02
Brea, CA 92821
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 09 October 2014 (09.10.2014)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 12US00.40-WO	
International application No. PCT/US2013/043453	International filing date (day/month/year) 30 May 2013 (30.05.2013)

1. The following indications appeared on record concerning:

☒ the applicant ☐ the inventor ☐ the agent ☐ the common representative

Name and Address CYTOJENE CORP. 4087 Cartona Court San Jose, California 95135 United States of America	State of Nationality US	State of Residence US
	Telephone No.	
	Facsimile No.	
	E-mail address	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address IRIS INTERNATIONAL, INC. 9172 Eton Avenue Chatsworth, California 91311 United States of America	State of Nationality US	State of Residence US
	Telephone No.	
	Facsimile No.	
	E-mail address ☐ Notifications by e-mail authorized	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

☒ the receiving Office ☐ the International Preliminary Examining Authority
☐ the International Searching Authority ☐ the designated Offices concerned
☐ the Authority(ies) specified for supplementary search ☒ the elected Offices concerned
☐ other:

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