



(51) International Patent Classification:

G01N 15/14 (2006.01) G01N 15/10 (2006.01)

(21) International Application Number:

PCT/US2017/025836

(22) International Filing Date:

04 April 2017 (04.04.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

15/399,172 05 January 2017 (05.01.2017) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR,

KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

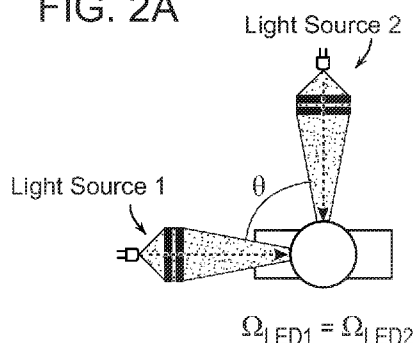
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

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

(54) Title: METHOD TO DISTINGUISH AND ANALYZE WHITE BLOOD CELLS IN THE PRESENCE OF RED BLOOD CELLS

FIG. 2A



(57) Abstract: Method for distinguishing between red blood cells and white blood cells. The method includes obliquely illuminating the blood sample with light from at least two rotational angles and analyzing light side scattered from cells in the sample to provide accurate discrimination of white blood cell types based on the anisotropy of red blood cell side scatter as compared to more isotropic white blood cell side scatter.



METHOD TO DISTINGUISH AND ANALYZE WHITE BLOOD CELLS IN THE PRESENCE OF RED BLOOD CELLS

This application claims priority to United States Application serial number
5 15/399,172 filed January 5, 2017, the contents of which are incorporated herein by reference
in its entirety.

This invention was made with Government support under Grant No. U54 EB015403
awarded by the National Institutes of Health. The Government has certain rights in the
10 invention.

Background of the Invention

This invention relates to a method for analyzing, at low magnification, white blood
15 cells in the presence of red blood cells that does not require lysing of the red blood cells.

Of paramount importance in a clinical setting is the analysis of blood and bone
marrow samples. Specifically in clinics and hospitals, analysis of patient blood and marrow
samples is used to diagnose or rule out a vast number of conditions. In 2014, there were 41.5
20 million complete blood counts and automated differential white blood cell counts combined
performed in the USA. Of central importance to this test is the analysis and discrimination of
white blood cell types by flow cytometers and hematology analyzers. Analyzing white blood
cell types and counts gives insight to the clinician into immune system status, general health,
or clues for diagnosis. However, hematology analyzers and flow cytometers fail to
25 accurately analyze white blood cells in the presence of significant red blood cell numbers.
Red blood cells can be mistaken for white blood cells, infiltrate the white blood cell
measurement, or any number of other errors that can make the analysis of a sample
inaccurate. This is the reason that red blood cells are often lysed (or destroyed) when white
blood cells are to be analyzed. Lysing red blood cells allows for more reliable analysis of
30 white blood cells in the patient's blood. A primary concern in sample analysis is the
incomplete lysis of red blood cells. Red blood cells can be resistant to lysis in a large
population of patients including those with liver disease, sickle cell disease, those undergoing
chemotherapy, and in newborns to name a few. Because the red blood cells become difficult
to lyse, the samples must be re-run or analyzed manually via a peripheral blood smear.

An object of the invention is a method that can be added to an image based cytometer or hematology analyzer to reduce the impact of red blood cells on the analysis of white blood cells and allow for more accurate assessment of the white blood cell population. This
5 invention can also be used to increase measurement accuracy in cases where a lyse-free measurement is used in clinical devices, which currently is not common.

Summary of the Invention

10 The method according to the invention for distinguishing between red blood cells and white blood cells in a flowing or stationary blood sample including red and white blood cells includes obliquely illuminating the blood sample with light from at least two rotational angles. Light side scattered from cells in the sample is analyzed to provide accurate discrimination of white blood cell types for white blood cell analysis and counting based on a
15 difference in light scattering profile of red blood cell side scatter as compared to white blood cell side scatter. In a preferred embodiment the difference in scattering profile is the relative difference in isotropy/anisotropy for the red blood cells and white blood cells. In a preferred embodiment, the oblique illumination angle is approximately 80 degrees. A suitable range is 78-86 degrees. In another preferred embodiment the rotational angles are 90 degrees. A
20 suitable rotational angle range is 75-105 degrees. In this embodiment, the light has a wavelength anywhere in the ultraviolet-visible-near infrared electromagnetic spectrum. A suitable light wavelength is approximately 400 nm.

We thus disclose a set of methods to accurately analyze white blood cells in the
25 presence of red blood cells, specifically for applications in hematology analysis. The method distinguishes blood types by illuminating a flowing or stationary blood sample from multiple angles and analyzing the light interaction with each cell. Embodiments of this invention can be used for imaging based analysis (low resolution or high resolution alike) of flowing or stationary cells on a surface. This method works for any sample containing red and/or white
30 blood cells (e.g. bone marrow, peripheral blood) and can be used in samples with hard-to-lyse red blood cells or in samples without any lysis of red blood cells. This could also be applied to any cell types discussed within this disclosure. Since this method relies on scattering-

based contrast, it does not require dyes, although incorporating existing dye, fluorescence, scattering, absorption, etc. methods with the one presented here could aid in its accuracy.

Brief Description of the Drawing

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Fig. 1A is a top view of an embodiment of the side scatter imaging technique disclosed herein.

10 Fig. 1B is a front view of an embodiment of the side scatter imaging technique disclosed herein.

Fig. 1C constitutes representative data from experiments using the disclosed methodology.

15 Fig. 2A is a schematic illustration showing the use of two light sources in an embodiment of the invention.

Fig. 2B constitutes photomicrographs of a red blood cell and a white blood cell observed using the technology of the invention.

20

Fig. 3A is a graph of side scatter intensity versus CD45 fluorescence intensity for a red blood cell.

Fig. 3B is a graph of side scatter intensity for angle 1 and angle 2 versus CD45 fluorescence
25 intensity.

Description of the Preferred Embodiment

Of particular interest in this invention is the wide angle scatter of light by cells. When
30 light interacts with a cell, one of the possibilities is for the light to be deflected to any number of angles. When the light is deflected a wide or large angle (>5 degrees, most often centered at 90 degrees) away from the original axis, this can often be used to measure the internal complexity of a cell. In flow cytometry and hematology analyzers, this angle is often

centered around 90 degrees and referred to as side scatter. Side scatter measurements from white blood cells can aid in discriminating white blood cell types, an essential task in almost any analysis of blood in the clinical or research setting. Side scatter can be used in conjunction with forward scatter, fluorescence, light absorption, or any number of analysis methods to distinguish types of white blood cells or discriminate white blood cells from other blood constituents. When many red blood cells are present, the side scatter signal from red blood cells in the flow path or image plane can create false positive white blood cell-like signals or make measurements of nearby white blood cells inaccurate. The disclosed technology consists of a modular optical add-on to image based analyzers (or slit based cytometers) and analysis methods that can attenuate the effects of red blood cells and aid in accurate quantification of white blood cell signals and thus better discrimination of white blood cell types.

The technology disclosed herein uses the differences in the source of side scatter signal for white blood cells and red blood cells, and the directional anisotropy of the scatter found in red blood cells to distinguish background red blood cells from the white blood cells of interest. In previous literature, these observations have never been made or leveraged to differentiate the two cell types.

The invention disclosed herein describes a set of optical instrumentation and analysis methods that allow for white blood cells to be more accurately analyzed in the presence of un-lysed or hard-to-lyse red blood cells. The technology theories suggested here can be applied to imaging-based solutions to hematology analysis that use high or low resolution microscopy to image flowing samples or stationary cell samples on a surface. It can also be applied to slit-based cytometers that do not supply 2-dimensional images but still supply sub-cellular feature resolution.

The basis of the technology disclosed herein leverages wide angle “side” scatter commonly employed by flow cytometry, and also highly oblique illumination microscopy for image based methods. In hematology analyzers and flow cytometers, wide angle scatter is one method used to help differentiate cell types by their internal complexity, and can be one of a subset of angles collected by a hematology analyzer. Other methods to discriminate cell type include impedance, low and forward angle scatter, absorption by a dye, fluorescence,

high resolution imaging, etc. However, nearby red blood cells can pollute the measurement of a white blood cell making its measurement inaccurate, or be misconstrued as white blood cells by the majority of these measurement types. Our technology limits this effect and better discriminates and analyzes white blood cells by leveraging the anisotropy of red blood cell side scatter and more isotropic white blood cell side scatter. Also, because the technology in this patent application utilizes scatter-based measurements, it can be employed on label-free samples, although additional labels can certainly aid in better discrimination of cell groups.

The optical embodiment of this technology begins with a setup similar to highly oblique illuminators for scatter-based discrimination of cells in a planar microscopy setting (Figs. 1a, 1b and 1c). Any form of imaging detection scheme can be used (high/low resolution, high/low numerical aperture, upright/inverted detection configuration, etc.) that can image cells that are flowing in single file, as a group, or stationary cells on a surface. A highly oblique illuminator that allows for side scatter data to be collected from all of the cells in the field of view is mounted adjacent to a microscope detection path at an angle Ω as shown in Figs. 1a and 1b. The illuminator and detector can have any number of numerical apertures, but a low numerical aperture illuminator is more practical for homogenous illumination. The angle Ω between the illuminator and detecting microscope can be any number of angles, but we have found highly oblique angles approaching 80 degrees allow for homogenous side scatter signal over a wide field of view with minimal concern of imaging lens working distance. In Fig. 1a a highly oblique illuminator is placed next to a planar sample that is positioned underneath a microscope imaging objective as shown. At low magnification, many cells can be imaged, and the size of the cell and signal intensity of the cell can be used to differentiate white blood cell types. At low magnification, many cells can be imaged, and the size of the cell and signal intensity of the cell can be used to differentiate white blood cell types. At high magnification, the spatial features of the cells can be used to differentiate white blood cells. The wavelength of the illuminator can fall anywhere in the ultraviolet-visible-near infrared electromagnetic spectrum. Wavelengths where peak red blood cell absorption occurs due to hemoglobin (~400 nm for example) are preferred as they allow for better separation of red blood cell and white blood cell signals.

In white blood cells, the primary source of side scatter is internal granularity and structure of a specific spatial periodicity. This source of contrast exists to a fairly constant

amount and from a similar spatial position within the cell regardless of the incoming illumination's rotational angle with respect to the sample and detection axis. Red blood cells, however, have a side scatter signal that originates primarily from refractive index mismatches with the surrounding fluid. This means that the source of the red blood cell side scatter signal becomes focused at the entry and exit sides of illumination. Therefore, illuminating at two distinct rotational angles causes changes in the resulting image (due to spatial changes in where the detected signal originates from the cell). In imaging based cytometry (i.e. highly oblique illumination microscopy as in Figs. 1a and 1b), this is easily visible as focused spots at two opposing directions on the circular edge of the red blood cell. In Fig. 2a we have added a second oblique illuminator that has the same angle of incidence on the sample (i.e. $\Omega_1 = \Omega_2$), but the second illuminator has been rotated $\Theta = 90$ degrees around the detection axis of the system. The two rotationally offset illuminators were sequentially used to image a white blood cell and a red blood cell under 40X magnification (Fig. 2b), and the detected imaging information was overlaid and color coded by blue and purple pixels depending on the direction of illumination. It is clear that the two color channels (i.e. the two illumination directions) significantly overlap spatially in the white blood cell due to the internal granularity structure being the source of contrast whereas for red blood cells the refractive index mismatch at the edges causes concentrated signals only along the axis of illumination.

The observed differences in response (signal/image) under two rotationally offset angles of illumination is the method to differentiate cell types. Although the 40X image shows features that should distinguish between white blood cell and red blood cell types, most analyzers operate at lower magnification to increase throughput, and when red blood cells and white blood cells are physically touching, separating the signals is a different task. Using multi-angle illumination can aid in attenuating the effects of red blood cells that overlap with neighboring white blood cells. Note that this idea does not hold only for two illuminators rotationally offset by 90 degrees. This preferred embodiment allows for the greatest difference of mutual information between images for red blood cells in our experience, but any number of illuminators greater than or equal to 2 and any angular offset could potentially lead to better detection of white blood cells and attenuation of red blood cell signal. It is important to note that although this disclosure has been described in the context of wide angle scatter (Ω approaching 90 degrees from the detection axis), the idea works for any side angle of illumination, so long as the illumination is rotated perpendicular to the

detection axis. We have chosen very wide angle scatter since there is a long history of side scatter being used to distinguish white blood cell types. Therefore, using very wide angle scatter allows for discriminating white blood cells from red blood cells as well as differentiating white blood cell types. That does not mean, however, that other angles of
5 interrogation (e.g., $\Omega = 5$ degrees) are not incorporated into this idea.

The optical embodiment of this disclosure allows for distinct images with differing interrogating angles for the varying highly oblique or side scatter illuminators. A further analysis step then uses the information from the two or more images to:

10

a) attenuate the effects of red blood cells on further analysis of cells present in the sample (e.g., white blood cells)

15

b) better identify only white blood cells for further analysis (e.g., have a better delineation of the white cell boundary within the image)

c) better analyze white blood cells (e.g., better interrogate pixels on the sensor that correspond to the white blood cell and not the red blood cell).

20

This can be achieved in any number of ways, some of which are listed below as a number of varying embodiments. The following analysis embodiments are examples and includes any form of publicly available analysis that utilizes the information from more than one angle of highly oblique or side scatter illumination is covered by this application.

25

One way to attenuate the effects of the red blood cells on image analysis is to combine the information from the two or more images from different illumination angles that leverages the high degree of mutual information for the white blood cell images and the low degree of mutual information for the red blood images. For example, multiplying the two images together will attenuate out the red blood cell signal relative to the white blood cell.

30

Since the signals do not overlap significantly in two dimensional space, the red blood cell signal is greatly attenuated, while the white blood cell signal remains due to significant spatial overlap of the two images. The images can be blended or fused using any number of methods (e.g., multiplication, addition, subtraction, weighted combination operations,

combination of manipulations, etc.) to reduce the effect of red blood cells, and we have found that multiplying the images is quite effective and elegant. This can be especially advantageous for removing imaging partial volume effects of red blood cell and white blood cells nearby to one another.

5

One can then do any number of analysis methods on the high or low magnification image and better segment and analyze the white blood cells. For low magnification images, cell event detection using linear discriminant analysis models followed by 2-dimensional Gaussian fitting for each cell event using an expectation maximization model that accounts for background signal has proven a very effective method to quantify size and side scatter intensity that can be used to later differentiate white blood cell types. The effect of attenuating the red blood cells is shown in Figs. 3a and 3b. For this example, overlapping HOIM images from two 90 degree rotationally offset illuminators and CD45 fluorescence (white blood cell control) at 4X were acquired from an unlysed human blood sample. Each cell event in the images were identified, fit to a 2-D Gaussian, and the integrated intensity of the signal for each cell was calculated. This was first done for the scatter information from one angle HOIM image and compared to CD45 fluorescence which shows white blood cells. It is clear that if the CD45 axis is ignored as would be required in label free measurements, the red blood cell cluster overlaps significantly with the white blood cell clusters.

Multiplying the images from the two HOIM images with rotationally offset illuminators and then estimating the scatter signal (Y-axis, bottom) results in a downward shift in the scatter signal from red blood cells. This attenuation of the red blood cell signal moves towards better discrimination between white blood cells and red blood cells in the label-free scatter channel.

25

Further improvements could conceivably separate the white and red blood cell populations without the need for any fluorescent/absorption labels. It is worth noting that multiplying the two HOIM images resulted in more accurate white blood cell classification due to reduced partial volume effects and increased contrast to noise of white blood cells.

30

Another method to discard red blood cells from further analysis is to analyze the different illumination angle images separately and compare. For example, if all cells (white and red cells alike) are identified from one image and segmented or analyzed, that image

region or analysis can be directly compared at the same locations in the image from another illumination angle. The actual image, pixel, or signal information can be directly compared for similarities by any number of standard methods. For example, mutual information of the image regions could be assessed to see if the image information is rotated or remains in the same location. Another example includes using structural similarity indices to assess how similar pixel intensities at corresponding locations compare. In our experience, white blood cells have a high similarity index between images from different illumination angles while red blood cells have similarity indices much lower. There are a number of methods for this that are publicly available. These similarity indices can also be used on fits that may be calculated for the image data, 2-dimensional Gaussians fit to the cell, for example. The metrics that define the fit or a set of pixels can also be compared. For example, the covariance matrix for the 2-dimensional Gaussian fit could be assessed for differences using the Euclidean distance or other distance metrics. One can imagine any set of available image analysis algorithms to compare the mutual or shared information between images of different illumination angles.

A final example embodiment includes using the information from multiple angle images to more accurately fit or segment white blood cells in the presence of red blood cells (by better identifying and fitting red blood cells). For example, in low magnification microscopy, white blood cells can be modeled as 2-dimensional Gaussians. However, these fits become inaccurate in the presence of red blood cells. Comparing mutual information or dissimilar information between images from two or more illumination angles could aid the fit to accurately converge to the proper cell location, shape, and intensity. This concept works for high magnification images to help rule out red blood cells from segmentation and analysis of white blood cells, a task that otherwise could be very challenging.

The scatter-based methods proposed here to analyze white blood cells in the presence of red blood cells requires little additional, low cost, optical instrumentation, no additional need for dyes or modifications to the existing instrumentation to attenuate the effect of red blood cells on the analysis of close-by white blood cells. This technology works for image-based cytometry in the low magnification regime where throughput is greatly increased compared to standard high resolution microscopy, and also for more standard high magnification microscopy (i.e. 0.5X-100X).

It is important to note that this technology is not limited to the analysis and discrimination of white blood cells and red blood cells. Utilizing directional scatter anisotropy could be useful in discriminating or better analyzing a number of cell and sample types. An example includes the discrimination of circulating tumor cells from red blood cells. Further example cells and biological structures whose analysis could benefit from this disclosure include any immune cell type, stem cells, fibroblasts, hepatocytes, gametocytes (malaria), exosomes and microvesicles, any form of cancer cells, epithelial cells, adipocytes, muscle cells, platelets, etc.

It is recognized that modifications and variations of the present invention will be apparent to those with ordinary skill in the art. It is intended that all such modifications and variations be included within the scope of the appended claims.

What is claimed is:

What is Claimed is:

- 1 1. Method for distinguishing between red blood cells and white blood cells in a flowing
2 or stationary blood sample including red and white blood cells comprising:
3
4 obliquely illuminating the blood sample with light from at least two rotational angles;
5 and
6
7 analyzing light side scattered from cells in the sample to provide accurate
8 discrimination of white blood cell types for white blood cell analysis and counting based on a
9 difference in light scattering profile of the red blood cells as compared to the white blood
10 cells.
- 1 2. The method of Claim 1 wherein the difference in the light scattering profile is a relative
2 difference in isotropy/anisotropy of the red blood cells and white blood cells.
- 1 3. The method of Claim 1 wherein the difference in the light scattering profile comprises
2 scatter intensity of the red blood cells and white blood cells.
- 1 4. The method of Claim 1 wherein the difference in light scattering profile results from
2 different spatial locations within a cell that scatter the light.
- 1 5. The method of Claim 1 wherein the difference in light scattering profile results for
2 differences of polarization or phase of the scattered light.
- 1 6. The method of claim 1 wherein the oblique illumination angle is in the range of 78-86
2 degrees.

- 1 7. The method of claim 1 wherein the light has a wavelength anywhere in the ultraviolet-
2 visible-near infrared electromagnetic spectrum.
- 1 8. The method of claim 7 wherein the wavelength is approximately 400 nm.
- 1 9. The method of Claim 1 wherein the rotational angle is in the range of 75-105 degrees.
- 1 10. The method of Claim 1 wherein the rotational angle is in the range of 50-120 degrees.
- 1 11. The method of Claim 1 wherein the rotational angle is in the range of 45-135 degrees.
- 1 12. Method for distinguishing between two or more cell types in a flowing or stationary
2 sample comprising:
3
4 obliquely illuminating the sample with light from at least two rotational angles; and
5
6 analyzing light side scattered from the two or more cell types in the sample to
7 discriminate the cell types based on a difference in light scattering profile of the cell types.
- 1 13. The method of Claim 12 wherein the difference in light scattering profile is a relative
2 difference in isotropy/anisotropy of the two or more cell types.
- 1
- 1 14. The method of Claim 12 wherein the difference in light scattering profile is scatter
2 intensity of the two or more cell types.
1

1 15. The method of Claim 12 wherein the difference in light scattering profile results from
2 different spatial locations within the two or more cell types.

1 16. The method of Claim 12 wherein the difference in light scattering profile results from
2 differences of polarization or phase of the scattered light.

1 17. The method of claim 12 wherein the oblique illumination angle is in the range of 78-86
2 degrees.

1 18. The method of claim 12 wherein the light has a wavelength anywhere in the ultraviolet-
2 visible-near infrared electromagnetic spectrum.

1 19. The method of claim 18 wherein the wavelength is approximately 400 nm.

1 20. The method of Claim 12 wherein the rotational angle is in the range of 75-105 degrees.

1 21. The method of Claim 12 wherein the rotational angle is in the range of 50-120 degrees.

1 22. The method of Claim 12 wherein the rotational angle is in the range of 45-135 degrees.

1 23. The method of Claim 12 wherein the cell types are tumor cells and red blood cells.

1/3

FIG. 1B

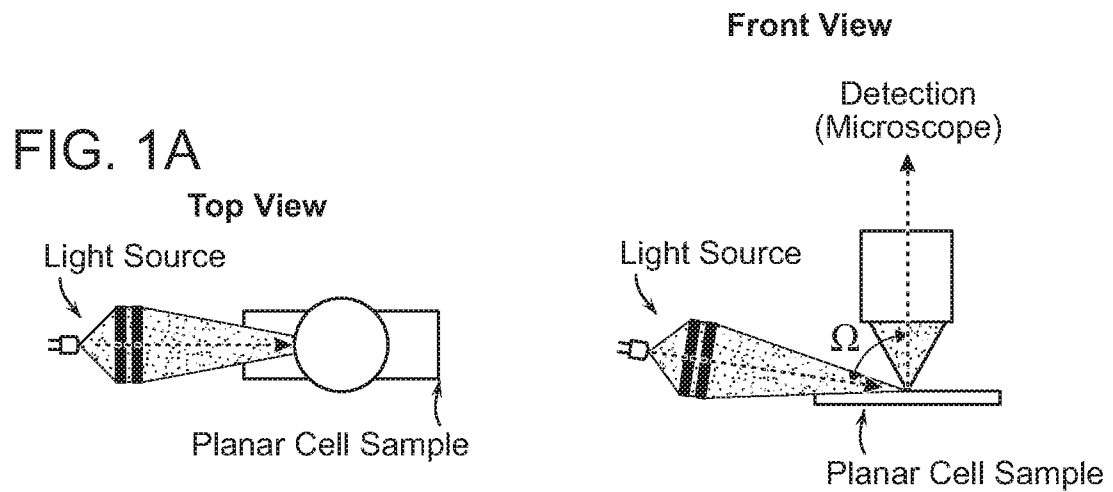
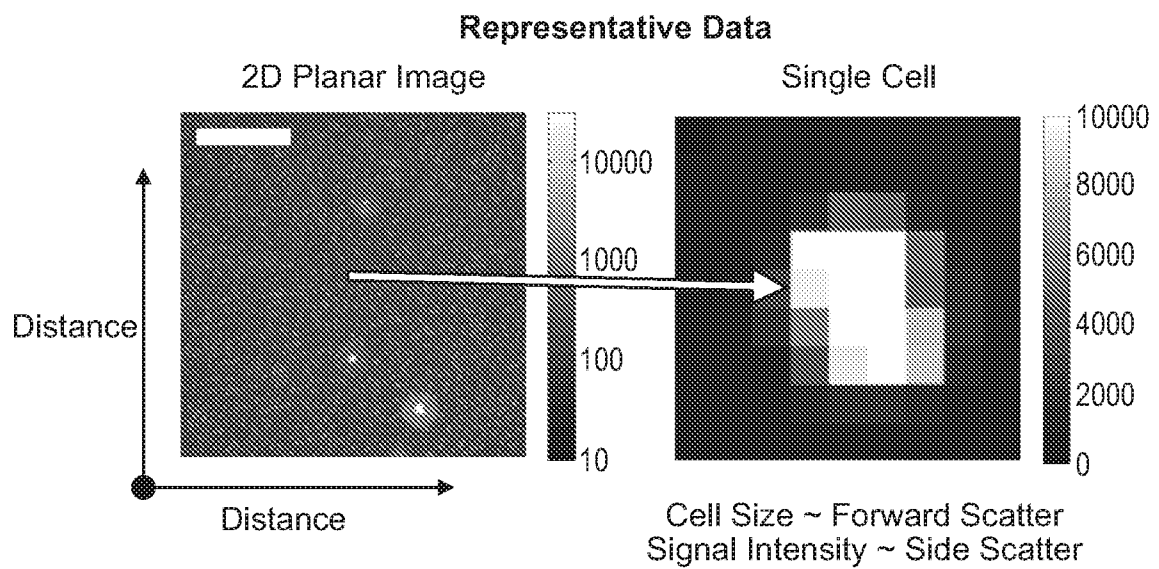


FIG. 1C



2/3

FIG. 2A

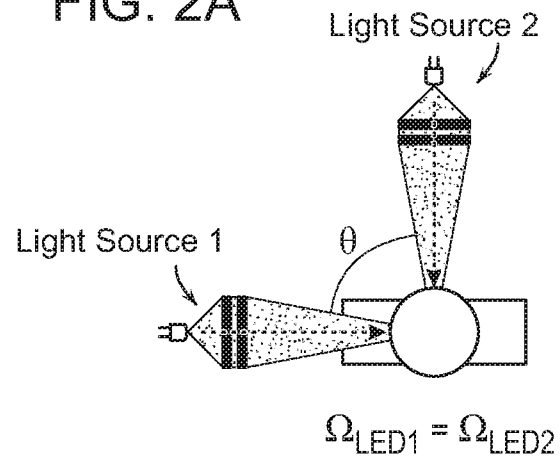
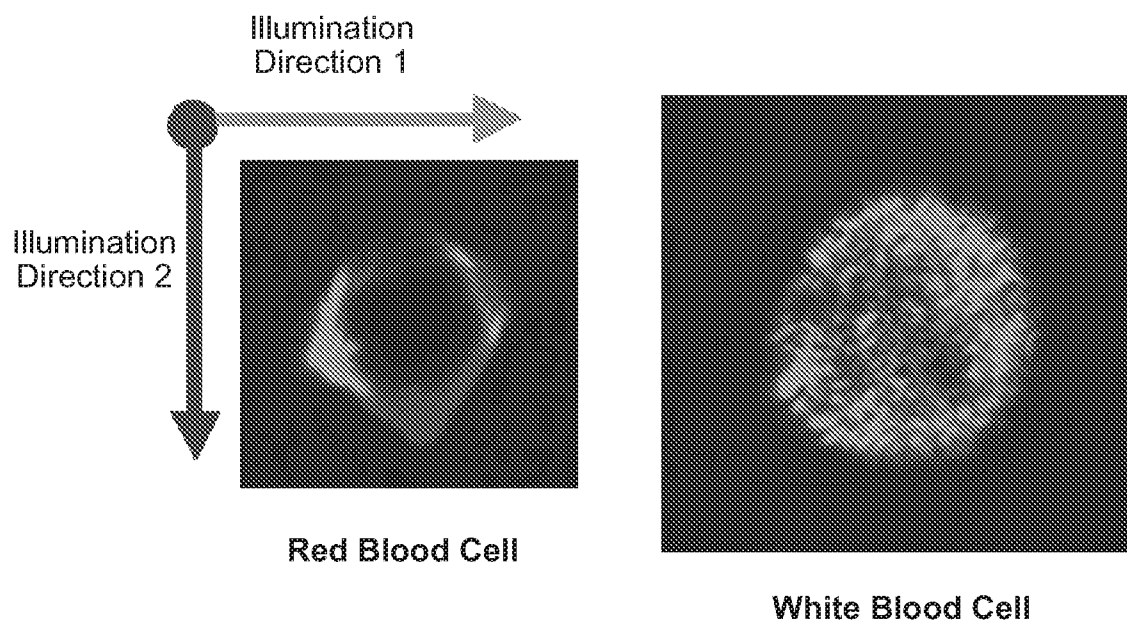


FIG. 2B



3/3

FIG. 3A

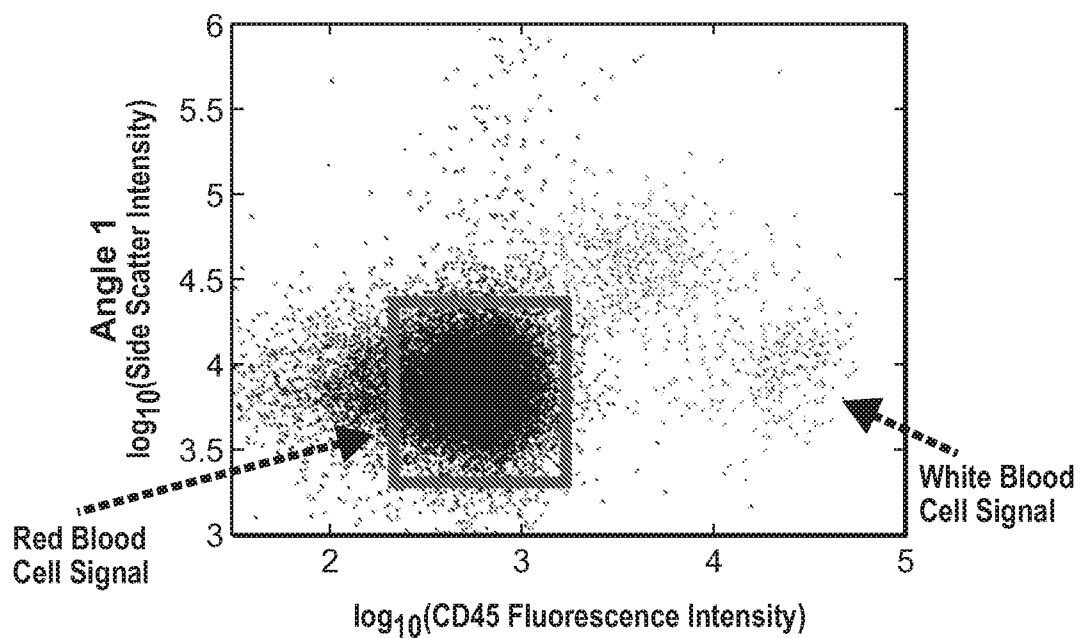
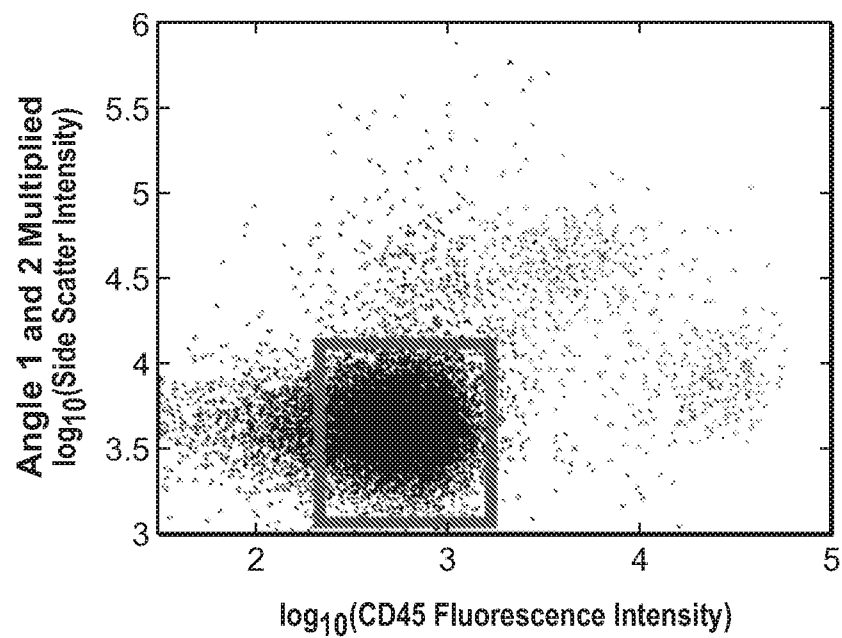


FIG. 3B



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/025836

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N15/14
ADD. G01N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/308122 A1 (MERCHEZ BENOIT [FR] ET AL) 21 November 2013 (2013-11-21) figure 1 paragraphs [0014], [0025], [0028], [0029], [0097], [0124] -----	1,3, 6-12,14, 17-23
X	US 2008/317325 A1 (ORTYN WILLIAM [US] ET AL) 25 December 2008 (2008-12-25) figure 1B paragraphs [0010], [0067], [0079], [0086] ----- -/-	1,6-12, 17-23



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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"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

15 September 2017

Date of mailing of the international search report

26/09/2017

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Stitou, Adel

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/025836

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Information on patent family members

International application No

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