



- (51) International Patent Classification:  
G01N 33/49 (2006.01) G01N 15/14 (2006.01)  
G01N 33/72 (2006.01)
- (21) International Application Number:  
PCT/US2011/021292
- (22) International Filing Date:  
14 January 2011 (14.01.2011)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
12/657,218 15 January 2010 (15.01.2010) 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, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, 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, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, 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, ML, MR, NE, SN, TD, TG).

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(54) Title: METHOD FOR DETERMINING VOLUME AND HEMOGLOBIN CONTENT OF INDIVIDUAL RED BLOOD CELLS

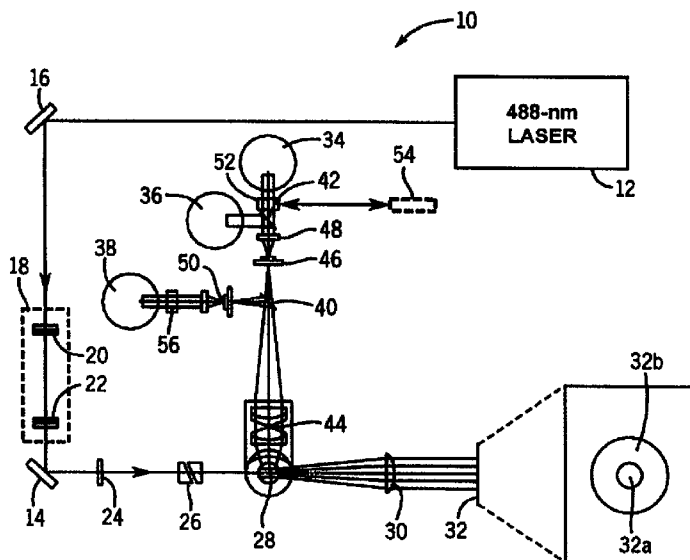


Fig. 1

(57) Abstract: Provided herein is a method for determining the volume or hemoglobin content of an individual red blood cell in a sample containing a population of red blood cells. The method may be performed on a hematology analyzer. Also provided are a hematology analyzer for performing the method and a computer-readable medium containing programming for performing the method.

WO 2011/088314 A1

**Published:**

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

## **METHOD FOR DETERMINING VOLUME AND HEMOGLOBIN CONTENT OF INDIVIDUAL RED BLOOD CELLS**

### **BACKGROUND OF THE INVENTION**

5 Variations in the morphological and physiological characteristics of red blood cells in a patient's blood provide valuable information concerning the pathological condition of many specific types of red cell disorders or anemias. In diagnosing such disorders, the mean cellular hemoglobin concentration (MCHC) and the mean cell volume (MCV) may be measured to provide valuable insight into the condition of a patient. Such information  
10 may be used in conjunction with the microscopic evaluation of the distribution of sizes, shapes and color of red cells in a stained blood smear by a trained hematologist and with other biochemical tests. Variations in the refractive index of individual red cells are highly correlated with their hemoglobin concentration, and this information can be combined with size measurements to provide diagnostic value. For example, in microcytic anemias, the  
15 size of the red cells and, therefore, also the MCV are significantly reduced, but the optical density (related to the refractive index) and the MCHC are somewhat elevated. In megaloblastic anemias, both the size (macrocytes) and the MCHC are somewhat increased.

This disclosure relates in part to a method for determining the volume and/or hemoglobin content of individual red blood cells using a hematology analyzer, and a  
20 hematology analyzer for performing the method.

### **SUMMARY OF THE INVENTION**

Provided herein is a method for determining the volume or hemoglobin content of an individual red blood cell of a blood sample using a hematology analyzer, and a  
25 hematology analyzer for performing the method. In general terms, the method involves obtaining a set of data points for a sample comprising a population of red blood cells using a hematology analyzer; and calculating the volume or hemoglobin content of an individual red blood cell in said sample containing using: (I) measurements of individual red blood cells in a plurality of optical scattering channels (e.g., axial light loss, intermediate angle  
30 scattering, and/or polarized side scattering channels, etc.); (II) the mean cell volume of the population of red blood cells calculated using measurements from a separate impedance transducer, (III) the bulk hemoglobin concentration of the sample measured on a separate colorimetric transducer; (IV) the number of cells in the population of red blood cells; (V) the mean values of the measurements of the population of red blood cells on the plurality of

said optical scattering channels; and (VI) the median-of-means value of prior populations of red blood cells analyzed on at least one of said optical scattering channels on said hematology analyzer. Computer-related embodiments are also provided.

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### **BRIEF DESCRIPTION OF THE DRAWINGS**

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

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Figure 1 is a schematic illustration of the optical subassembly of an exemplary hematology analyzer.

Figure 2 is a plot of blood cells collected in the reticulocyte assay on a CELL-DYN Sapphire hematology analyzer from Abbot Diagnostics.

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Figure 3 is a graph showing exemplary thresholds applied to a red blood cell (RBC)/reticulocyte (Retic) distribution.

Figure 4 is a table that maps the relationships in the volume and cellular concentration models.

Figure 5 is a graphical representation of the model used to calculate cell volume.

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Figure 6 is a plot showing the data points from a study, representing the mean scaled and normalized axial light loss and intermediate angle scattering values of all the cells of a particular sample. Data points were used to create the model used to calculate cell volume. The internal lines show the extent of the volume model surface.

Figure 7 is a graphical representation of the local model used to calculate cellular hemoglobin concentration (the CHC local model).

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Figure 8 is a plot showing the data points used to create the CHC local model. The internal lines show the extension of the CHC model surface.

Figure 9 is a plot of the percentages of RBC/Retic points that fall outside the CHC model surface for 977 samples in a study.

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Figure 10 is a plot of the percentages of RBC/Retic points that fall outside of the CHC model surface for the 977 samples from a study as a function of optical MCHC values.

5 Figure 11 is a plot of all RBC/Retic events in the plane of scaled, normalized intermediate angle and polarized side scattering for the sample in the study with the most events (6%) outside of the CHC model surface.

Figure 12 is a plot of all RBC/Retic events in the of scaled, normalized intermediate angle and polarized side scattering plane for another sample in the study with an MCHC of 32 g/dL.

10 Figure 13 schematically depicts the algorithm used for classifying reticulated platelets (rPLT) in a normal sample. rPLTs are the circled events.

## DETAILED DESCRIPTION

Before the present invention is described in detail, it is to be understood that this invention is not limited to particular variations set forth and may, of course, vary. Various  
5 changes may be made to the invention described and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications can be made to adapt a particular situation, material, composition of matter, process, process act(s) or step(s), to the objective(s), spirit or scope of the present invention. All such modifications are intended to be within the scope of the claims made  
10 herein.

Steps of any method recited herein can be carried out in any order of the recited events which is logically possible, as well as the recited order of events. Furthermore, where a range of values is provided, it is understood that every intervening value, between the upper and lower limit of that range and any other stated or intervening value in that  
15 stated range is encompassed within the invention. Also, it is contemplated that any optional feature of the inventive variations described can be set forth and claimed independently, or in combination with any one or more of the features described herein.

The referenced items are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the  
20 present invention is not entitled to antedate such material by virtue of prior invention.

Reference to a singular item, includes the possibility that there are plural of the same items present. More specifically, as used herein and in the appended claims, the singular forms “a,” “an,” “said” and “the” include plural referents unless the context clearly dictates otherwise. For example, if a value is compared to “a criterion”, the value may be  
25 compared to one or more criteria, i.e., a single criterion or multiple criteria. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

30 The following abbreviations may be used in this disclosure: CBC (complete blood count), RBC (red blood cell or erythrocyte), rRBC (lysis-resistant red blood cell or erythrocyte), Retic (reticulocyte, an immature red blood cell), HGB (hemoglobin), MCHC (mean corpuscular, or cell, hemoglobin concentration), MCV (mean corpuscular, or cell, volume), RDW (red blood cell distribution width), PLT (platelet), WBC (white blood cell

or leukocyte), fWBC (fragile white blood cell or leukocyte), NEU (neutrophils), LYM (lymphocytes), MON (monocytes), DSS (depolarized side scatter), CLL (chronic lymphocytic leukemia), ALL (axial light loss), IAS (intermediate angle scatter), PSS (polarized side scatter), DSS (depolarized side scatter), and FCS (flow cytometry standard).

5 Generally, uppercase acronyms are used to indicate a measurement method, assay, or detection channel (e.g., HGB, IAS), while lowercase italics are used to indicate values obtained from such measurements (e.g., *mcv*, *pss*). In addition, the term “cell” may be used to refer to any of the formed bodies commonly or pathologically found in peripheral blood, e.g., RBCs and WBCs, and also including PLTs. Other abbreviations may be defined  
10 below.

In this disclosure, the term “FCS file” is used to describe a digital representation of the collection of detected events captured by the analyzer and classified (as, e.g., RBCs, lymphocytes, etc.) by automated internal algorithms. The events in the FCS file may also be referred to as “list mode” data, reflecting one aspect of the FCS file format, in which  
15 events are arranged in a list ordered sequentially by time of detection.

### **Hematology analyzers**

As noted above, a hematology analyzer that provides the volume and/or the amount of hemoglobin of individual red blood cells of a sample is provided. In general terms, the  
20 hematology analyzer comprises: a) a flow cell; b) a light source for directing light to said flow cell; c) a plurality of detectors for detecting light scattering by cells on a plurality of optical scattering channels; d) an apparatus for measuring bulk hemoglobin concentration in a cell lysate; e) an impedance transducer for measuring changes in impedance attendant to the passage of individual cells; and f) a data analysis workstation comprising  
25 programming to calculate the volume or hemoglobin content of an individual red blood cell in a sample containing a population of red blood cells using: (I) measurements of said individual red blood cells on a plurality of said optical scattering channels; (II) the mean cell volume of said population of red blood cells calculated using impedance values; (III) the bulk hemoglobin concentration of said sample; (IV) the number of cells in said  
30 population of red blood cells; (V) the mean values of the measurements of said population of red blood cells on said plurality of said optical scattering channels; and (VI) the median-of-means value (i.e., the median of the means) of prior populations of red blood cells analyzed on at least one of said optical scattering channels on said hematology analyzer.

The methodology described below include several calculations that may be generally employed on any suitable flow cytometer in the form of programming, including a hematology analyzer, examples of which are known on the art and described in, e.g., U.S. Patents 5,017,497, 5,266,269, 5,378,633, 5,631,165, and 6,524,858, as well as published  
5 U.S. Patent Applications US20080153170, US20080158561 and US20080268494, the disclosures of which are incorporated herein by reference in their entirety. Hematology analyzers analyze samples of whole blood to determine, among other results, the concentration of erythrocytes, platelets, and hemoglobin. The optical subassembly of an exemplary hematology analyzer is schematically illustrated in Fig. 1. One of skill in the art  
10 would recognize that the choice, number and design of the components (e.g., the type of laser used, the number and specifications of the optical components, etc.) can vary greatly between analyzers and, as such, the hematology analyzer of Fig. 1 is provided as an example and should not be used to limit this disclosure. For example, in certain cases a hematology analyzer may or may not detect fluorescence. In addition to the optical  
15 components shown in Fig. 1, a hematology analyzer may contain an impedance-measuring device for measuring changes in impedance as cells pass through (e.g., enter or exit) the flow cell. Such an apparatus includes an impedance meter, examples of which are described in U.S. Pat. Nos. 2,656,508, 3,810,011 and 5,125,737, which are incorporated by reference herein in their entirety. Electrical impedance measurements may be used to count  
20 and size (e.g., calculate the volume of) cells passing through the flow cell.

Referring now to Fig. 1, exemplary hematology analyzer **10** comprises a source of light **12**, a front mirror **14** and a rear mirror **16** for beam bending, a beam expander module **18** containing a first cylindrical lens **20** and a second cylindrical lens **22**, a focusing lens **24**, a fine beam adjuster **26**, a flow cell **28**, a forward scatter lens **30**, a bull's-eye detector **32**, a  
25 first photomultiplier tube **34**, a second photomultiplier tube **36**, and a third photomultiplier tube **38**. The bull's-eye detector **32** has an inner detector **32a** for measuring extinction of the forward-propagating beam (the data produced therefrom being referred to as "axial light loss" or "ALL") and an outer detector **32b** for light scattering in an annulus of 3° to 10° from forward (otherwise referred to as "intermediate angle scatter" or "IAS"). The  
30 source of light **12** can be a vertically polarized 488-nm air-cooled argon-ion laser or a vertically polarized blue (488 nm) solid-state laser. Other laser wavelengths can be substituted, with attendant changes in the optical design layout (i.e., selection, positioning and characteristics of the optical components). Additional details relating to the laser, the flow cell, the lenses, the focusing lens, the fine-beam adjust mechanism and the laser



focusing lens can be found in U.S. Patent No. 5,631,165, incorporated herein by reference, particularly at column 41, line 32 through column 43, line 11.

The forward optical path system of the hematology analyzer shown in Fig. 1 includes a plano-convex lens **30** and a two-element photodiode detector **32** located in the back focal plane of the lens. In this configuration, each concentric ring within the outer photodiode detector **32b** maps to a specific incremental angular collection annulus of light from cells moving through the flow cell **28**. The detector **32** can be a bull's-eye detector capable of detecting axial light loss (ALL) and intermediate angle forward scatter (IAS). U.S. Patent No. 5,631,165 describes various alternatives to this detector at column 43, lines 12-52.

The first photomultiplier tube **34** (PMT1) measures depolarized side scatter (DSS) or green fluorescence (FL1). The second photomultiplier tube **36** (PMT2) measures polarized side scatter (PSS) or yellow to orange fluorescence (FL2) and the third photomultiplier tube **38** (PMT3) measures red fluorescence (FL3). FL1, green fluorescence, is detected between about 515 to 545 nm. FL2, yellow to orange fluorescence, is detected between about 565 to 595 nm. FL3, red fluorescence, is detected between about 615 to 645 nm. Side-scatter and fluorescent emissions are directed to these photomultiplier tubes by dichroic beam splitters **40** and **42**, which transmit and reflect efficiently at the required wavelengths to enable efficient detection. U.S. Patent No. 5,631,165 describes various additional details relating to the photomultiplier tubes at column 43, line 53 through column 44, line 4.

Sensitivity is enhanced at photomultiplier tubes **34**, **36**, and **38**, when measuring fluorescence, by using an immersion collection system. The immersion collection system is one that optically couples the first lens of condenser assembly **44** to the flow cell **28** by means of a refractive index-matching layer, enabling collection of light over a wide angle. U.S. Patent No. 5,631,165 describes various additional details of this optical system at column 44, lines 5-31.

The condenser **44** is an optical lens system with aberration correction sufficient for diffraction-limited imaging used in high resolution microscopy. U.S. Patent No. 5,631,165 describes various additional details of this optical system at column 44, lines 32-60.

The functions of other components shown in Fig. 1, i.e., a slit **46**, a field lens **48**, and a second slit **50**, are described in U.S. Patent No. 5,631,165, at column 44, line 63 through column 45, line 15. The photomultiplier tubes **34**, **36**, and **38** detect either side-scatter (light scattered in a cone whose axis is approximately perpendicular to the incident

laser beam) or fluorescence (light emitted from the cells at a different wavelength from that of the incident laser beam). A slider assembly placed in front of photomultiplier **34** allows dual use of photomultiplier **34**: to detect depolarized side scatter (DSS) when polarizer **52** is moved in the light path, and to detect green fluorescence (FL1) when filter **54** is moved  
5 in the light path. A similar slider assembly (not shown) placed in front of photomultiplier **36** allows the dual use of detecting polarized side scatter (PSS) and yellow-orange fluorescence (FL2). Photomultiplier **38** is configured with filter **56** to detect red fluorescence (FL3) only.

As would be readily apparent, numerous variations of the above-described analyzer  
10 are possible. For example, the two cylindrical lenses may be replaced by an anamorphic prism pair, the bull's-eye detector can be replaced by separate detectors and a holed mirror, and other wavelengths of light may be employed.

An example of a hematology analyzer that does not detect fluorescence is described in U.S. Patent 5,378,633, particularly at col. 24, line 47 to col. 25, line 36 and Figs. 6 and 7.  
15 This description is incorporated by reference herein.

A suspension of blood containing erythrocytes may be propelled from a sample nozzle where it comes into contact with a fast-moving, laminar-flow sheath stream. In a process known as hydrodynamic focusing, the sample stream is squeezed into a thin central core. This arrangement usually ensures that only a single cell is in the sensing region of the  
20 laser beam at any given time.

The measurement process begins as the cell core stream passes through the flow cell **28**, having been diluted with the diluent so that the cells pass through the laser-illuminated volume substantially in single file, in a laminar flowing sample stream surrounded by a sheath fluid. The illuminated volume is bounded by the intersection of the  
25 laser beam and the sample stream, and in one embodiment it has the approximate dimensions of 80  $\mu\text{m}$  along the laser propagation direction, 20  $\mu\text{m}$  along the sample stream flow direction, and about 5-10  $\mu\text{m}$  in a direction transversal to both sample flow and laser beam propagation.

Light scattered at 90 degrees to the axis of the laser beam may be collected using  
30 photomultipliers (PMTs). Photomultipliers, not photodiodes, are used in the 90-degree channels because relatively little light is scattered at high angles, and because they can also be used to detect the generally much lower-intensity fluorescence emissions. If the impinging polarized light undergoes optical scattering mainly from the cell membrane and nucleus (if present), it generally retains its original vertical plane of polarization. However,

if it interacts with certain subcellular components that may be present in the cytoplasm, e.g., granules or anisotropic structures, then the scattered light can have an altered angle of polarization. In order to exploit this phenomenon, one of the PMTs may have a horizontal polarizer in front of it. This polarizer prevents vertically polarized light from striking the photomultiplier. Therefore, any light detected by the “90-degree depolarized” PMT is light  
5 that has been depolarized by its interaction with a cellular substructure—usually a leukocyte, and particularly a eosinophil. The second photomultiplier (the “90-degree polarized” PMT) may receive the scattered light reflected off a beam splitter that is angled at 45 degrees and designed to mostly reflect vertically polarized light and mostly transmit  
10 horizontally polarized light at the excitation (laser) wavelength. The major portion of the light detected by this second photomultiplier is vertically polarized side-scattered light and carries information correlated with the conformation of the nucleus. The scattering light detection scheme briefly summarized here is a proprietary design referred to as Multi-Angle Polarized Scattering Separation (MAPSS), and described in fuller detail in U.S.  
15 Patent 5,017,497. This description is incorporated by reference herein.

Data obtained from the photosensors may be used to construct a multi-dimensional scattergram (comprising two to five or more dimensions, and typically four). Any three dimensions can be viewed using the computer graphics capabilities of the instrument which enable a three-dimensional "solid" representation to be rotated in space—color being used  
20 to identify the different classifications of cell populations effected by automated algorithms programmed on the data station, or alternately used to map the values from a fourth dimension. For purposes of paper documentation, the four-dimensional scattergram can be examined by six user-selectable pairs of two-dimensional scatter plots or projections and by numerous user-selectable one-dimensional histogram projections.

25 The data may be analyzed in order to enumerate, for example, the erythrocytes and platelets in the sample, as well as to enumerate, for example, the immature erythrocytes (e.g., reticulocytes) in the sample. Reticulocytes may be distinguished from mature erythrocytes by use of a nucleic-acid dye or stain in the reagent solution which, upon binding to the RNA contained in the immature cell (but absent from a mature one), so  
30 labels the reticulocyte to allow its identification by fluorescent or light-scattering means. The data may be further analyzed in accordance with methods described in greater detail below.

The bulk hemoglobin concentration (HGB) of a blood sample is measured on an automated hematology analyzer by mixing a separate aliquot of a blood sample with a lytic

reagent, and measuring the hemoglobin concentration of the resultant cell lysate using a colorimetric transducer. Upon exposing to the lytic reagent, the red blood cells are completely lysed, and hemoglobin is released to the sample mixture, which upon reacting with a ligand in the lytic reagent forms a chromogen. The hemoglobin chromogen is then  
5 measured by colorimetry at a predetermined wavelength, and HGB is calculated from the measurement. One lysing reagent system suitable for measuring HGB comprises an isotonic blood diluent, such as the diluents described in U.S. Pat. Nos. 4,521,518, 4,528,274, 5,935,857 and 6,706,526, and a lysing reagent, such as the lysing reagents described in U.S. Pat. Nos. 5,763,280, 5,834,315 and 6,573,102; these are hereby  
10 incorporated by reference in their entirety. Alternatively, the reagent system can also be a single lysing reagent as described in U.S. Pat. No. 5,882,934 which is hereby incorporated by reference in its entirety. Furthermore, various lytic reagents known in the art for measurement of hemoglobin can be used for the purpose of the present invention.

Impedance may be measured using a non-focused flow aperture, and the blood  
15 sample can be highly diluted, for example with a dilution ratio of 6250:1. When a focused flow cell is used for the measurement, the dilution ratio can be substantially lower, such as 290:1. To maintain the volume and morphology of the red blood cells during their measurements on a hematology analyzer, an isotonic diluent is used for diluting the blood sample. Typically, the diluent contains one or more alkaline metal salts. Various  
20 commercially available isotonic blood diluents can be used for diluting the blood sample. Suitable examples include, but are not limited to, the diluents described in U.S. Pat. Nos. 4,521,518, 4,528,274, 5,935,857 and 6,706,526. When a particle or a blood cell, suspended in a conductive solution, passes through an aperture, an electrical signal, or a pulse, can be measured due to the increase of impedance. The electrical pulses can be used for counting  
25 the number of blood cells of a blood sample. Also, the pulse shape, height and width are directly related to the volume or size of a particle, and can be converted to the volume of the cell measured. When a sample that contains two or more different blood cells having different volumes is measured, a histogram obtained from the measurement can represent the volume distribution of these blood cells. The detection methods and apparatus used for  
30 blood cell counting and sizing by a blood analyzer equipped with an impedance meter are generally described in U.S. Pat. Nos. 2,656,508, 3,810,011 and 5,125,737, which are hereby incorporated by reference in their entirety.

### **Methodology**

In the discussion that follows below and in the Examples section, certain calculations may be described using the phrase “inputting measurements into a formula” or a grammatical equivalent thereof. As would be recognized, any single calculation may be performed as a multi-step method, and many multi-step calculations can be described using a single formula. As such, any method that has a step that “inputting measurements into a formula” or the like is not intended to be limited to only those embodiments that involve inputting measurements into a single formula. As would be recognized and as illustrated in the Examples section herein, such a calculation may be done using a number of different steps, each using a different formula, that lead to the same result as if a single formula were employed. All methods, including those that employ different measurement units and different formulae that lead to the same results similar to or the same as those described below are contemplated herein. All steps of the calculating method may be performed using a computer, and the various steps of the calculation may be embodied on a computer-readable medium in the form of programming. Further, in several embodiments described below, the method may be described as a method of calculating the volume or hemoglobin content of an individual red blood cell in a sample “using” multiple variables. As would be understood, the term “using” includes inputting the variables, or a derivative thereof, into a formula that, when executed, outputs a result, i.e., the volume or hemoglobin content of an individual red blood cell in a sample. The terms “algorithm” and “formula” are used synonymously and, as noted above, are not intended to imply that any calculation can be solved using a single step.

In certain embodiments, certain metrics of a red blood cell population (e.g., the number of cells in the population, the mean cellular hemoglobin concentration of the population, the mean cell volume, etc.) may be calculated without using optical scattering measurements, e.g., calculated using impedance and bulk hemoglobin measurements. Such methods are known in the art, and are described in detail in references cited above.

Provided herein is a method of sample analysis. In general terms, the method comprises obtaining a set of data points for a sample comprising a population of red blood cells using a hematology analyzer; and calculating the volume or hemoglobin content of an individual red blood cell in the sample containing using: (I) measurements of individual red blood cells on a plurality of a optical scattering channels; (II) the mean cell volume of the population of red blood cells calculated using impedance values; (III) the bulk hemoglobin concentration of the sample; (IV) the number of cells in the population of red blood cells; (V) the mean values of the measurements of the population of red blood cells on the

plurality of the optical scattering channels; and (VI) the median-of-means value of prior populations of red blood cells analyzed on at least one of the optical scattering channels on the hematology analyzer. In certain embodiments and as will be described below the measurements of whichever optical scattering channels are used may be filtered to remove  
5 data points generated by noise and coincidences prior to use. Also as will be described below, the measurements may be scaled (i.e., multiplied by a suitable factor in order to minimize differences between analyzers and the effect of drift on any one analyzer) and normalized (i.e., mathematically transformed to yield quantities centered around 0 and generally distributed between -1 and 1) as part of the method.

10 In certain embodiments and as described in greater detail in the Examples section of this disclosure, the volume of individual red blood cells in the sample may be calculated using: (I) measurements of individual red blood cells on ALL and IAS channels; (II) the mean cell volume of the population of red blood cells calculated using impedance values; (III) the mean cellular hemoglobin concentration of the population, calculated using the  
15 mean cell volume, the bulk hemoglobin concentration, and the number of cells in the population; (IV) the mean values of the measurements of the population of red blood cells on the ALL and IAS channels; and (V) the median-of-means IAS value for prior populations of red blood cells analyzed on the IAS channel on the hematology analyzer.

In these embodiments, the ALL and IAS measurements may be scaled ALL and  
20 IAS measurements, i.e., ALL and IAS measurements that are adjusted relative to the initial ALL and IAS measurements prior to calculating the volume of the cell. In certain cases, the initial IAS measurement may be scaled to provide a scaled IAS value using: (I) the mean cellular hemoglobin concentration of the population of red blood cells, (II) the mean value of the measurements of the population of red blood cells on the IAS channel; and (III) the  
25 median-of-means IAS value obtained from prior samples run on the hematology analyzer. An exemplary formula for scaling the initial IAS measurement to provide a scaled IAS value is set forth in the Examples section of this disclosure. The initial ALL measurement may be scaled to provide a scaled ALL measurement using: (I) the scaled IAS value; (II) the mean cell volume of the population of red blood cells calculated using impedance  
30 values; and (III) the mean value of the measurements of the population of red blood cells in the ALL channel. An exemplary formula for scaling the initial ALL measurement to provide a scaled ALL measurement is set forth in the Examples section of this disclosure.

In certain embodiments and as described in greater detail in the Examples section of this disclosure, the hemoglobin content of individual red blood cells in the sample may be

calculated using: (I) measurements of individual red blood cells on IAS and PSS channels; (II) the mean cellular hemoglobin concentration of the population (which is calculated using the mean cell volume based on impedance values, the bulk hemoglobin concentration, and the number of cells in the population); (III) the mean values of the  
5 measurements of the population of red blood cells on the IAS and PSS channels; and (IV) the median-of-means IAS and PSS values for prior populations of red blood cells analyzed on the IAS and PSS channels on the hematology analyzer.

In these embodiments, the IAS and PSS measurements may be scaled IAS and PSS measurements, i.e., IAS and PSS measurements that are adjusted relative to the initial IAS  
10 and PSS measurements prior to calculating the hemoglobin content of the cell. In certain cases, the initial IAS measurement may be scaled to provide a scaled IAS value using the method described above, i.e., by using: (I) the mean cellular hemoglobin concentration of the population of red blood cells, (II) the mean value of the measurements of the population of red blood cells on the IAS channel; and (III) the median-of-means IAS value obtained  
15 from prior samples run on the hematology analyzer. An exemplary formula for scaling the initial IAS measurement to provide a scaled IAS value is set forth in the Examples section of this disclosure. The initial PSS measurement may be scaled to provide a scaled PSS measurement using: (I) the mean cellular hemoglobin concentration of the population of red blood cells; (II) the mean value of the measurements of the population of red blood  
20 cells on the PSS channel; (III) the median-of-means PSS value obtained from prior samples run on the hematology analyzer. An exemplary formula for scaling the initial PSS measurement to provide a scaled PSS measurement is set forth in the Examples section of this disclosure.

In certain cases, in order to remove data points produced by noise (e.g., cell debris  
25 or other particles) and coincidences (i.e., unresolved movement of two cells across the light source of the hematology analyzer at very nearly the same time), the data points for whichever optical scattering channels are used may be filtered prior to use in the method, e.g., prior to any scaling that may occur. In some embodiments, the filtering removes high- and low-end measurements that are expected to result from noise and coincidences. In one  
30 embodiments, the data points for whichever channels are used (e.g., data for the ALL, IAS and/or PSS channels) are plotted on a histogram, and the boundaries of acceptable data can be set at defined percentages of the histogram peak height, as described in Fig. 3. For example, the boundaries of acceptable data can be set at a percentage in the range of 1% to 10%, e.g., 5%, of the histogram peak height. For removing coincidences and noise from

IAS data for reticulocytes, the histogram bin used as upper boundary can be increased by 5% to 15%, e.g., 10% as compared to that used for the entire population of red blood cells, because the reticulocytes tend to be larger than the red blood cells, which means that data produced by coincidences tend to have higher values than for the entire population of red blood cells. Other methods are known for identifying and removing noise (at the lower range) and coincidences from optical data obtained from a hematology analyzer, and may be employed herein.

In some embodiments, the method may further comprise, as part of the calculating step, normalizing the measurements, e.g., after scaling the measurements. The normalization comprises adjusting the measurements relative to a constant so as to obtain quantities approximately 1, and further subtracting 1 so as to obtain normalized measurements distributed around 0. In particular embodiments, the initial data for each channel may be divided by a normalization constant (which is different for each channel), which constant may be empirically determined. The normalization constant may not change from sample to sample. Rather, it may be stable and not subject to change between samples unless a need arises.

As such, in particular embodiments, the method may include: a) obtaining a set of data points for a sample comprising a population of red blood cells using a hematology analyzer; b) removing data points corresponding to noise and coincidences to provide a filtered dataset; and c) calculating the volume or hemoglobin content of an individual red blood cell in the sample containing using: (I) measurements of individual red blood cells on a plurality of a optical scattering channels; (II) the mean cell volume of the population of red blood cells calculated using impedance values; (III) the bulk hemoglobin concentration of the sample; (IV) the number of cells in the population of red blood cells; (V) the mean values of the measurements of the population of red blood cells on the plurality of the optical scattering channels; and (VI) the median-of-means value of prior populations of red blood cells analyzed on at least one of the optical scattering channels on the hematology analyzer. The calculating includes scaling and normalizing the measurements, and inputting the so scaled, normalized measurements into a formula to obtain the volume and/or hemoglobin concentration of the cell.

Measurement of the volume of individual red blood cells and/or the amount of hemoglobin in individual red blood cells of a red blood cell population permits the analysis of further characteristics of the population. In one embodiment, the method may further comprise calculating the proportion (which may be expressed as a percentage, fraction or



another number, for example), of red blood cells having a defined characteristic. For example, the method may be employed to calculate the proportion of red blood cells having a volume above and/or below a defined volume (e.g., the percentage of cells larger than 120 fL, i.e., the percentage of “macrocytic” red blood cells; or the percentage of cells smaller than 60 fL, i.e., the percentage of “microcytic” red blood cells). In another embodiment, the method may be employed to calculate the proportion of red blood cells having a hemoglobin concentration above and/or below a defined volume (e.g., the percentage of red blood cells having a cellular hemoglobin concentration of less than 28 g/dL, i.e., the percentage of “hypochromic” red blood cells; or the percentage of red blood cells having a cellular hemoglobin concentration of greater than 41 g/dL, i.e., the percentage of “hyperchromic” red blood cells). Likewise, the volumes and/or hemoglobin concentrations of individual red blood cells of a population may be statistically analyzed to identify other statistical measures that describe, e.g., the shape of the distribution or variation of the volume or hemoglobin concentration of individual RBCs within the population. In one exemplary embodiment, the width of the distribution of hemoglobin concentration in the population of red blood cells is calculated.

In a further embodiment, the method may further involve identifying reticulocytes, which are a subset of red blood cells that are distinguishable from other red blood cells by fluorescence, in the sample. The method may also be used to analyze the reticulocytes by, for example, calculating the mean amount of hemoglobin in the reticulocytes, the mean concentration of hemoglobin in the reticulocytes, or the mean volume of the reticulocytes, in a sample.

The hematology analyzer described above can be employed, for example, to investigate red blood cell disorders or anemias, and to make treatment decisions, if necessary. Examples of anemia include iron deficiency anemia, anemia of chronic disorder, and megaloblastic anemia caused by vitamin B<sub>12</sub> or folic acid. For example, administration of iron supplement is extremely effective as a treatment for iron deficiency anemia, but not for anemia of chronic disorder. The cause of the anemia is therefore important to the treatment of the anemia.

Iron deficiency is the most prevalent single deficiency state on a worldwide basis. It is important economically because it diminishes the capability of affected individuals to perform physical labor, and it diminishes both growth and learning in children.

Absolute iron deficiency, with anemia or without anemia, and functional iron deficiency are high-frequency clinical conditions, and these patients have iron-deficient

erythropoiesis. Absolute iron deficiency is defined as a decrease in total iron body content. Iron deficiency anemia occurs when iron deficiency is sufficiently severe to diminish erythropoiesis and cause the development of anemia. Functional iron deficiency describes a state where the total iron content of the body is normal or even elevated, but the iron is  
5 “locked away” and unavailable for the production of red blood cells. This condition is observed mainly in patients with chronic renal failure who are on hemodialysis, and in patients with chronic inflammation or chronic infections.

Iron status can be measured using hematological and biochemical indices. Each parameter of iron status reflects changes in different body iron compartments and is  
10 affected at different levels of iron depletion. Specific iron measurements include hemoglobin, mean cell volume, hematocrit, erythrocyte protoporphyrin, plasma iron, transferrin, transferrin saturation levels, serum ferritin, soluble transferrin receptors and red-cell distribution width.

Hemoglobin has been used longer than any other iron status parameter. It provides a  
15 quantitative measure of the severity of iron deficiency once anemia has developed. Hemoglobin determination is a convenient and simple screening method and is especially useful when the prevalence of iron deficiency is high, as in pregnancy or infancy. The limitations of using hemoglobin as a measure of iron status are its lack of specificity (as factors such as vitamin B<sub>12</sub> or folate deficiency, genetic disorders and chronic infections  
20 can limit erythropoiesis) and its relative insensitivity due to the marked overlap in values between normal and iron deficient populations. To identify iron deficiency anemia, hemoglobin is measured together with more selective measurements of iron status.

A reduction in mean cell volume occurs when iron deficiency becomes severe, at about the same time as anemia starts to develop. It is a fairly specific indicator of iron  
25 deficiency once thalassemia and the anemia of chronic disease have been excluded. A cut-off value of 80 fl is accepted as the lower limit of the normal range in adults. The red-cell distribution width (RDW) has been used recently in combination with other parameters for the classification of anemias. It reflects the variation in the size of the red cells and can be used to detect subtle degrees of anisocytosis.

30 The most commonly used iron status parameters at present are transferrin saturation (TSAT) and serum ferritin. However, both are indirect measures of iron status. Transferrin is a transport protein that contains two iron binding sites by which it transports iron from storage sites to erythroid precursors. TSAT (i.e., the percentage of total binding sites that are occupied by iron) is a measure of iron that is available for erythropoiesis. TSAT is

calculated by dividing the serum iron by the total iron binding capacity, a measurement of circulating transferrin, and multiplying by 100. Ferritin is a storage protein that is contained primarily within the reticuloendothelial system, with some amounts released in the serum. Under conditions of iron excess, ferritin production increases to offset the increase in plasma iron. The level of ferritin in the serum, therefore, reflects the amount of iron in storage.

Reticulocytes are immature red blood cells with a maturation time of only 1 to 2 days before turning into mature red blood cells. When these are first released from the bone marrow, measurement of their hemoglobin content can provide the amount of iron immediately available for erythropoiesis. A less-than-normal hemoglobin content in these reticulocytes is an indication of inadequate iron supply relative to demand. The amount of hemoglobin in these reticulocytes also corresponds to the amount of hemoglobin in mature red blood cells. The hemoglobin content of reticulocytes (CHr) has been evaluated recently in numerous studies as a test for iron deficiency and functional iron deficiency and has been found to be highly sensitive and specific. However, exact threshold values have not been established, as the threshold values vary depending on the laboratory and instrument used.

Erythropoietin is effective in stimulating production of red blood cells, but without an adequate iron supply to bind to hemoglobin, the red blood cells will be hypochromic, i.e., low in hemoglobin content. Thus, in states of iron deficiency, a significant percentage of red blood cells leaving the bone marrow will have a low hemoglobin content. By measuring the percentage of red blood cells with hemoglobin content less than 28 g/dL, iron deficiency can be detected. A percentage of hypochromic cells greater than 10% has been correlated with iron deficiency, and hence has been used as a diagnostic criterion for detection of iron deficiency.

### **Programming**

In one embodiment, a physical memory containing instructions (i.e. “programming”) for performing the method described above is provided. In some embodiments, the memory can comprise a physical computer-readable medium comprising programming to calculate: a) the volume of individual red blood cells of the sample using a method described above: and/or b) the amount of hemoglobin in individual red blood cells of the sample a the method described above.

In one embodiment, data from the hematology analyzer is collected, and programming containing an algorithm for the calculation is executed, using inputs from an FCS file.

The programming can be provided in a physical storage or transmission medium. A computer receiving the instructions can then execute the algorithm and/or process data obtained from the subject method. Examples of storage media that are computer-readable include floppy disks, magnetic tape, CD-ROM, a hard disk drive, a ROM or integrated circuit, a magneto-optical disk, or a computer readable card such as a PCMCIA card and the like, whether or not such devices are internal or external to the computer. A file containing information can be “stored” on computer readable medium, where “storing” means recording information such that it is accessible and retrievable at a later date by a computer on a local or remote network.

The method described above can be automatically executed each time a sample is run.

15

### **EXAMPLES: ALGORITHM IMPLEMENTATION**

Methods for measuring parameters of individual red blood cells (RBCs) are described herein. Certain parameters that can be measured include the fraction of RBCs smaller than 60 fL (percent microcytic, %Micro), the fraction larger than 120 fL (percent macrocytic, %Macro), the fraction of RBCs with a cellular HGB concentration of less than 28 g/dL (percent hypochromic, %Hypo) and those higher than 41 g/dL (percent hyperchromic, %Hyper). Other parameters of interest include the width of the distribution of HGB concentration in RBCs (Hemoglobin Distribution Width, HDW), the mean amount of HGB in reticulocytes (Mean Corpuscular Hemoglobin for Reticulocytes, MCHr), the mean concentration of HGB in reticulocytes (Mean Corpuscular Hemoglobin Concentration for Reticulocytes, MCHCr), the mean volume of reticulocytes (Mean Corpuscular Volume for Reticulocytes, MCVr), and the fraction of reticulated platelets (percent reticulated platelets, %rPLT).

The RBC parameters were derived from optical cell-by-cell scatter data in ALL, IAS and PSS using optical cellular hemoglobin concentration (CHC) and optical volume models. The following examples describe in detail the implementation of these models.

#### **Data Event Filtering**

A Retic assay (which comprises collecting RBC, PLT, and Retic cellular events, as well as some WBC cellular events) was performed and ALL, IAS, PSS, and FL1 data were collected. The RBC algorithms described below used data from all of these sources. Figure 2 illustrates the log FL1 vs. linear IAS forms of the data from the Retic assay.

5           The Retic assay separates RBCs (red blood cells) and Retics (reticulocytes) from PLTs (platelets) and WBCs (white blood cells). The same classifications were used for the present example, but with a closer examination of the RBCs and Retics. As shown in Fig. 2, the RBCs presented a certain amount of coincidences (quantified around a few percent of the total RBC count), which should be eliminated before any calculations. Therefore, the  
10           highest point of the distributions of the population of RBCs and Retics was found in the three relevant detection channels (ALL, IAS and PSS), and then the boundaries of acceptable data were set at 5% of the histogram peak height. These thresholds served to eliminate noise (at the low end) and coincidence events (at the high end), and were derived from the data in a prior study. For removing coincidences and noise in the Retics, the top  
15           level IAS boundary previously derived was increased by 10 % because the Retics tend to be larger than the RBCs. Figure 3 shows the thresholds used to gate the RBCs and Retics. The horizontal line above the x axis is the 5% level relative to the maximum. The internal vertical lines are the RBC thresholds, which are placed at the first bins outside the 5% threshold. In this example, the lower thresholds for both RBCs and Retics were at channel  
20           380. The upper RBC threshold was at channel 630. Adding 10%, channel 693 was obtained, which was used as the upper threshold for the Retics (vertical line on right).

### **Models and Channel Scaling Schemes**

25           The way the channels were scaled is closely linked to the way the volume and CHC models were constructed. Figure 4 maps those relationships.

Referring to Fig. 4, there were variables, as well as functions, that were built from and apply to sets of individual cell-by-cell measurements (left column); and other variables and functions that were related to means of such sets (right column).

30           The quantities in the table of Fig. 4 are defined and discussed in the following passage. After applying the filtering boundaries as described in “Data Event Filtering” above, the mean values (in IAS, ALL and PSS) of the remaining RBCs and Retics were determined. These mean values were used to build the volume and CHC models, and were used to calibrate the channel scaling factors.

Since RBC parameters can be used on analyzers with different gain settings, the algorithm contains a mechanism to normalize and to scale the measurements from IAS, PSS, and ALL before they were used in the calculations. The optical normalization and scaling (on a cell-by-cell basis) was as follows:

5

$$\begin{aligned}
 al &= \left( \frac{\alpha \text{ all}}{\text{ALLC}} \right) - 1 \\
 ia &= \left( \frac{\text{median}(\beta) \text{ ias}}{\text{IASC}} \right) - 1 \\
 ps &= \left( \frac{\text{median}(\gamma) \text{ pss}}{\text{PSSC}} \right) - 1
 \end{aligned}$$

Here *al*, *ia*, and *ps* are the scaled and normalized scatter values and *all*, *ias*, and *pss* are the measured values of each cell on the respective detection channels ALL, IAS, and PSS. The measured values were first normalized by the constants ALLC, IASC, and PSSC, respectively. After normalization, they were multiplied by their respective channel scaling factor ( $\alpha$ ,  $\beta$ , and  $\gamma$ , respectively; for IAS and PSS the value used is a median from a history log, described below). The resulting value should be approximately 1. Then 1 is subtracted to have values that are distributed around 0 for a normal event.

15 The values of the normalization constants were ALLC = 13235.25, IASC = 6041.75, and PSSC = 11500; these values were derived from a training set of data, which was archived, and are not subject to change for analyzers with the same hardware and firmware configuration; they simply normalize the values to around 1.

20 The channel scaling factors, on the other hand (which are of order unity), were used to compensate for slight inter-analyzer differences and intra-analyzer drifts in optical performance. The analyzer kept a running log of the samples run on it; for IAS and PSS, the algorithm used the latest 51 valid samples to compute the channel scaling factor medians used above, while for ALL, the channel scaling factor was computed newly for each sample (based on impedance MCV data), without reference to old samples. See 25 "Standardization" below on how the scaling factors were derived and managed.

Because the RBC parameters were calculated from absolute channel values, it may be important to track the optical calibration of the instrument. The optically derived parameters were adjusted based on the reported impedance MCV and colorimetric HGB

results. The adjustment was implemented by changing channel scaling factors on a sample-by-sample basis.

The IAS and PSS channel scaling factors,  $\beta$  and  $\gamma$ , were calculated from the impedance and colorimetric MCHC measurements,  $mchc_{imp,col}$ :

5

$$mchc_{imp,col} = \frac{hgb_{col}}{rbc_{imp} * mcv_{imp}}$$

where  $hgb_{col}$  is the numerical value of HGB concentration from the colorimetric transducer,  $rbc_{imp}$  indicates the concentration of RBCs in the sample obtained from impedance data, and  $mcv_{imp}$  is the mean RBC volume from impedance data. Since MCHC exhibits a lower reproducibility and lower repeatability than other parameters (as, e.g., MCV), a simple linear relation was used to tie the IAS and PSS values to MCHC. The equations that were used on a sample-by-sample basis were:

15

$$\beta = IASC \left( \frac{48.5 - mchc_{imp,col}}{13.9 iasm} \right)$$

$$\gamma = PSSC \left( \frac{mchc_{imp,col} - 25.4}{7.1 pssm} \right)$$

The numerical constants in the above equations follow from a first order linear fit to data collected on four CELL-DYN Sapphires™ during the Feasibility Study. The  $iasm$  and  $pssm$  variables are the mean IAS and PSS values, respectively, for all RBCs in any particular sample. The IASC and PSSC constants were used as above to normalize the  $iasm$  and  $pssm$  variables so that the scaling factors were around unity.

The ALL channel scaling factor,  $\alpha$ , was calculated from the measured impedance MCV, the scaled mean IAS value and the ALL measurement. The equations were derived from the optical volume model (see "Volume Model" below):

25

$$\alpha = \frac{\sqrt{b^2 - 4ac} - b}{2a}$$

where

$$\begin{aligned}
 a &= 327.41 \text{ allnorm}^2 \\
 b &= (213.51 - 605.78 \text{ iam} - 2 * 327.41) \text{ allnorm} \\
 c &= 84.02 - 213.51 + (605.78 - 125.32) \text{ iam} \\
 &\quad + 260.64 \text{ iam}^2 + 327.41 \text{ allnorm} - \text{mcv}_{\text{imp}}
 \end{aligned}$$

and

$$\begin{aligned}
 \text{iam} &= \left( \frac{\beta \text{ iasm}}{\text{IASC}} \right) - 1 \\
 \text{allnorm} &= \frac{\text{allm}}{\text{ALLC}}
 \end{aligned}$$

where *iasm* and *allm* are the sample means of all the cell-by-cell IAS and ALL measurements, respectively, and *mcv<sub>imp</sub>* is the impedance MCV value.

Essentially, these formulae take the quadratic volume model dependent on mean ALL (*allm*) and IAS (*iasm*); insert the IAS scaling obtained through the MCHC linear fit above ( $\beta$ ); constrain the volume result to equal the impedance value (*mcv<sub>imp</sub>*); and solve for the ALL scaling factor required to satisfy the equation ( $\alpha$ ). Since the ALL channel scaling factor is tied to the impedance MCV, which exhibits good reproducibility and good repeatability, there is no need to take a median of the last 51 samples.

15

### **Volume Model**

The volume model is based on the measurement of each cell in IAS and ALL. The formula used to calculate the volume *vol* (on a cell-by-cell basis) is as follows:

$$\begin{aligned}
 \text{vol} &= 84.02 - 125.32 \text{ ia} + 213.51 \text{ al} - 605.78 \text{ ia} * \text{al} \\
 &\quad + 260.64 \text{ ia}^2 + 327.41 \text{ al}^2
 \end{aligned}$$

20

where *ia* and *al* are the cell-by-cell scaled measurements defined in “Models and Channel Scaling Schemes” above. The coefficients in this second-order global model (meaning an analytical model that applies to all the cells in the sample) were derived from the earlier study. To get the correct volume, the channel scaling factors for IAS and ALL were known (see equations in “Models and Channel Scaling Schemes” above). Figure 5 shows a graphical representation of the volume model. Figure 6 shows the 977 data points from a study plotted on the *iam* vs. *alm* (means of *ia* and *al*, respectively) plane. Each data point

25



represents the mean *al* and *ia* of all the cells of that particular sample; the various points comprise the entire set of samples from the study. The rectangle around the data points shows the extent in parameters for which the surface was calculated. The areas with the least data coverage lie along the *iam* boundaries. It is noted that the optical volume model was not used to determine %Micro and %Macro (which were extracted from the impedance MCV data), but only as a basis to calculate MCVr and MCHr and to plot individual RBCs in a *chc-vol* scattergram.

**Local CHC Model**

The cell-by-cell optical CHC model is based on the measurement of each cell in IAS and PSS. The model is based on a local fit. In order to implement the local model in the code, the model was sampled on a regular grid. The grid of numbers was imported into the software of the hematology analyzer used. The software finds all 4 nearest grid points, and does a linear interpolation between those 4 points.

The axes of the grid were normalized IAS and PSS measurements. The cell-by-cell IAS and PSS measurements were normalized and scaled as described already in “Models and Channel Scaling Schemes” above and reported below:

$$ia = \left( \frac{\text{median}(\beta) \text{ ias}}{\text{IASC}} \right) - 1$$

$$ps = \left( \frac{\text{median}(\gamma) \text{ pss}}{\text{PSSC}} \right) - 1$$

The IAS and PSS channel scaling factors  $\beta$  and  $\gamma$ , which are applied to all cells in the sample under test, were calculated using the median of the scaling factors obtained for the latest 51 samples. The model values at the grid points are shown in Fig. 7.

If the *ia* and *ps* values were outside of the range for which the CHC model was defined, the closest edge value of the model was used. This was supported by the analysis presented below.

Figure 8 shows the 977 data points, from the same study used in the volume model, which were used to derive the surface model of Fig. 7, plotted on the *psm vs. iam* (mean of *ps* and *ia*, respectively). As in Fig. 6, each data point represents the mean *ps* and *ia* of all the cells of that particular sample; the various points comprise the entire set of samples from the study. The rectangle around the data points shows the extent in parameters for

which the surface has been calculated. The areas with the least data coverage lie along the *iam* boundaries.

Figure 9 shows a graph with the percentage of cells falling outside of the model boundaries for each of the 977 samples from the study. The maximum was around 6%.

5 Figure 10 shows the same results plotted against the optically derived MCHC value of the sample.

Figure 11 is a *ps vs. ia* plot (i.e., a plot, for a single sample, of the scaled and normalized values of PSS and IAS of all the individual cells in the sample; contrast with the plots in Figs. 6 and 8, which plot sample means) for the sample with 6% of events  
10 outside of the model boundary. This particular sample had a much broader PSS distribution than was usually observed. For comparison, Fig. 12 shows the distribution pattern for a typical sample with an MCHC value of about 32 g/dL.

Those relatively few events (in those relatively very few samples) that were outside of the model surface were mostly leaving the surface on the high- *ps* boundary. Looking at  
15 the shape of the model surface in Fig. 7, the gradient in *ps* along the high- *ps* boundary of the surface was generally small (compared to the overall CHC variation across the entire model surface). Based on these results, it was acceptable to use the values of the model on the high- *ps* boundary for those events that extend beyond it. This approach, further  
generalized to the other boundaries, was implemented in the algorithm.

20

### Calculated Parameters

With optical cell-by-cell models for CHC and volume for each RBC and Retic event, the desired parameters can be calculated.

25 **HDW:** The robust standard deviation (*rSD*) of the CHC distribution was calculated for all Retics and RBCs. The *rSD* was calculated as

$$rSD = 1.4826 \text{ median}(chc - \text{median}(chc))$$

The factor of 1.4826 ensured that for normally distributed data the *rSD* and the normal *SD*  
30 were identical. The HDW was given as a percentage by dividing the *rSD* by the median and multiplying by 100.

**%Hypo, %Hyper:** All RBCs and Retics were counted with *chc* < 28 g/dl (%Hypo) and *chc* > 41 g/dl (%Hyper). The count was divided by the total number of RBCs and Retics and multiplied by 100.

**MCHCr:** Only the CHC values (*chc*) of the Retics were used and their mean calculated.

**MCVr:** Only the volume (*vol*) of the Retics was used and their mean calculated.

**MCHr:** The product of *chc* and *vol* for each Retic were determined, their mean  
5 calculated and divided by 100.

### **%Micro and %Macro**

The %Micro and %Macro parameters were measured from the impedance histogram. The %Micro was the fraction of RBCs that were smaller than 60 fL. The  
10 %Macro was the fraction of RBCs that were larger than 120 fL. RBC coincidences were removed and the numbers of the cells above (%Macro) and below (%Micro) the limits were summed up, respectively.

Removing the coincidences was important for samples which had a low number of %Macro events. The impedance count typically had 3% coincidences, which were recorded  
15 at a volume of roughly twice the MCV value. So for an MCV of 80 fL, it was expected to see 3% of the RBC events as coincidences around a volume of 160 fL. In order to give a good estimate of the %Macro, those 3% coincidences should be eliminated because they would otherwise be counted as macrocytic. The algorithm looked for a valley in the volume histogram and limited the valid RBC events to those to the left (i.e., with volumes  
20 smaller than that) of the bottom of the valley. Accordingly, coincidence events were generally correctly excluded from the calculation of macrocytic RBCs.

### **Reticulated Platelets**

Also provided is a method of enumerating reticulated platelets (rPLT). In general  
25 terms, the method relies on the same principle used for the reticulated RBC assay, whereby a suitable cell-permeable nucleic-acid dye or nucleic-acid stain binds to the RNA in the reticulocytes and makes them distinguishable from mature RBCs, in which RNA is absent. Reticulated PLTs also present a nonzero RNA content, which sets them apart from mature PLTs. The nucleic-acid dye or stain confers to reticulated PLTs similar signal-  
30 differentiation characteristics as it does to reticulated RBCs; for example, in the case of a fluorescent-conjugated cell-permeable nucleic-acid dye, reticulated RBCs typically exhibit higher fluorescence than mature RBCs, and reticulated PLTs typically exhibit higher fluorescence than mature PLTs. This elevation of fluorescence can be exploited by suitably designed algorithms.

An algorithm with the best straight-line fit through the platelet population (in  $\log fll$  vs.  $\log ias$ ) was used. Another line with the same slope and a positive offset of 30 channels was drawn. The number 30 was previously determined using a training set so that a set of normal samples had an average %rPLT of about 2%, which is typical in healthy subjects. Any platelet event that was above the second line was classified as a reticulated PLT. Figure 13 illustrates the algorithm for classifying rPLTs in a normal sample. The platelets are shown in blue, and the dash-dotted line through the population is the best-fit line. The dashed line is the line offset by 30 and all rPLT events above it are circled. In this particular sample the rPLT percentage was 1.0%.

In case there were less than 150 platelets in the FCS file, a default slope of 0.5 was used, which was only fit for the offset of the line through the platelet population.

### **Standardization**

The gain settings for ALL and PSS in the Retic assay were set according to the following procedure.

The gains for the WBC differential assay were first standardized. A value of 20V was subtracted from the PSS PMT voltage setting for the WBC differential assay. The resulting value was used as the PSS PMT voltage setting for the Retic assay (e.g.:  $V_{PMT_{PSS,WBC/diff}} = 387V$ ;  $V_{PMT_{PSS,Retic}} = 387V - 20V = 367V$ ). The PSS amplifier gain settings were copied from the WBC differential assay to the Retic assay.

The ALL pre-amplifier gain setting for the WBC differential assay was multiplied by 2, and used as the ALL pre-amplifier gain setting for the Retic assay (e.g.:  $preampgain_{ALL,WBC/diff} = 16$ ;  $preampgain_{ALL,Retic} = 32$ ). The ALL amplifier gain setting was copied from the WBC differential assay to the Retic assay.

After the voltages and gains were set, 3.3- $\mu$ m standard reference particles (SRPs) at a concentration of  $250 \times 10^6/mL$  were used to establish the initial channel scaling factors. The SRPs were run in the Retic RBC Reference SRP mode.

The algorithm used the means of the SRPs in the ALL, IAS and PSS detection channels to obtain the initial channel scaling factors. The initial channel scaling factors were calculated as

$$\alpha = \frac{4893}{\text{mean}(all_{SRP})}$$

$$\beta = \frac{2328}{\text{mean}(ias_{SRP})}$$

$$\gamma = \frac{31311}{\text{mean}(pss_{SRP})}$$

where  $\alpha$ ,  $\beta$ , and  $\gamma$ , the channel scaling factors, again represent the ALL, IAS, and PSS channel scaling factors, respectively. The mean channels here were represented at full resolution (15 bit). The initial scaling factors were stored in a configuration file. For each sample, a new determination of the scaling factors was obtained, following the procedure detailed in “Models and Channel Scaling Schemes” above. The 51 most recent estimates of the scaling factors were stored in the configuration file.

10 **Deconvolution with instrument profile**

From the impedance transducer, a measurement of  $RDW_{imp}$  (the width of the RBC volume distribution histogram) was obtained and compared with the optically-derived  $RDW_{opt}$  from the cell-by-cell scattering measurements to get an estimate of how much instrument-dependent degradation there might be in the optical RDW measurement.

15 An empirical model was constructed relating the ratio of  $RDW_{opt}/RDW_{imp}$  to the instrument profiles in ALL and IAS. The model allowed the deconvolution of the optical degradation out of the IAS and ALL signals.

The implemented model assumed the instrument profile as well as the sample profile to be Gaussian. Under this assumption the deconvolution was performed by reducing the IAS and ALL distributions widths around their respective means. Each was multiplied by the ratio of the desired to existing total distribution width.

20 The instrument distribution width was estimated by the ratio of the measured optical distribution width of the RBC volumes to the impedance distribution width (RDW).

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes can be made and equivalents can be substituted without departing from the true spirit and scope of the invention. In addition, many modifications can be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A hematology analyzer comprising:
  - a) a flow cell;
  - 5 b) a light source for directing light to said flow cell;
  - c) a plurality of detectors for detecting light scattering by cells on a plurality of optical scattering channels;
  - d) a colorimetric transducer for measuring hemoglobin concentration in a cell lysate;
  - 10 e) an impedance transducer for measuring changes in impedance; and
  - f) a data analysis workstation comprising programming to calculate the volume or hemoglobin content of an individual red blood cell in a sample containing a population of red blood cells using:
    - i. measurements of said individual red blood cells on a plurality of said
    - 15 optical scattering channels;
    - ii. the mean cell volume of said population of red blood cells calculated using impedance values,
    - iii. the bulk hemoglobin concentration of said sample;
    - iv. the number of cells in said population of red blood cells;
    - 20 v. the mean values of the measurements of said population of red blood cells on said plurality of said optical scattering channels; and
    - vi. the median-of-means value of prior populations of red blood cells analyzed on at least one of said optical scattering channels on said hematology analyzer.
- 25 2. The hematology analyzer of claim 1, wherein said data analysis workstation comprises programming to calculate the volume of said individual red blood cell in said sample using:
  - i. measurements of said individual red blood cells on ALL and IAS
  - 30 channels;
  - ii. the mean cell volume of said population of red blood cells calculated using impedance values,

iii. the mean cellular hemoglobin concentration of said population, calculated using said mean cell volume, said bulk hemoglobin concentration, and said number of cells in said population;

5 v. the mean values of the measurements of said population of red blood cells on ALL and IAS channels; and

vi. the median-of-means IAS value for prior populations of red blood cells analyzed on said IAS channel on said hematology analyzer.

3. The hematology analyzer of claim 2, wherein said data analysis workstation  
10 comprises programming to scale said ALL and IAS measurements prior to calculating said volume, wherein:

a) said IAS measurement is scaled to provide a scaled IAS value using:

15 i. said mean cellular hemoglobin concentration of said population of red blood cells;

ii. the mean value of said measurements of said population of red blood cells on said IAS channel;

iii. the median-of-means IAS value obtained from prior samples run on said hematology analyzer; and

b) said ALL measurement is scaled to provide a scaled ALL measurement using:

20 i. said scaled IAS value; and

ii. said mean cell volume of said population of red blood cells calculated using impedance values; and

25 iii. the mean ALL value of said measurements of said population of red blood cells in said ALL channel..

4. The hematology analyzer of claim 1, wherein said data analysis workstation  
comprises programming to calculate the hemoglobin concentration of said individual red  
blood cell in said sample using:

30 i. measurements of said individual red blood cells on IAS and PSS channels;

ii. the mean cellular hemoglobin concentration of said population, calculated using said mean cell volume based on impedance values, said bulk hemoglobin concentration, and said number of cells in said population;



iii. the mean values of the measurements of said population of red blood cells on the IAS and PSS channels; and

iv. the median-of-means IAS and PSS values for prior populations of red blood cells analyzed on said IAS and PSS channels on said hematology analyzer.

5

5. The hematology analyzer of claim 4, wherein said data analysis workstation comprises programming to scale said IAS and PSS measurements prior to calculating said hemoglobin concentration, wherein:

a) said IAS measurement is scaled to provide a scaled IAS value using:

10 i. said mean cellular hemoglobin concentration of said population of red blood cells;

ii. the mean IAS value of said measurements of said population of red blood cells on said IAS channel;

15 iii. the median-of-means IAS value obtained from prior samples run on said hematology analyzer; and

b) said PSS measurement is scaled to provide a scaled PSS value using:

i. said mean cellular hemoglobin concentration of said population of red blood cells;

20 ii. the mean PSS value of said measurements of said population of red blood cells on said PSS channel;

iii. the median-of-means PSS value obtained from prior samples run on said hematology analyzer.

6. The hematology analyzer of claim 1, wherein said programming comprises instructions for filtering said measurements to remove data points produced by noise and coincidence.

7. The hematology analyzer of claim 1, wherein said programming comprises instructions for normalizing said measurements, wherein said normalizing comprises adjusting said measurements relative to a constant.

8. The hematology analyzer of claim 1, wherein said programming further comprises instructions for calculating a portion of red blood cells having a defined characteristic.

9. The hematology analyzer of claim 8, wherein said calculating comprises calculating a percentage of red blood cells having a volume above or below a defined volume.
10. The hematology analyzer of claim 8, wherein said calculating comprises calculating a percentage of cells having a hemoglobin concentration above or below a defined concentration.
11. The hematology analyzer of claim 1, wherein said programming further comprises instructions for calculating the width of the distribution of hemoglobin concentration in said red blood cells.
12. The hematology analyzer of claim 1, wherein said programming further comprises instructions for identifying reticulocytes in said sample.
13. The hematology analyzer of claim 12, further comprising calculating the mean amount of hemoglobin in said reticulocytes, the mean concentration of hemoglobin in said reticulocytes, or the mean volume of said reticulocytes.
14. The hematology analyzer of claim 1, further comprising instructions for calculating the fraction of reticulated platelets in said sample.
15. A method of sample analysis comprising:  
obtaining a set of data points for a sample comprising a population of red blood cells using a hematology analyzer; and  
calculating the volume or hemoglobin content of an individual red blood cell in said sample containing using:  
i. measurements of said individual red blood cells on a plurality of a optical scattering channels;  
ii. the mean cell volume of said population of red blood cells calculated using impedance values,  
iii. the bulk hemoglobin concentration of said sample;  
iv. the number of cells in said population of red blood cells;  
v. the mean values of the measurements of said population of red blood cells on said plurality of said optical scattering channels; and

vi. the median-of-means value of prior populations of red blood cells analyzed on at least one of said optical scattering channels on said hematology analyzer.

5 16. The method of claim 15, wherein said volume of said individual red blood cell in said sample is calculated using:

i. measurements of said individual red blood cells on ALL and IAS channels;

10 ii. the mean cell volume of said population of red blood cells calculated using impedance values,

iii. the mean cellular hemoglobin concentration of said population, calculated using said mean cell volume, said bulk hemoglobin concentration, and said number of cells in said population;

15 v. the mean values of the measurements of said population of red blood cells on ALL and IAS channels; and

vi. the median-of-means IAS value for prior populations of red blood cells analyzed on said IAS channel on said hematology analyzer.

17. The method of claim 15, wherein said hemoglobin content of said individual red blood cell in said sample is calculated using:

i. measurements of said individual red blood cells on IAS and PSS channels;

20 ii. the mean cellular hemoglobin concentration of said population, calculated using said mean cell volume based on impedance values, said bulk hemoglobin concentration, and said number of cells in said population;

25 iii. the mean values of the measurements of said population of red blood cells on IAS and PSS channels; and

iv. the median-of means IAS and PSS values for prior populations of red blood cells analyzed on said IAS and PSS channels on said hematology analyzer.

30 18. The method of claim 15, wherein said calculating employs a simple quadratic formula that is empirically derived from a training set of samples.

19. The method of claim 15, further comprising calculating a percentage of red blood cells having defined characteristics.

20. A physical computer-readable medium for use in a hematology analyzer to analyze a sample, comprising:
- programming to calculate the volume or hemoglobin content of an individual red
- 5 blood cell in a sample containing a population of red blood cells using:
- i. measurements of said individual red blood cell on a plurality of said optical scattering channels;
  - ii. the mean cell volume of said population of red blood cells calculated using impedance values;
  - 10 iii. the bulk hemoglobin concentration of said sample;
  - iv. the number of cells in said population of red blood cells;
  - v. the mean values of the measurements of said population of red blood cells on said plurality of said optical scattering channels; and
  - 15 vi. the median-of-means value for prior populations of red blood cells analyzed on said optical scattering channels on said hematology analyzer.

20

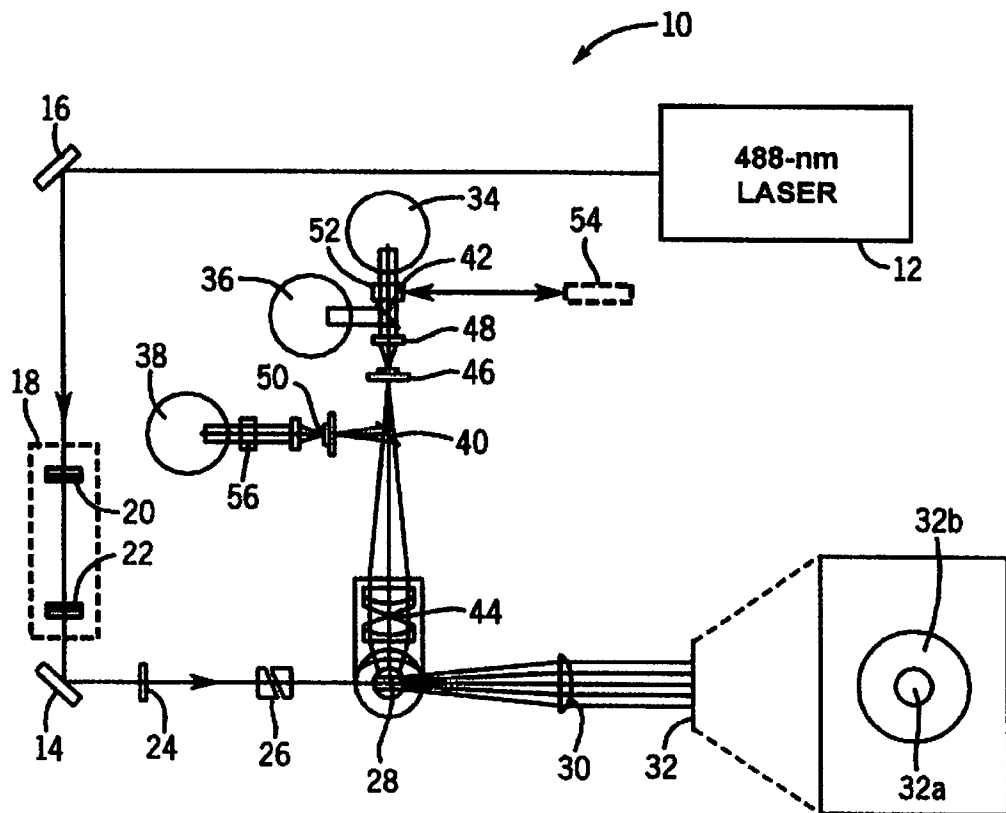
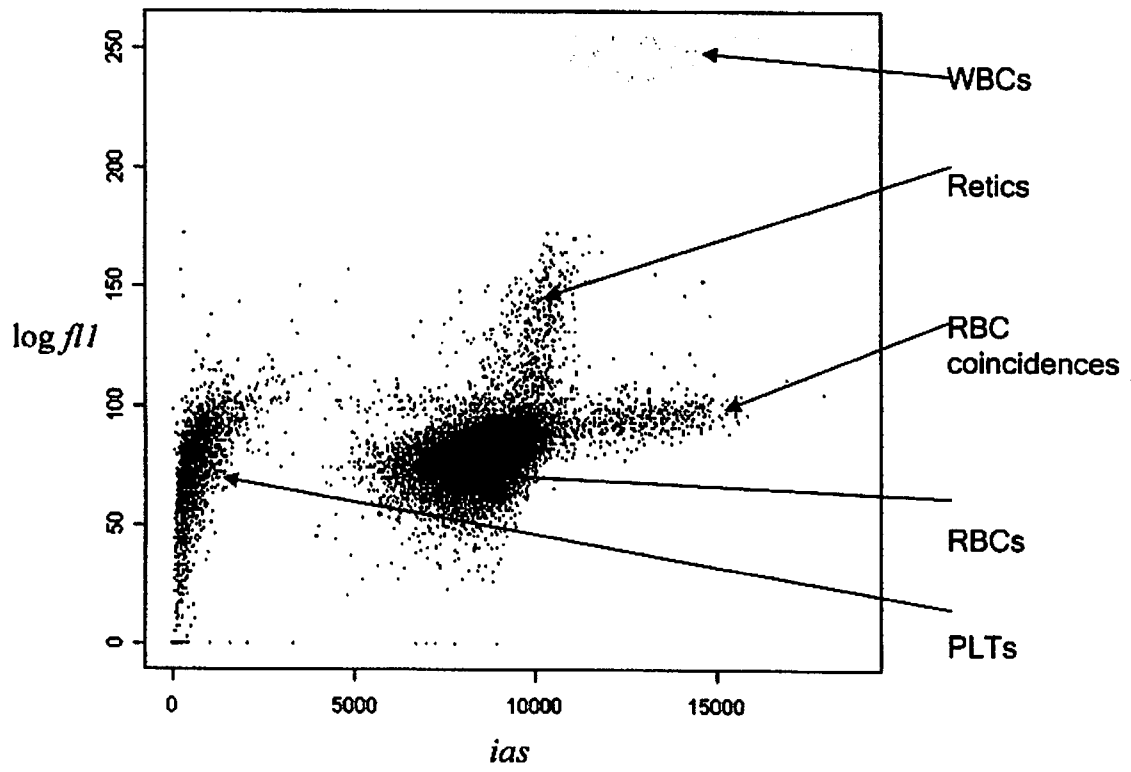
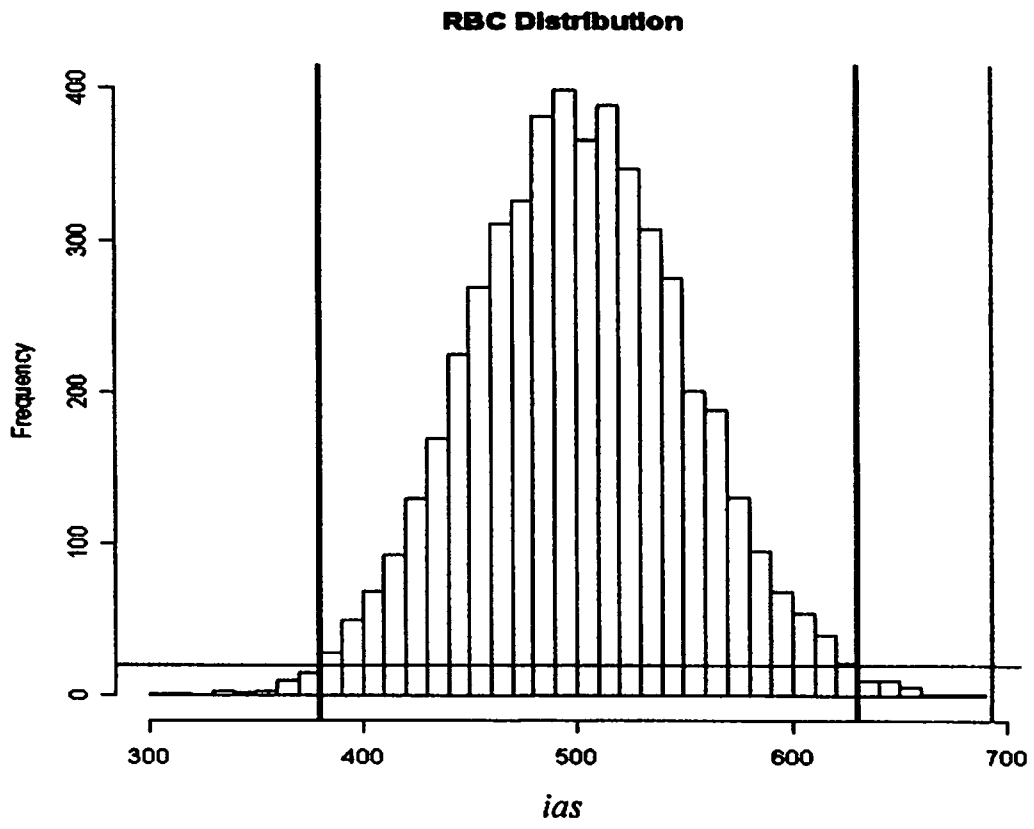


Fig. 1



**Fig. 2**

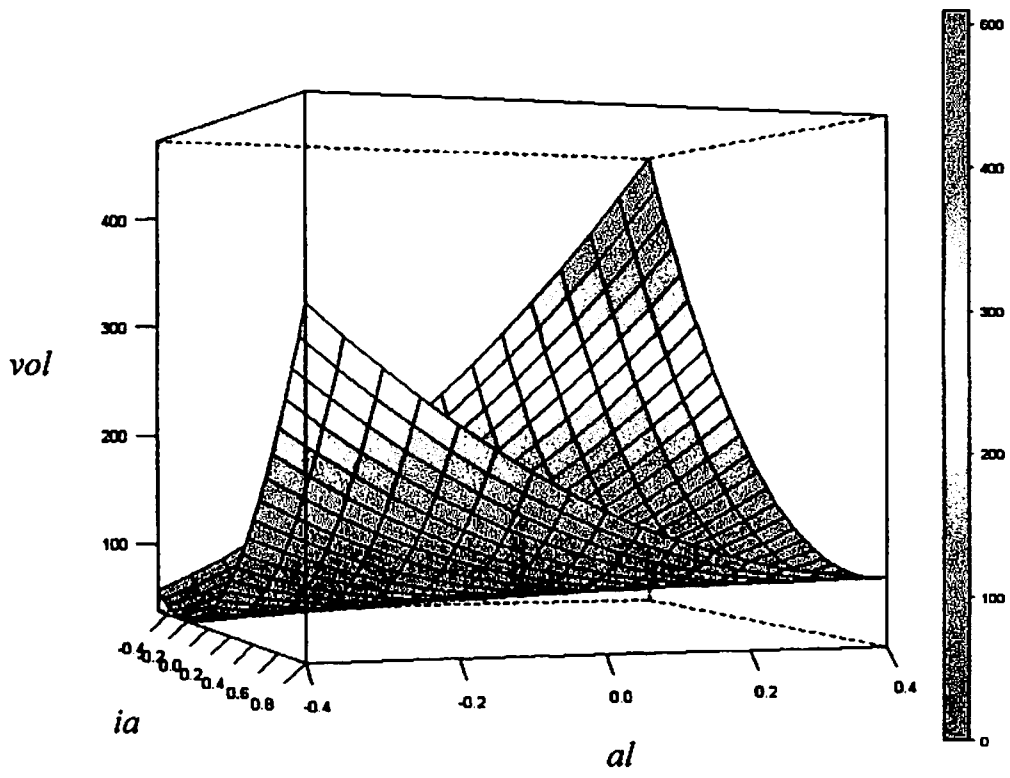


**Fig. 3**

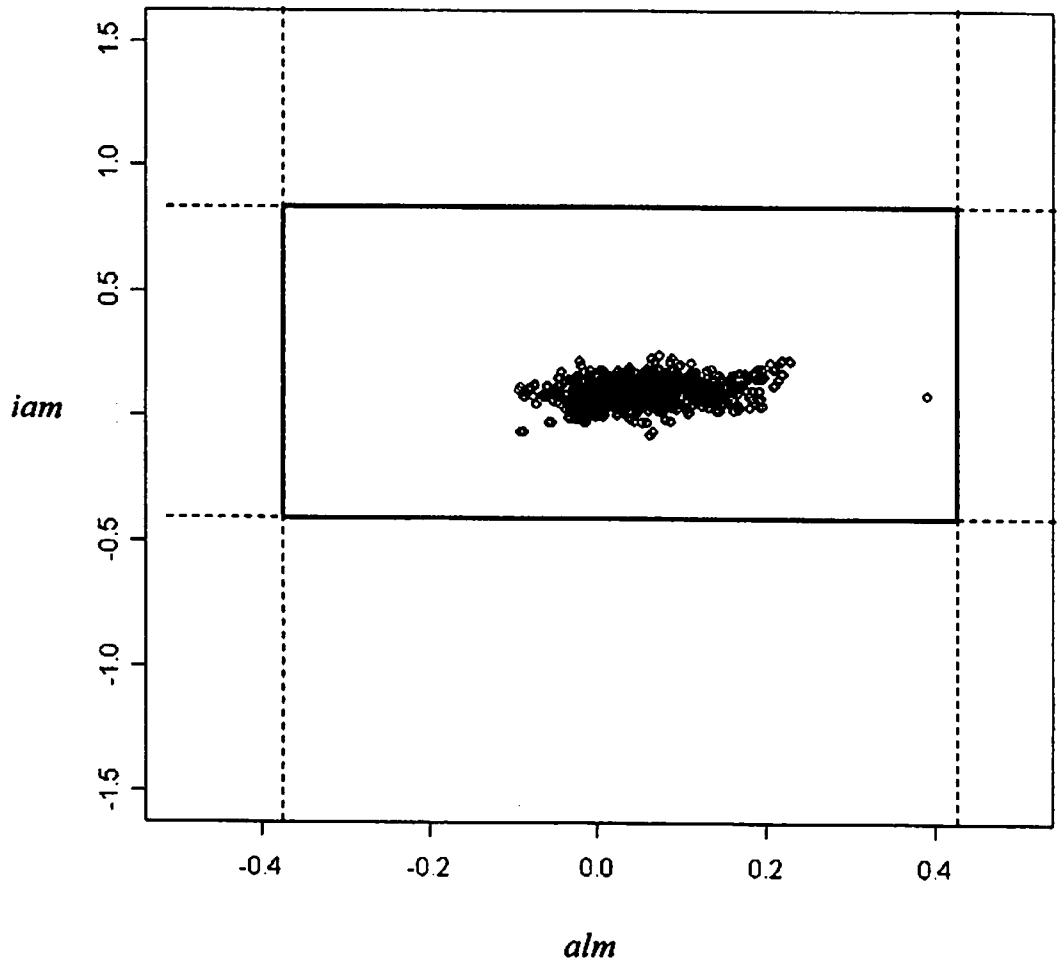
	cell-by-cell measurements	sample averages
<b>volume model</b>	$vol = f_1(all, ias)$ find $f_1...$ impedance	$mcv_{opt}$ $mcv_{imp}$ ... such that optical MCV and impedance MCV are equivalent
<b>CHC model</b>	$chc = f_2(ias, pss)$ find $f_2...$ impedance colorimetry	$mchc_{opt}$ $mchc_{imp,col}$ ... such that optical MCHC and impeded/color. MCHC correlate
<b>IAS channel scaling</b>	$ias$ $ia = \frac{\langle \beta \rangle ias}{IASC} - 1$	$iasm$ find $\beta$ from linear MCHC fit $\beta = \frac{IASC}{iasm} f_3(mchc)$
<b>PSS channel scaling</b>	$pss$ $ps = \frac{\langle \gamma \rangle pss}{PSSC} - 1$	$pssm$ find $\gamma$ from linear MCHC fit $\gamma = \frac{PSSC}{pssm} f_4(mchc)$
<b>ALL channel scaling</b>	$all$ $al = \frac{\alpha all}{ALLC} - 1$	$allm$ $mcv_{opt} = f_1(\alpha allm, \beta iasm)$ adjust $\alpha...$ ... so optical MCV equals impedance MCV $mcv_{opt} = mcv_{imp}$

Fig. 4





**Fig. 5**



**Fig. 6**

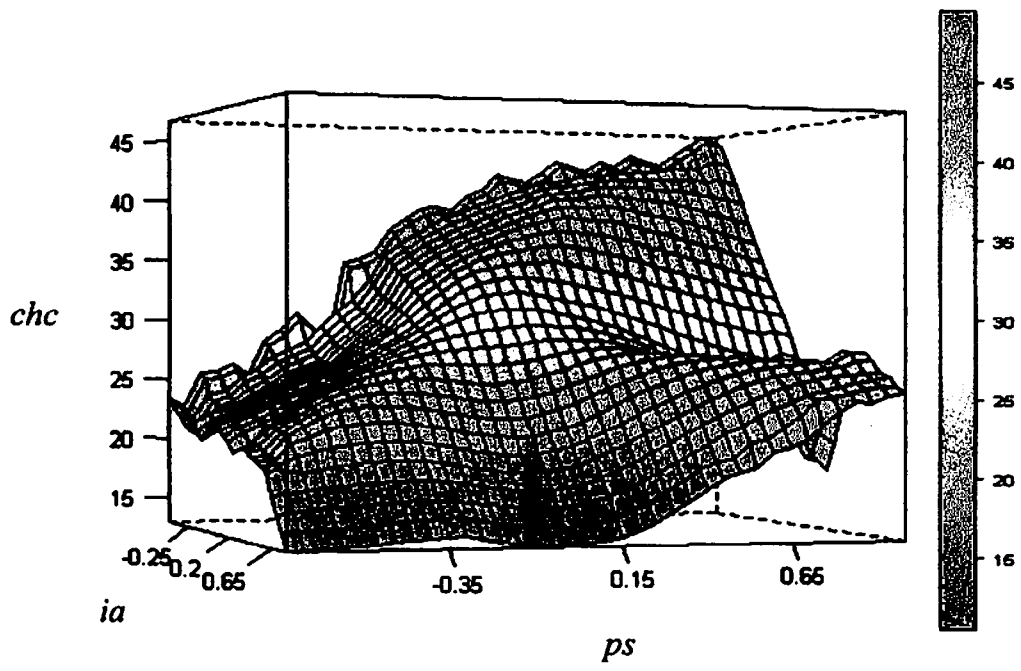
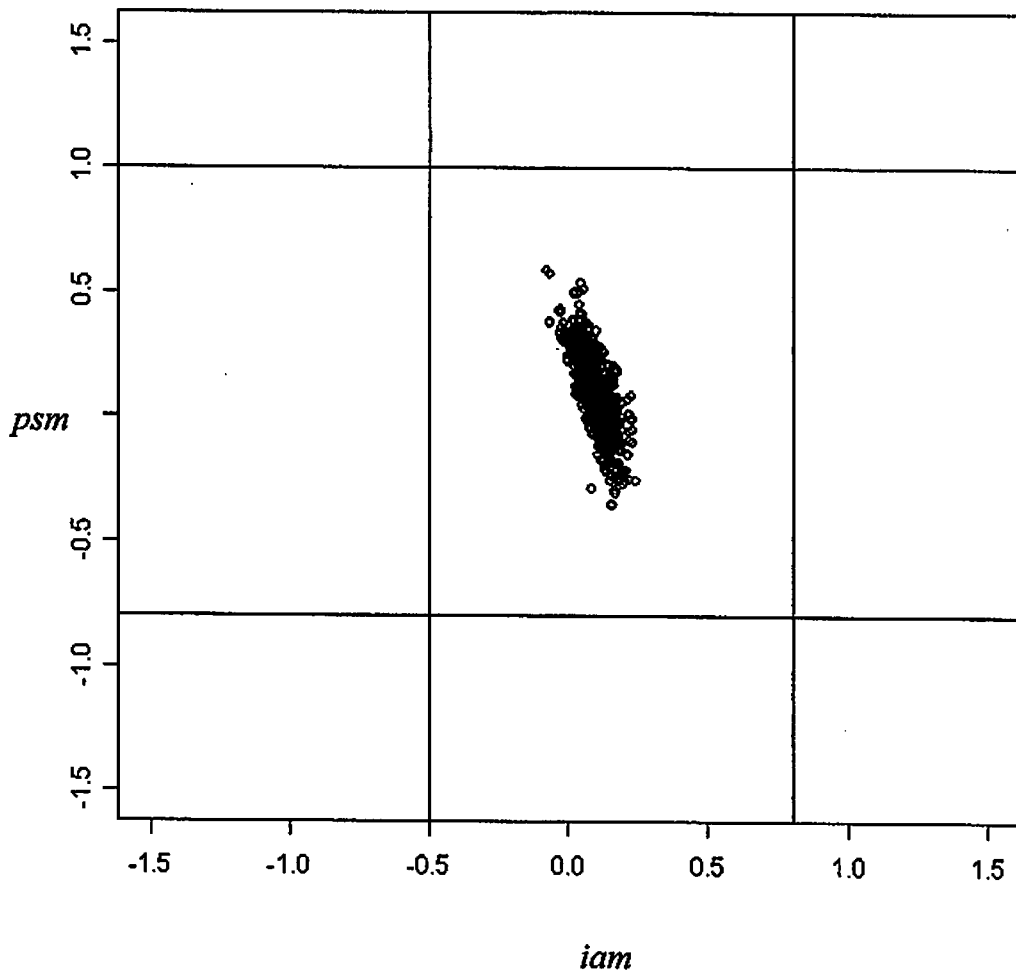
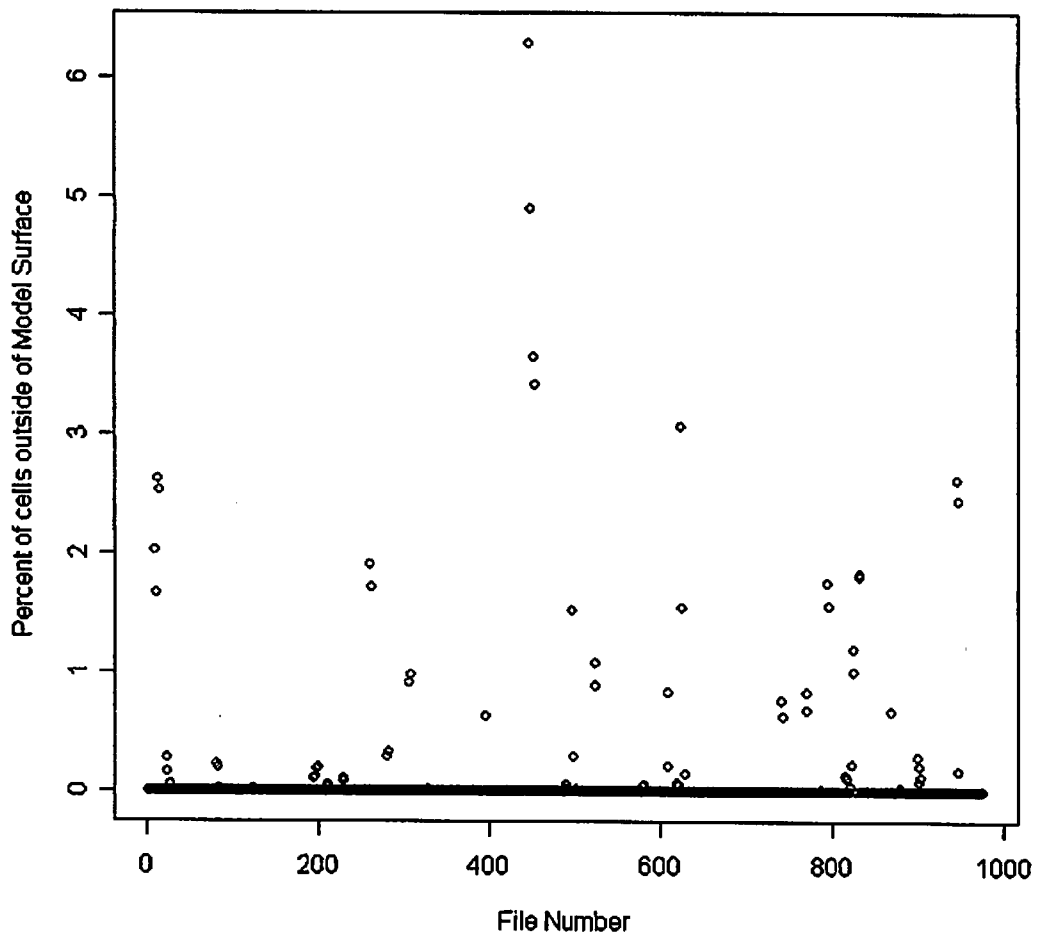


Fig. 7



**Fig. 8**



**Fig. 9**

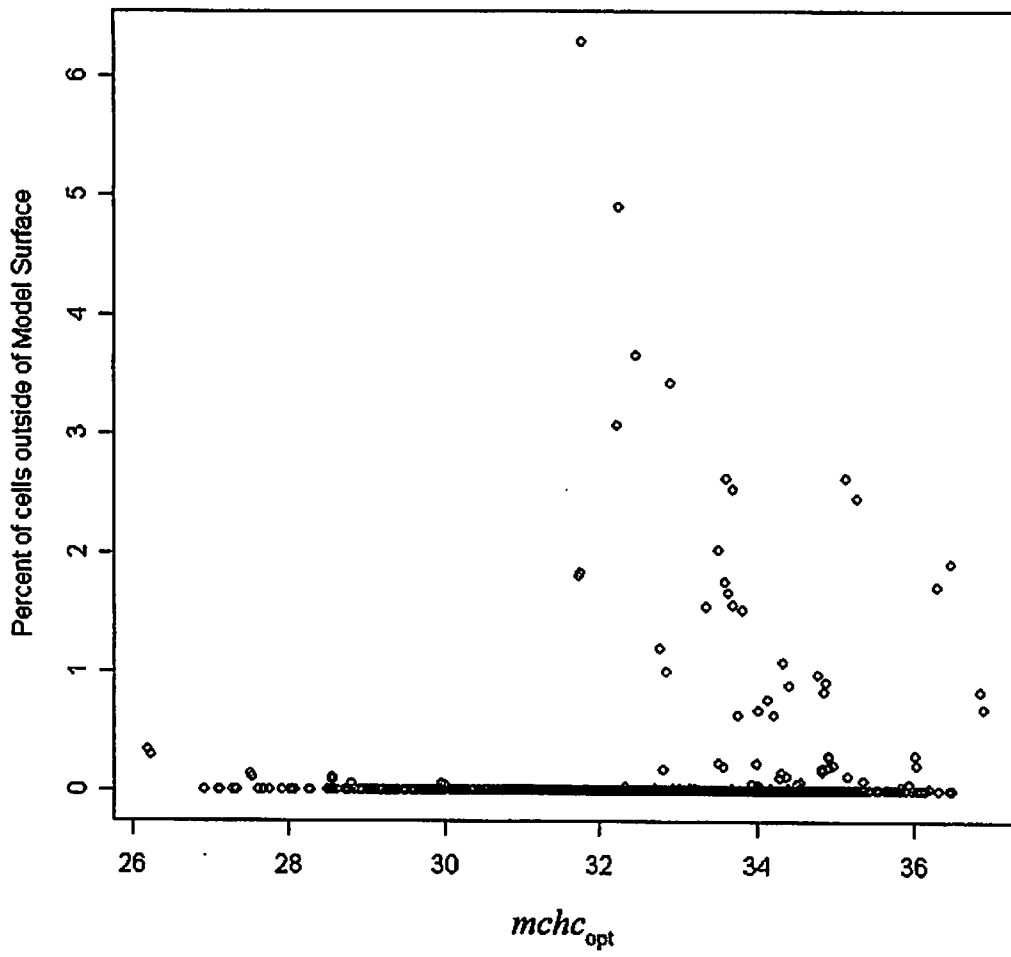
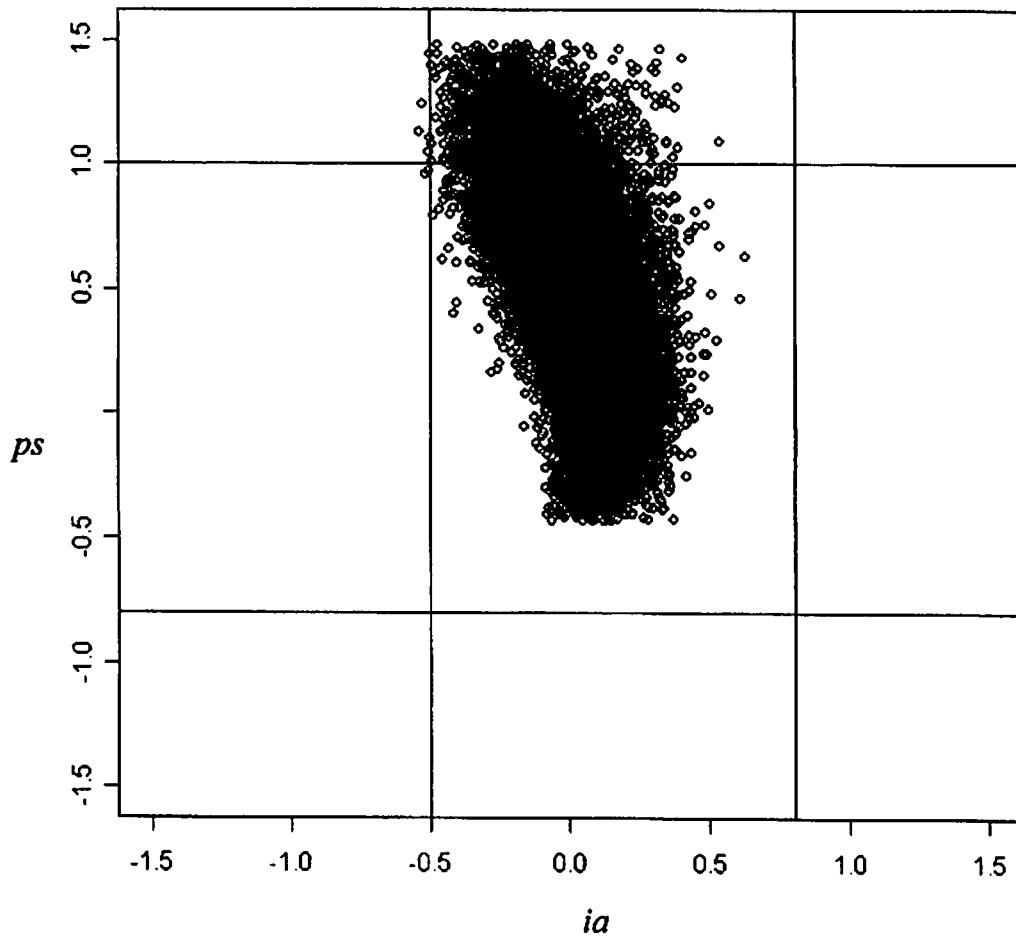
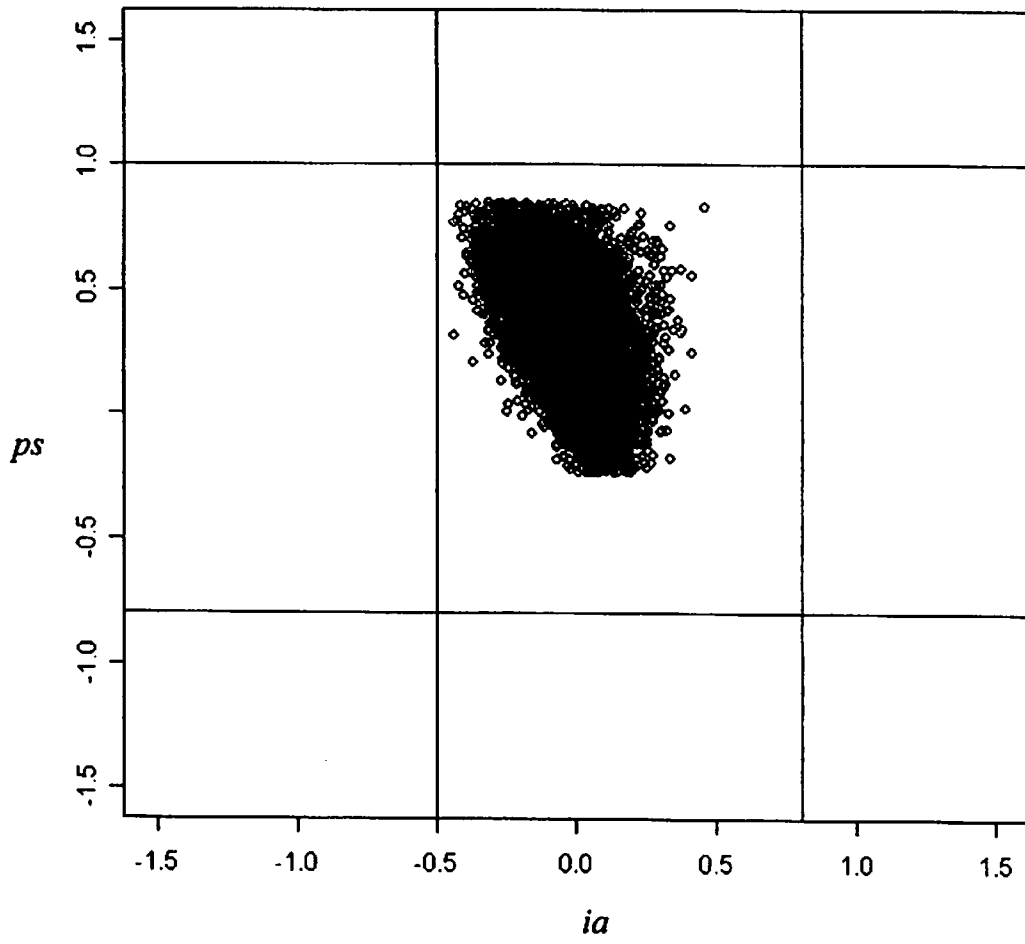


Fig. 10

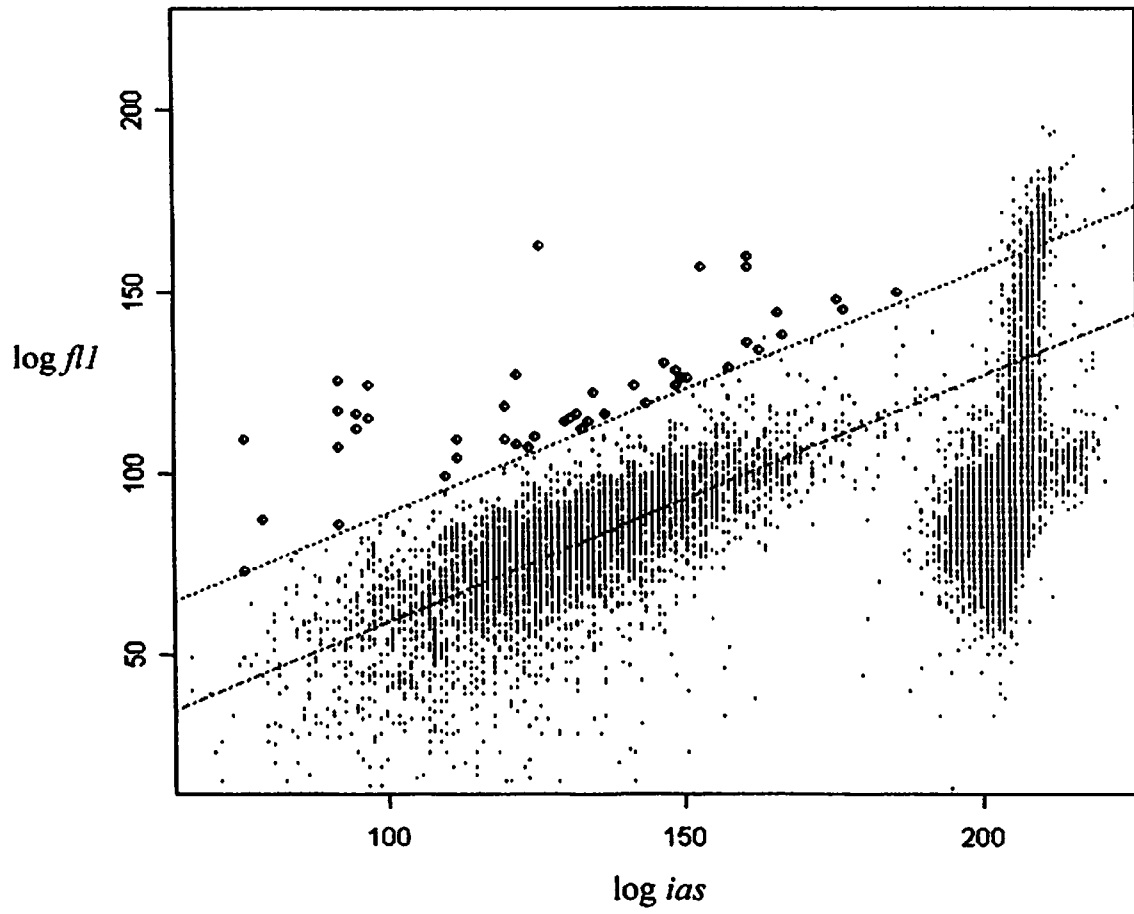


**Fig. 11**



**Fig. 12**





**Fig. 13**

INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2011/021292

A. CLASSIFICATION OF SUBJECT MATTER  
INV. G01N33/49 G01N33/72 G01N15/14  
ADD.  
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED  
Minimum documentation searched (classification system followed by classification symbols)  
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 practical, search terms used)  
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"ABSTRACTS OF THE XXIST INTERNATIONAL SYMPOSIUM ON TECHNOLOGICAL INNOVATIONS IN LABORATORY HEMATOLOGY", INTERNATIONAL JOURNAL OF LABORATORY HEMATOLOGY, vol. 30, no. Suppl. 1, June 2008 (2008-06), page 58,66,67, XP002632192, page 66, paragraph 119 - page 67 -----	1-3,6-9, 12-16, 18-20
X	WO 02/099380 A2 (ABBOTT LAB [US]) 12 December 2002 (2002-12-12)  page 6, line 8 - page 7, line 5 page 9, line 18 - page 16, line 24 ----- -/--	1-3,6-9, 12-16, 18-20

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  
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 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
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 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.  
 "&" document member of the same patent family

Date of the actual completion of the international search  14 April 2011	Date of mailing of the international search report  04/05/2011
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Lazar, Zala

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/US2011/021292

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN HOVE ET AL: "Anemia Diagnosis, Classification, and Monitoring Using Cell-yn Technology reviewed for the New Millenium", LABORATORY HEMATOLOGY, vol. 6, 2000, pages 93-108, XP002632223, page 95, right-hand column, paragraph 1 - page 97, right-hand column, paragraph 1 -----	1-3,6-9, 12-16, 18-20

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2011/021292

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		CA 2449401 A1	12-12-2002
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		JP 4184258 B2	19-11-2008
		JP 2004535570 T	25-11-2004
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		US 2003025896 A1	06-02-2003
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