United States Patent

Adams et al.

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[54] **METHOD FOR ANALYSIS OF BLOOD BY OPTICAL ANALYSIS OF LIVING CELLS**

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[51] **Int. Cl.**...........................**G01n 1/30,** G01n 33/16

[58] **Field of Search**......356/36, 39; 250/83.3 UV, 71

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[57] **ABSTRACT**

Dye composition for differential blood analysis of living white cells including an aqueous solution of acridine orange, and having a pH factor and osmolality within normal physiological ranges for human blood. The white cell analysis is made by subjecting a suspension of fresh blood in the above solution to radiation from a blue laser, distinguishing the white cells from other blood particles by detecting green fluorescence emitted from the white cells in response to the laser radiation, and measuring the magnitudes of red fluorescence emitted from individual white cells in response to the laser radiation.

25 Claims, 4 Drawing Figures

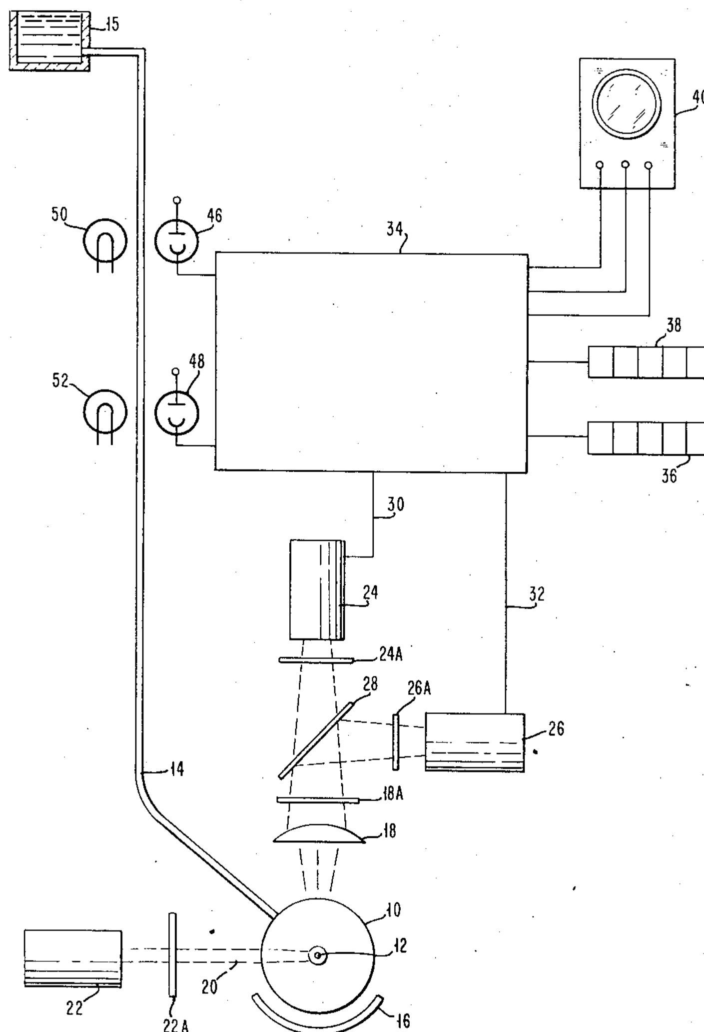


FIG. 1

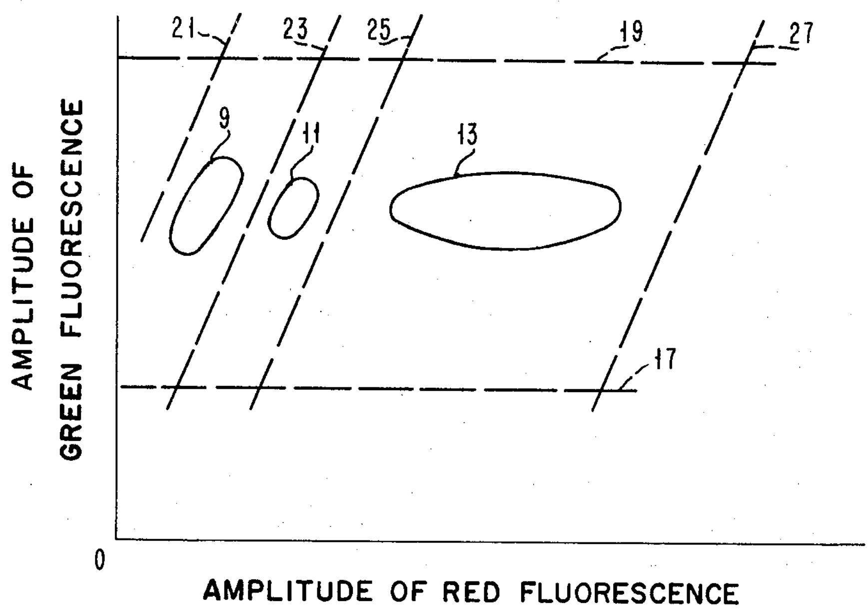
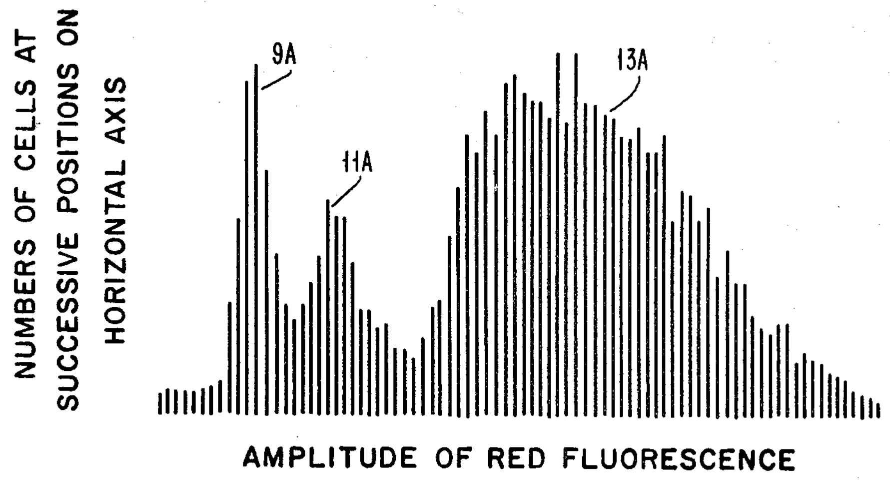


FIG. 2



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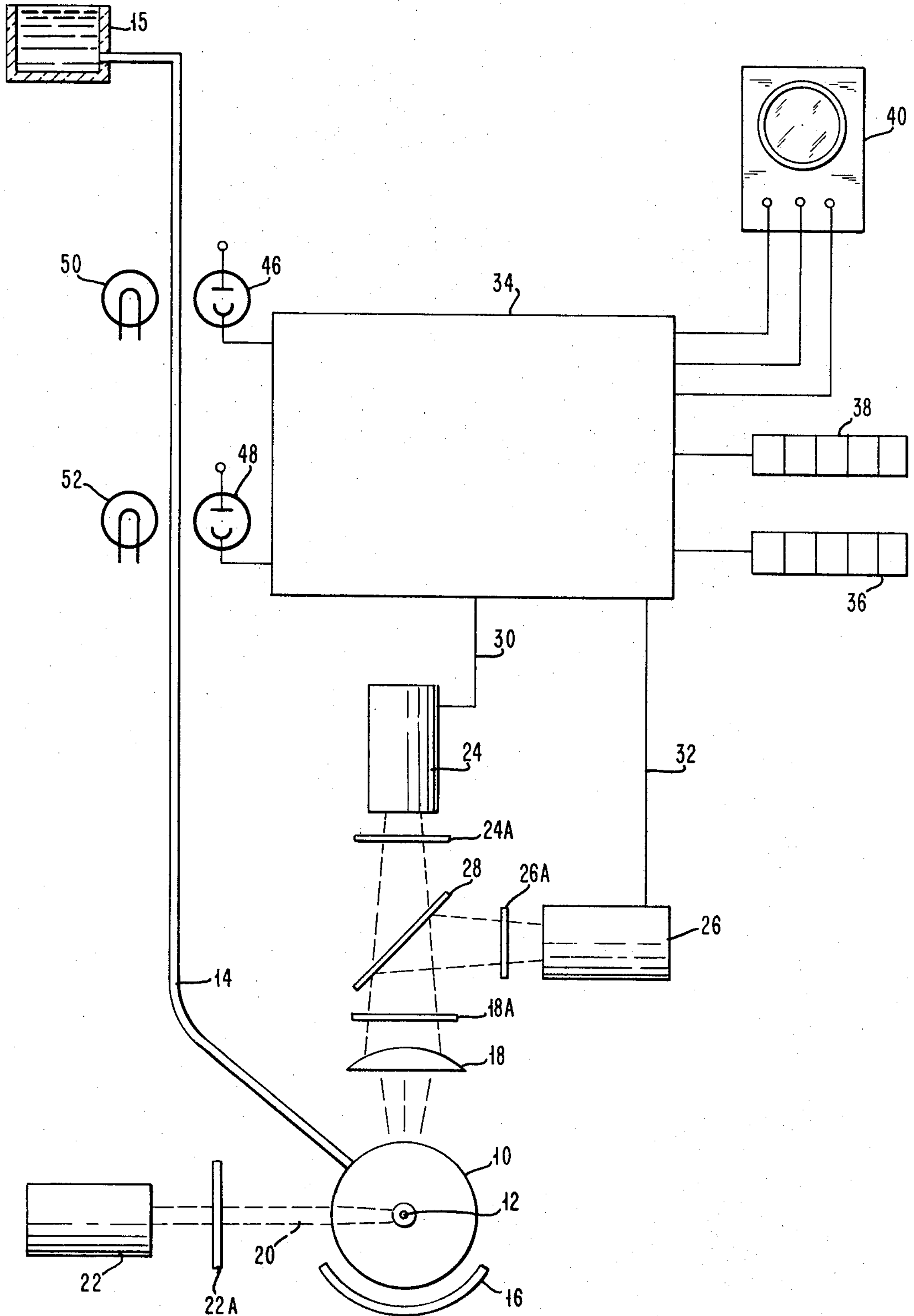
FIG. 3
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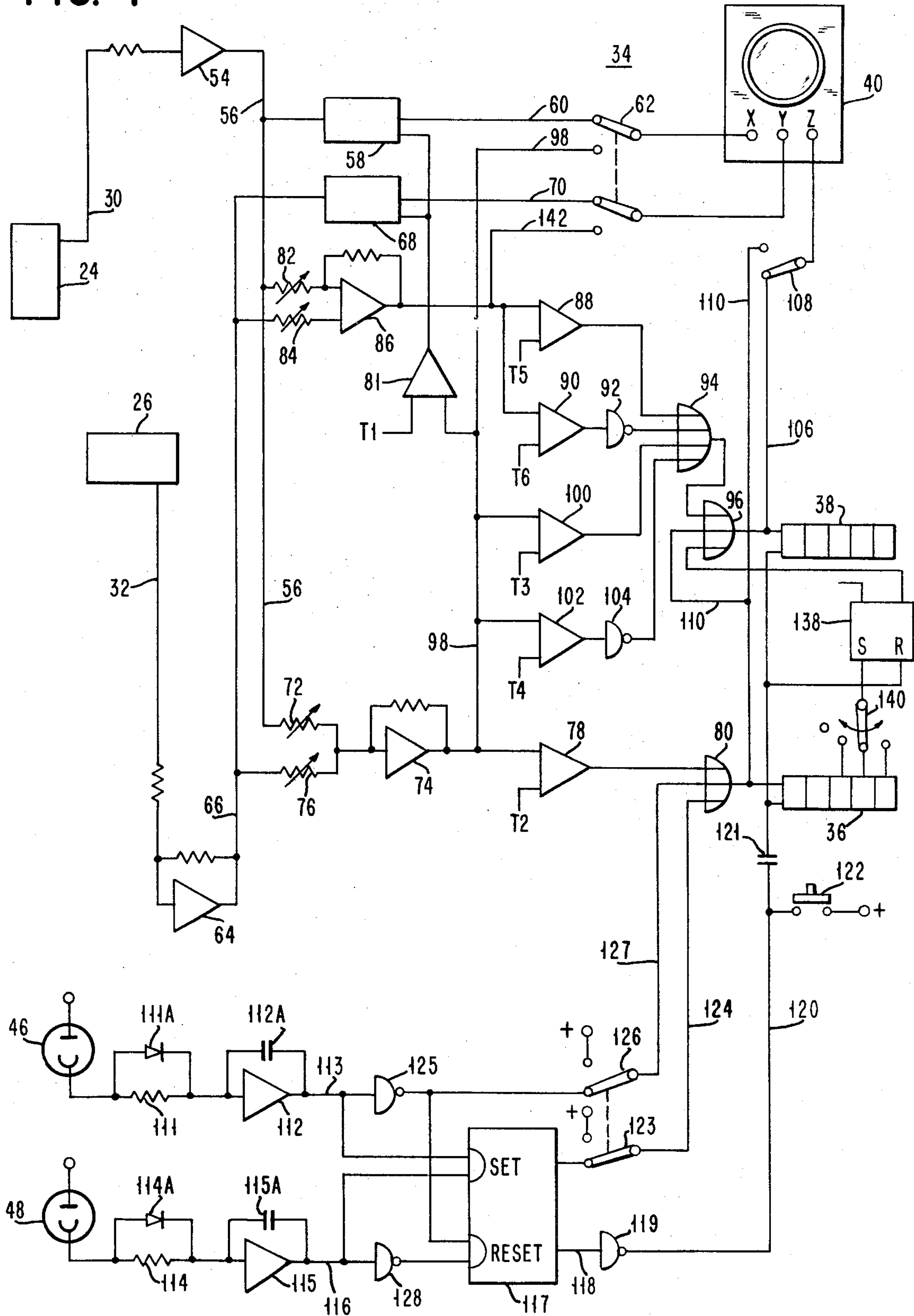
FIG. 4
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T1
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T5
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92
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100
T3
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38
56
102
104
T4
110
138
S R
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72
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74
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T2
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140
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66
64
121
122
127
124
120
46
111A
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112A
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125
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123
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114A
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115A
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128
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RESET
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METHOD FOR ANALYSIS OF BLOOD BY OPTICAL ANALYSIS OF LIVING CELLS

This invention relates to an improved composition for staining white blood cells, and to an improved method for obtaining an analysis of blood for white cells.

It is well known that white blood cells (also sometimes referred to as white corpuscles, and leucocytes) normally exist in the blood in different forms which ma be classified into major groups and in which each group forms a percentage of the total within the limits as shown in the following table:

Polymorphonuclear leucocytes
- Neutrophiles 40 to 60 percent
- Eosinophils (acidophils) **1** to **3** percent
- Basophiles **0** to **1** percent

Mononuclear leucocytes
- Lymphocytes **20** to **40** percent
- Monocytes **4** to **8** percent

The polymorphonuclear leucocytes not only have segmented nuclei, but they are also characterized by having granules in the cytoplasm, and they are therefore sometimes referred to as granulocytes.

Even though they the are far outnumbered by the red blood cells (erythrocytes) by a ratio of approximately **700** to **1**, the leucocytes are extremely important to the body in fighting disease and infections. Furthermore, probably because of that function, it has been observed that abnormal conditions of disease or infection in the body often result in marked changes in the leucocytes in the blood stream. These changes may include a marked increase in the total number of white cells in proportion to the number of red cells in the blood stream. Marked changes in the proportions of different types of leucocytes in the blood stream have been found to be characteristic and unique with respect to particular diseases. Thus, a differential blood count, a count which reveals the relative percentages of the different types of white cells in the blood is an extremely valuable diagnostic and medical research procedure.

In the usual procedure for obtaining a differential analysis of white cells within a blood sample, a smear of the blood sample is dried upon a clean glass microscope slide and is then treated with an appropriate reagent which typically contains a fixative such as methyl alcohol in combination with a mixture of stains, or the preparation procedure may include treatment with the fixative as a separate step. The treated blood smear is then examined by microscope under oil immersion. Sample counts are then taken and recorded in various different areas of the blood smear. An even distribution of the white cells is difficult to obtain and therefore it is recommended that counts be taken at the edges of the blood smear as well as at the central portion, and that a minimum of 200 cells be counted. It is by this means that a differential analysis of white blood cells, and a quantitative white cell count is normally taken. This method involves many difficulties and limitations which seriously affect the accuracy, including lack of uniformity in the blood smear sample, the extremely small size of the sample actually counted, the tediousness of the task — which encourages the laboratory technician to cut corners and to make short counts, and the inconsistencies in the skills of different technicians in recognizing and accurately recording all of the unique cells which he actually sees.

Because this procedure is time consuming for a highly skilled technician, the cost of each analysis is necessarily high, and the speed at which urgent tests can be obtained is limited. Furthermore, because of the drying procedure and the treatment with alcohol in conjunction with stains, the white cells are no longer alive when they are examined. This is believed to be a serious disadvantage because only the dead remains of the cells are examined, whereas it is desired to determine as accurately as possible the actual conditions existing in the living blood of the patient. Furthermore, particularly because of the rough handling of the blood sample in conjunction with the formation of the blood "smear" it is believed that there is a strong possibility of serious damage to the white cells, including the generation of artifacts, and that this may cause substantial errors in the observations and measurements.

So far as is known, it has not been possible in the past to devise a satisfactory machine method for differential analysis of white blood cells. One of the reasons for this is that no stain has been previously found which would provide for satisfactory machine detection of differences between different types of white cells.

In accordance with the present invention a stain has been discovered which will provide a clear basis for distinguishing between different types of white cells in a machine analysis.

An important object of the invention is to provide a new automatic machine method for accomplishing a differential analysis of white blood cells which is characterized by a high degree of accuracy and a very low cost.

Another object of the present invention is to provide a machine method for producing a differential count of white blood cells under conditions in which the cells are maintained in the living state.

Furthermore, it is believed that the method of the present invention provides for a measurement of the vitality of the white cells as well as of their total numbers and types.

Another extremely important problem in devising a machine method for obtaining a white blood cell differential count is that of providing signals to the machine to enable the machine to recognize and distinguish all white cells from all other bodies within the blood such as red cells or platelets (sometimes also referred to respectively as erythrocytes and thrombocytes). The machine recognition and distinguishment of all white cells is essential in the problem of classifying all of the white cells to provide the differential analysis.

Accordingly, it is another object of the present invention to provide an improved composition for staining white cells, and an improved machine method for recognizing and distinguishing all white cells within a blood sample in conjunction with a machine method for obtaining a white cell differential analysis.

As mentioned above, abnormal body conditions such as disease or infection can result in changes in the number of white cells in each unit of volume of blood. Accordingly, one of the most useful diagnostic tests which can be performed entails counting the total number of white cells within a specified volume of a blood sample. This is normally expressed in terms of the number of white blood cells per cubic millimeter of blood. The usual medical laboratory procedure is to

combine a precise volume of a blood sample with a precise volume of a weak acetic acid solution, thereby destroying the red cells, and then to place a portion of the sample upon a "counting chamber" microscope slide and to visually count the number of white cells appearing in several squares of the counting chamber. This is a tedious procedure and involves making a count on such a small sample that inaccuracies are very likely to occur. Furthermore, the skill of the technician is extremely critical in achieving accurate results. Because of these problems, and because of the high costs of these tests, machine methods have been devised for obtaining a white cell count. However, those machine methods have generally involved the procedure of destroying all of the red cells by providing a sample suspension having an osmolality which is adjusted so as to destroy the red cells without destroying the white cells. All of the remaining cells are then counted, and the assumption is made that they are all white cells. This prior machine method leaves much to be desired because the destruction of the red cells is not always complete, and any remaining red cells are then counted as white cells, thus hurting the accuracy of the count. Also, the osmolality condition which destroys the red cells may damage the white cells, and such damage often interferes with the subsequent identification of the white cells.

Accordingly, it is another object of the present invention to provide an improved composition for staining white blood cells so that they are clearly distinguishable from red blood cells in a machine method, without destroying the accompanying red blood cells and damaging the white blood cells.

It is another object of the invention to provide an improved machine method for obtaining a white blood cell count while maintaining normal physiological osmolality and pH conditions and thereby maintaining the white cells in a live and substantially undamaged condition so as to provide the greatest possible accuracy.

Further objects, advantages, and features of the invention will be apparent from the following description and the accompanying drawings.

In carrying out the invention, there is provided a vital dye composition for differential blood analysis of living white cells consisting essentially of acridine orange in an aqueous solution in a concentration between 10^{-7} and 10^{-5} grams of acridine orange per cubic centimeter of solution, said solution having a pH factor and an osmolality within the normal physiological ranges for human blood plasma.

In carrying out the method of the present invention, the following steps are taken: combining a fresh blood sample with an aqueous solution of acridine orange to thereby form a suspension, said solution having a concentration of acridine orange sufficient to provide a concentration of acridine orange in said suspension in the neighborhood of 10^{-6} (0.000001) grams per cubic centimeter of suspension, the pH and osmolality of the solution being at values required to maintain the pH factor and the osmolality of the suspension within the normal ranges for human blood. The suspension is allowed to stand for a period of about 10 minutes and mildly stirred front time to time, and then the suspension is subjected to radiation from a blue laser and the cells are differentially classified based upon the differences in the magnitudes of red fluorescence emitted from individual cells in response to excitation from the blue laser radiation.

In the accompanying drawings:

FIG. 1 is an illustration of a cluster display which may be produced in the practice of the method in accordance with the present invention.

FIG. 2 is an illustration of a histogram display which may be produced in the practice of the method in accordance with the present invention.

FIG. 3 is a schematic diagram illustrating an apparatus which may be employed in carrying out the machine method in accordance with the present invention.

And FIG. 4 is a more detailed schematic diagram illustrating circuit features of the apparatus of FIG. 3 which may be employed in carrying out the method of the present invention.

The dye acridine orange, which is an important constituent of the dye composition of the present invention, is sometimes referred to in abbreviated form as simply "AO". This material is an organic compound for which the chemical name can be expressed as follows: 3,6-bis-dimethyl-amino-acridiniumchloride. This material is also identified by color index specification 46,005 from the publication entitled "COLOR INDEX", Second Edition — of 1956 and 1957, published jointly by the Society of Dyers and Colorists of Great Britain, and by the American Association of Textile Chemists and Colorists of Lowell, Mass. As previously mentioned above, the composition consists essentially of acridine orange in an aqueous solution in a concentration between 10^{-7} and 10^{-5} grams of acridine orange per cubic centimeter of solution. The solution includes other additives to provide a pH factor and an osmolality within the normal physiological ranges for human blood plasma. In the preferred concentrations of acridine orange solution, the mixture does not appear to be a true solution, but rather a colloidal solution or, perhaps more properly a colloidal dispersion, in which extremely minute undissolved particles are in suspension in the liquid. However, this composition is referred to as a solution throughout this specification. The mixture of the acridine orange solution with a blood sample is then referred to as a suspension. Thus, while the acridine orange "solution" may not be a true solution, the use of that term serves to distinguish the composition from the liquid suspension formed after the addition of the blood sample.

The normal physiological pH value for human blood is generally considered to be 7.40, plus or minus 0.05. Since it is desired to provide a physiological environment for the blood sample, such that the cells will not be damaged or killed, the pH of the solution is preferably adjusted, within practical limits, to 7.4, plus or minus 0.01. Similarly, the osmolality, which is a function of the concentration of salts in the solution, is adjusted to the physiological level of approximately 0.30 osmolality units. This may preferably be accomplished with sodium chloride, since natural blood plasma is itself saline.

EXAMPLE I.

One acceptable dye composition in accordance with the present invention was produced with the following composition: an aqueous solution having 10^{-6} grams per cubic centimeter of acridine orange, and having the osmolality adjusted by the addition of 0.85 percent of sodium chloride, and being buffered to a pH value of 7.40 with a phosphate buffer such as a combination of Na H_2PO_4 and Na_2H PO_4 at a combined phosphate molality level of 0.0025.

This composition may be produced by the following steps. A one liter container may be partially filled with distilled water and 8.5 grams of sodium chloride may be added together with 0.090 grams of Na $H_2PO_4 \cdot H_2O$ and 0.496 grams of Na_2H $PO_4 \cdot 7H_2O$. The mixture is stirred or agitated until the salts go into solution, and the container is then topped up to a total volume of one liter by adding additional distilled water. In a separate container, 100 milligrams of acridine orange powder are combined with sufficient distilled water to produce 100 cubic centimeters of acridine orange solution. This combination is stirred or agitated until the acridine orange is dissolved in the water. This results in a clear solution with a reddish-orange tinge of color. One cubic centimeter of the 100 cubic centimeters of acridine orange solution is then added to the previously mixed one liter of saline-buffer solution to produce the acridine orange composition described above. The combination with a saline buffer appears to cause some of the acridine orange to form a colloidal suspension.

Preferably, the combined composition is checked with a pH meter, and if further adjustment is necessary, a drop or two of one-tenth normal hydrochloric acid is added to lower the pH, or a drop or two of one-tenth normal sodium hydroxide is added to raise the pH.

This dye composition was used in accordance with the teachings of this invention and was found to produce a very satisfactory result.

EXAMPLE II.

Example I was repeated, substituting for the phosphate buffer, a buffer commonly referred to as "-HEPES" (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) at a molality level of 0.005.

This composition was also found to provide satisfactory results when employed in the method of the present in invention.

EXAMPLE III.

Example I was again repeated, substituting for the phosphate buffer and saline, a buffer commonly referred to as "TRIS" (2-amino-2-(hydroxy-methyl)-1,3-propanediol) at a molality level of 0.15.

This composition was also found to provide satisfactory results when employed in the method of the present invention.

EXAMPLE IV.

Example I was again repeated with the exception that the amount of acridine orange was quadrupled to provide a concentration of four times 10^{-6} grams per cubic centimeter. At this concentration, the results were found to be very good in the method of the present invention.

EXAMPLE V.

Example I was again repeated, except that the concentration of acridine orange was again increased to a level of 10^{-5} grams of acridine orange per cubic centimeter of solution, all of the other conditions being as set forth in Example I. This composition was found to provide results which were satisfactory, but which were not quite as effective as the lower concentrations of acridine orange described in the preceding examples. Accordingly, this is believed to represent the approximate upper limit of acridine orange concentration which is effective in accordance with the present invention. The staining effect appears to be too great to provide good differentiation between different classes of white cells.

EXAMPLE VI.

Example I was again repeated, except that the acridine orange concentration was reduced to eight times 10^{-7} grams of acridine orange per cubic centimeter of solution, all other conditions remaining the same as in Example I.

This composition was found to provide results which were satisfactory for the purposes of this invention. Thus, it was satisfactory for providing a clear distinction between white cells and other blood particles. However, the results in terms of distinguishing one white cell group from another appeared to be somewhat less satisfactory than Example I. Accordingly, this concentration of acridine orange is believed to represent the lower limit of the preferred range of concentration.

EXAMPLE VII.

Example I was repeated, except the acridine orange concentration was reduced to 10^{-7} grams of acridine orange per cubic centimeter of solution, all other conditions remaining the same as in Example I.

This composition was found to provide results which were satisfactory for some of the purposes of this invention. Thus, it was satisfactory for providing a clear distinction between white cells and other blood particles. However, the results in terms of distinguishing one white cell group from another were seriously impaired. Thus, it appears that this is the minimum concentration of acridine orange which is truly useful in the practice of the present invention.

In all of the Examples II through VII, the acridine orange appears to form a colloid when added to the saline-buffer solution, just as described in connection with Example I.

EXAMPLE VIII.

In practicing the method of the present invention, a quantity of 0.20 milliliters of a fresh blood sample is added to 5 milliliters of the acridine orange solution of Example I. The resulting suspension is mildly agitated from time to time and allowed to stand for a period of about 10 minutes to permit the dye to be taken up by the white cells of the blood sample. The sample is then introduced into an apparatus having a flow system which provides for a flow of the sample liquid in an exceedingly fine stream through an optical chamber. The fine stream is so narrow as to be capable of confining the flow so that the individual cells (both red cells and

white cells) usually traverse the stream one at a time, in single file. In the optical chamber, the cells are caused to pass through a uniform light field provided from a blue laser beam, which may preferably be an argon ion laser at a wave length of 4,880 angstrom units. The uniform light field from the laser preferably has a very short dimension in the direction of travel of the cell sample stream. That dimension is of the same order of magnitude as the maximum dimension of an individual cell so that the cells are subjected to radiation one at a time.

Green fluorescent radiation from the white cells at a range of wave lengths centered at about 5,300 angstrom units resulting from the optical excitation of the white cells by the argon laser beam is then detected, and the total number of white cells is counted in terms of optical green fluorescent pulses. This counting is continued for a carefully measured volume of the suspension sample to obtain an exact white cell count per unit of volume.

It has been discovered that at the acridine orange concentrations noted above there is substantially no stake-up of the acridine orange dye by red cells so that the red cells remain substantially invisible in the detection method just described. There is an exception to this statement, as noted below. By contrast, each white cell nucleus takes up a concentration of the acridine orange dye which results in a substantially uniform green fluorescent emission characteristic. Thus, a rapid and accurate count of white cells is produced. Immature red cells, referred to as reticulocytes, do take up significant concentrations of the acridine orange dye, but the dye is taken up in such a way that there is substantially no green fluorescence emission from the reticulocytes. Thus, the green fluorescence emission is an accurate basis for distinguishing white cells from all other blood particles.

EXAMPLE IX.

The method as set forth in Example VIII is repeated, with the exception that, in addition to detecting the green fluorescence characteristic, the red fluorescence of each cell is detected, at a range of wave lengths in the order of 6,500 angstrom units. The red and green signals are optically separated by a dichroic mirror and suitable filters, and amplified by separate photomultiplier tubes. The green signals are then used to obtain a total white cell count, and the red signals are used to provide a pattern display upon a cathode ray tube. Preferably, the green signals are also used to intensify the cathode ray beam to provide an individual display spot for each cell, and also to provide one coordinate signal upon the cathode ray tube display. Thus, the green signal may be used as the vertical displacement coordinate and the red signal as the horizontal displacement coordinate for a single display spot on the cathode ray tube for each white cell. Various amplitudes of red fluorescence from the different white cells are thus displayed upon the cathode ray tube. It is believed that the different red fluorescence amplitudes are due to the variation in characteristics of different types of white cells, the white cells having the largest number of granules in the cytoplasm displaying the largest red fluorescence signal. It has been discovered that each blood sample produces a display pattern having distinct clusters of points indicating the distribution of different types of white cells. By comparing the patterns produced by blood samples from normal individuals with patterns produced by blood samples from individuals who have diseases or infections which cause abnormalities in the balance of white cells, it has been determined that it is quite practical to detect the differences and to quickly recognize, in a qualitative sense, white cell imbalance conditions which are characteristic of particular diseases or infections. Although the green signal varies much less than the red signal, there is some individual signal variation in the green. Thus, a two-dimensional display pattern is produced upon the face of the cathode ray tube which provides qualitative information about the distribution of white cells within the sample.

The pattern displayed in connection with the last described example of the method is preferably photographed to thereby provide a permanent record which may be analyzed and studied, and which may be compared with later tests from the same patient.

EXAMPLE X.

Example IX is repeated, and threshold circuits are employed to select red fluorescence signals within individual narrow fields of clusters, and those signals are individually counted for a pre-selected total white cell count sample. The method is then repeated for other settings of the threshold circuits to successively count separate cluster portions of the display corresponding to different classes of white cells. By this means, individual counts of the quantities of white cells of each different type are obtained. Thus, the ratio of each type of white cells to the total white cell count may be obtained. These ratios are preferably presented as percentages.

Multiple threshold circuits may be employed to count several narrow fields of clusters at once.

Since the apparatus operates very rapidly, a white cell counting rate as high as 1,000 cells per second is achievable. Previously unattainable accuracies can be achieved by counting thousands of white cells of each type from each sample, by contrast to the usual manual microscope method of counting a very small total quantity in the order of two hundred individual cells. Furthermore, since the cells are actually living at the time they are counted, and since they have been undamaged by the use of any procedure to destroy the red cells, the measured cell population corresponds very accurately to the cell population in the living blood stream of the patient.

It has been discovered that the dye composition and the methods of the present invention are extremely effective in detecting and counting white cells, in distinguishing white cells from red cells on the basis of green fluorescence (essentially ignoring the presence of the red cells), and most particularly in distinguishing the different types of white cells. The different types are distinguishable in the cathode ray tube display by reason of creation of a discrete cluster of points for each distinct type. It has been determined thus far that at least the three most numerous classes of white cells, the granulocytes, the lymphocytes, and a class of cells intermediate in red fluorescence (probably identifiable as monocytes), are distinguishable on this basis. It ap-

pears that the shape of distribution of the granulocyte cluster may provide an index of the maturity of the granulocytes.

FIG. **1** depicts a cathode ray tube display of a white cell analysis characteristic produced in accordance with the above described methods, and with particular reference to Examples IX and X. In this display, the green fluorescence signals have been used for vertical deflection up from the bottom margin (origin), and the red fluorescence signals have been used for horizontal deflection to the right from the origin line on the left margin. Typical clusters of bright spot signal points for groups of cells are indicated at **9**, **11**, and **13**. The cluster **9**, indicating the lowest red fluorescence value, displays the group of lymphocytes. The cluster **11**, indicating a somewhat higher red signal represents the intermediate group, and the cluster **13**, indicating the highest value and the broadest range of red fluorescence, represents polymorphonuclear leucocytes (granulocytes). In certain cases, it has been found to be possible to distinguish sub-groups within these clusters, particularly if the horizontal gain of the oscilloscope deflection circuits is increased.

In order to avoid any possibility of the detection of false signals, the signal circuit for the detection of the green fluorescence is preferably operated with a threshold circuit, as indicated for instance by the horizontal threshold line **17**, so that only signals having a sufficient green fluorescence signal value to exceed the threshold line **17** are actually registered and indicated in the visual display. Similarly, an upper threshold limit may be established, as indicated at **19**. By the employment of additional threshold limits at positions as indicated by lines **21**, **23**, **25**, and **27**, individual point clusters may be picked out and displayed alone, or counted as mentioned above in Example X. For instance, cluster **9** may be selected by setting the upper limit at **23** and the lower limit at **21**. Similarly, cluster **11** may be selected by setting the upper limit at **25** and the lower limit at **23**.

EXAMPLE XI.

With the acridine orange composition of Example IV, the procedure of Example IX was repeated, allowing the fresh blood sample to be in suspension in the acridine orange solution for a period of 16 minutes before placing the sample in the optical chamber and exposing it to the laser beam. The result was a display as shown in FIG. **1**, having very distinct clusters of points for individual cells. However, the middle cluster **11** was, as usual, not so concisely delineated as clusters **9** and **13**.

EXAMPLE XII.

Example XI was repeated, except that the period of exposure of the blood sample to the acridine orange solution before optical testing was reduced to eleven minutes. The clusters were substantially as well defined as in Example XI.

EXAMPLE XIII.

Example XI was repeated, except that the period of exposure of the blood sample to the acridine orange solution before optical testing was reduced to 7½ minutes. The definition of the clusters was somewhat decreased.

EXAMPLE XIV.

Example XI was repeated, except that the period of exposure of the blood sample to the acridine orange solution before optical testing was reduced to 3 minutes. The definition of the clusters was substantially decreased.

In each of the last two examples, the impairment in the definition of the clusters primarily affected the middle cluster **11**, which is thought to represent monocytes.

EXAMPLE XV.

The method of Example IX was repeated employing the acridine orange solution of Example V having the 10^{-5} concentration. The white cells took up the stain very well, providing a very strong green fluorescence signal and strong red fluorescence signals. However, because of the high degree of take-up of the dye by the cells, the results were somewhat marginal in terms of providing red signals having the ability to illustrate distinctive clusters in order to differentiate the different types of cells. Thus, the middle cluster **11** again was not separated from the other clusters.

EXAMPLE XVI.

The method of Example XV was repeated, the only change being to increase the period of exposure of the fresh blood sample to the acridine orange solution to 18 minutes prior to the optical analysis. The result was that the uptake of dye by the cells was even more complete, and the fluorescent signals were even more intense, but the end result was essentially the same.

EXAMPLE XVII.

The method of Example IX was repeated employing an acridine orange composition having a concentration of four times 10^{-7} acridine orange and exposing the blood sample to the acridine orange solution for thirteen minutes before optical measurement. The result was a relatively low uptake of the dye by the cells, resulting in reduced red and green signals, and somewhat of an impairment in separating and identifying the different clusters, particularly the middle cluster **11**.

EXAMPLE XVIII.

The process of Example XVII was repeated, except that the period of exposure of the fresh blood sample to the acridine orange solution was increased to 24 minutes before optical measurement. The results were somewhat improved, but were not as good as prior tests, such as Example IX, employing a higher acridine orange concentration.

EXAMPLE XIX.

The method of Example IX was repeated employing the acridine orange solution composition as set forth in Example II, except that the acridine orange concentration was doubled to two times 10^{-6} grams of acridine orange per cubic centimeter of solution, and the period of exposure of the blood sample to the solution before

optical observation was 13 minutes. Excellent results were obtained.

EXAMPLE XX.

The process of Example IX was repeated, with the exception that instead of using a fresh blood sample, the sample of blood was first combined with an equal volume of a 10 percent aqueous formalin solution which was buffered to a pH of 7.0 with acetates consisting of a mixture of sodium acetate and acetic acid. The mixture of formalin and whole blood was allowed to stand for one hour. The combination with the formalin solution was effective to assure that none of the cells remained alive.

The process of Example IX was then followed, with the exception that the formalin-blood mixture was substituted for the fresh blood sample. The result was that there was very little uptake of acridine orange dye by the cells and consequently there was very little of the green fluorescence signal previously relied upon to distinguish the white cells from other blood particles. Furthermore, the red fluorescence signals were very small and indistinct and did not provide any basis for distinguishing one white cell from another. For all practical purposes, it appears that there was substantially no uptake of dye by the cells.

EXAMPLE XXI.

Example IX was repeated using a helium-cadmium laser instead of an argon laser. The results were marginally satisfactory, the distinctness of the first and third clusters being well maintained, but the distinctness of the second cluster not as well separated from the first cluster.

The reasons for the high degree of effectiveness of the composition and methods of the present invention are not fully known. However, the maintenance of the cells in the live state during the staining and analysis procedure is believed to be very important. It is believed that the white cells take up the acridine orange dye by means of their phagocytic action. Thus, it appears that they actually gobble up and store the colloidal particles of the dye. This belief is supported by the observation (Example XX) that the white cells do not take up the dye if they are not maintained in the vital state until after they are combined with the dye composition. Accordingly, the methods of the present invention appear to provide a measure of the vitality of the blood cells in terms of the effectiveness of this phagocytic action, in addition to providing a count of the cells and a means of distinguishing between different white cells.

It is believed that the reason for the distinctive green fluorescence which distinguishes the white cells from other blood particles is that the acridine orange dye combines with the DNA (deoxyribonucleic acid) of the cell nuclei in a distinctive way such that a green fluorescence can be elicited by excitation with the blue laser illumination. The major portion of the uptake of acridine orange dye by each cell, which does not combine with the nuclear DNA, appears to stain the cytoplasm, and is particularly concentrated in the cytoplasmic organelles known as granules. It is believed that this is the reason why the granulocytes, the white cells which are particularly distinguished by the presence of granules in the cytoplasm, apparently take up more of the acridine orange dye in the cytoplasm, and thus provide a greater red fluorescence signal. All of the acridine orange dye taken up by the cytoplasm (as distinguished from that taken up by the nuclei) appears to cause red fluorescence in response to the blue laser light. Furthermore, it is believed that the red fluorescence intensity is also a measure of the phagocytic activity and capacity of the individual cells. Thus, it appears that a vital cell action, phagocytosis, is instrumental in dye uptake to achieve red fluorescent coloration of white cells, that this activity falls into three distinct categories of red coloration intensity, and in the discovery of particular acridine orange dye compositions containing colloidal particles which appear to stimulate phagocytic action, and are subject to that phagocytic action.

Acridine orange has been recognized for some time as a fluorescent stain which is capable of staining nucleic acids such as ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). For instance, see Rudolf Rigler's article entitled "Acridine Orange in Nucleic Acid Analysis" in Volume 157, Article 1 of the Annals of the New York Academy of Sciences of Mar. 31, 1969, pages 211–224. However, so far as is presently known, the unique value of acridine orange as a vital dye for white blood cells have never been recognized. Since it is regarded primarily as a red fluorescence dye for RNA, and since the amount of RNA is generally considered to be negligible in white blood cells, acridine orange has not been considered as a likely candidate to provide meaningful information in blood cell analysis. However, it has been discovered that the unexpected result is achievable with acridine orange that a very distinctive staining is achievable which is extremely useful in distinguishing white cells from all other blood particles, in a machine method, and in differentiating different groups of white cells from one another.

The preferred concentration of acridine orange is in the near neighborhood of 10^{-6} grams of acridine orange per cubic centimeter of aqueous solution, and this near neighborhood range may extend, for instance, from about eight times 10^{-7} to four times 10^{-6}. It has been observed that because of various undetermined factors, probably including slight variations in the characteristics of the dye, variations in the characteristics of the other components of the composition, and variations due to aging of the dye compositions when they have been placed on the shelf for several days, that some variation in the concentration of the acridine orange in the composition is desirable in order to obtain the most effective dyeing result.

It appears that for high concentrations of acridine orange in the acridine orange solution, the center cluster display **11** in FIG. **1** tends to shift to the right, and to begin to merge with the cluster **13**. On the other hand, for lower concentrations of the solution, the cluster **11** tends to appear more to the left, tending in merge with the cluster **9**.

In the description of Example I, a procedure is followed in which separate solutions of acridine orange and the saline buffer combination are produced and then combined. It is mentioned that the combined solution tends to form colloidal particles which apparently

include the acridine orange dye. It has been observed that the combined solution containing the colloidal particles appears to have a rather limited shelf life. The colloids appear to precipitate out upon the walls and bottom of the container. Accordingly, it is preferred to store the solutions separately, one solution containing only acridine orange, and the other solution containing only the saline buffer combination. The two solutions are then combined in the proper proportions on the day when they are to be used. It has been determined that it is well to combine the solutions several hours before they are actually used. It is believed that the better results which have been observed under these conditions are due to the fact that the colloid particles become stabilized at a more uniform size.

Whether the two solutions are stored separately, or in a combined solution, it has been observed that there appears to be an aging process which occurs such that higher acridine orange concentrations may be necessary to achieve the desired results with older solutions as compared to relatively fresh solutions.

FIG. 2 is a histogram (frequency curve) which is produced from the same data displayed in FIG. 1. Here it is seen that the peak of the curve shown at **9A** corresponds to the center of the cluster **9** of FIG. **1**. The fact that this peak is quite high indicates that there are a large number of individual cells in this cluster within a very narrow range of red fluorescence.

By contrast, peak **11A**, corresponding to cluster **11** of FIG. **1** shows up with a lower value indicating a lesser concentration in numbers of cells having a red fluorescence value falling at the center of the cluster **11**. The peak **13A** has a broad shape indicating a relatively large number of cells falling in virtually the entire range of the cluster **13** at virtually all of the red fluorescence values within that cluster. This histogram is believed to provide very valuable and unique information for each individual whose blood is examined, the shape of the histogram being distinctive for each individual, and showing up individual physiological peculiarities within the normal range, as well as pathological conditions, or pathological changes from the normal for a particular individual.

All of the emphasis to this point has been upon the objective of distinguishing white cells from all other particles in the blood, and upon deriving useful information about the white cells. However, it has been determined that the discoveries of the present invention are also useful for deriving other important information about the blood. For instance, the present invention may be employed to detect and count the number of reticulocytes per unit of volume of blood. Reticulocytes are red blood cells which contain a network of granules or filaments representing an immature stage in development. Reticulocytes normally comprise about one percent of the total red blood cells, but the percentage of reticulocytes may change dramatically under abnormal conditions, and such a change may be symptomatic of disease.

It has been observed that the reticulocytes take up the acridine orange dye, under the conditions generally outlined in the above examples, to a much greater extent than the other red cells. The uptake of the acridine orange dye by the other red cells is insignificant, but the reticulocytes take up enough dye to provide a red fluorescence signal which is of substantially the same magnitude as the lymphocytes class of white cells. However, the reticulocytes do not provide an significant green fluorescence signal. Thus, it is possible to distinguish reticulocytes from all other blood particles by staining a blood sample with the physiological acridine orange composition, and by then distinguishing the reticulocytes by excluding from detection all of the green fluorescing white cells, and detecting those remaining cells which have a significant red fluorescence. The following example illustrates this modification of the process.

EXAMPLE XXII.

The process of Example VIII was repeated, except that the optical green fluorescent radiation from the white cells was employed as a discrimination signal to exclude any count of the white cells, and with the further exception that in addition to detecting the green fluorescence characteristic, the red fluorescence of each cell was detected at a range of wave lengths in the order of 6,500 angstrom units to thereby select all of those cells having a significant red fluorescence signal, and which at the same time do not produce any green fluorescence signal. As described in connection with Example IX, the green signal may be used as the vertical displacement coordinate, and the red signal as the horizontal displacement coordinate as a basis for setting a low green fluorescence threshold which excludes all of the white cells, and for setting red fluorescence thresholds which particularly select the reticulocytes.

Several attempts have been made to determine whether dye compositions containing dyes other than acridine orange might be effective for the purposes of carrying out the present invention. In each instance, the dye composition was prepared by a procedure similar to that described above in connection with Example I, with the exception that the new dye material was substituted for acridine orange. In each instance, the resultant saline dye solution was mixed with a blood sample and examined using the techniques described above in connection with Examples VIII and IX.

With dye compositions produced with each of the following dyes, there was substantially no fluorescence signal:

Erythrosine B, Rhodamine B, Safranine O, and Eosine. With the dye Phosphine, there was a very mild fluoresence, with apparently a faintly distinguishable two-color effect. The white cell nuclei appeared yellow with a slight green tinge, while the white cell granules appeared yellow with a slight orange tinge. None of the other dyes tested, other than acridine orange, produced any perceptible two-color fluorescence. The following dyes produced varying degrees of monochromatic fluorescence signals having intensities less than that produced with the phosphine: Phenosafranine, Neutral Red, Auromine O, and Acridine Yellow. An attempt was also made to use the dye Ba 0, 2,5-bis(4-amino phenyl)-1,3,4-oxdiazole, which is not soluble in water. In this instance, the initial dye solution was made with methyl alcohol to overcome the problem of the insolubility of the dye in water. This dye composition provided no fluorescent signal result at all.

A preferred apparatus for carrying out the method of the present invention, particularly with respect to the optical chamber, the sample flow system, and the optical system, is constructed in accordance with the teachings contained in a prior patent application Ser. No. 2,750 filed Jan. 14, 1970 by Mitchell Friedman, Louis A. Kamentsky, and Isaac Klinger for a PHOTOANALYSIS APPARATUS, and assigned to the same assignee as the present application. Other features of a preferred apparatus for carrying out the method of the present invention, and particularly relating to the arrangement of the counters and the cathode ray oscilloscope apparatus and associated circuits, are carried out in accordance with the teachings of another prior patent application Ser. No. 25,931 filed Apr. 6, 1970 by Louis A. Kamentsky and Isaac Klinger for a PARTICLE ANALYSIS METHOD AND APPARATUS and assigned to the same assignee as the present application. The disclosures of both of these prior patent applications are hereby incorporated herein by reference. However, for the sake of completeness, portions of the disclosures of the last mentioned patent applications are reproduced here, and specifically related to the process of the present invention.

Referring particularly to FIG. **3**, there is illustrated a schematic diagram of an apparatus which may be employed in carrying out the method in accordance with the present invention. The apparatus includes an optical chamber **10** through which a stream **12** of cells may be passed while entrained in the suspension and supplied through a pipe **14** from a reservoir **15**. The suspension is preferably surrounded by a sheath of water in order to confine the particles (cells) to a very fine stream. As the stream **12** of particles passes through the chamber **10**, it passes through a narrow beam of light **20** from a light source **22**. Light source **22** is preferably an argon laser, and may include a cylindrical lens **22A** for shaping and directing the light.

Different optical reactions of the individual blood cells to the light beam **20**, in the form of fluorescent radiation from each cell, are detected by photoelectric pick-up elements **24** and **26**, which may preferably be photomultiplier tubes. The signals detected by the photoresponsive pick-up elements **24** and **26** are converted by those elements to electrical signal pulses which are supplied through connections **30** and **32** to an evaluation and utilization circuit **34**. The pick-up element **24** is arranged to respond to red fluorescence signals, and element **26** is arranged to respond to green fluorescence signals. The transmission of optical fluorescence signals to the pick-up elements is enhanced by a reflector **16** and a lens **18**. A filter **18A** may be provided which passes all light having wave lengths longer than about 5,000 angstrom units. This excludes substantially all radiation reflected from the argon laser at the wave length 4,880 angstrom units. Filter **18A** thus helps to assure that all radiation received by the pick-up units **24** and **26** is due to fluorescent radiations from the cells. A dichroic mirror **28** is provided which has a nominal cut-off wave length for light at about 5,800 angstrom units. Thus, it reflects light of all wave lengths below that limit, through a filter **26A**, to the pick-up **26**. All optical signals above 5,800 angstroms in wave length are transmitted through the dichroic mirror **28**, and through an optical filter **24A** to the pick-up **24**. The pick-up **24** receives the red fluorescence signals and the filter **24A** passes a red band of radiation in the neighborhood of **6,300** angstrom units wave length. Similarly, the green filter **26A** passes a green band of optical signals in the range in the neighborhood of 5,300 angstrom units wave length.

Analysis of the optical reaction signals in the circuit **34** causes that circuit to energize two counters **36** and **38**. Counter **36** provides a count of the total number of particles within a predetermined sample, and counter **38** indicates the number of particles within the sample having a particular characteristic which is to be distinguished, such as a particular range of red fluorescence signal amplitude. The circuit **34** is also preferably connected to provide signals to a cathode ray oscilloscope **40**.

The liquid sample containing the particles to be analyzed may be supplied to the pipe **14** from a source such as a reservoir **15**. In order to provide a precise volume measurement for a particular volume of sample liquid to be analyzed, photocells **46** and **48** are provided at spaced points along the pipe **14**, which is preferably composed of glass, to detect the presence or absence of liquid at the respective positions opposite those photocells. At the respective photocells there are provided separate light sources **50** and **52**. When there is liquid in the portion of the pipe **14** directly between light source **50** and photocell **46**, the liquid tends to focus the light from source **50** upon the photocell **46** to provide a higher level signal. However, when that portion of pipe **14** is empty, and occupied only by air, the illumination is de-focused and the optical signal to photocell **46** is correspondingly reduced. This change in signal level at photocell **46** is detected within circuit **34**. Photocell **48** reacts in a similar manner to illumination from light source **52**. The portion of pipe **14** between photocells **46** and **48** may be referred to hereinafter as an elongated container having an entrance end at photocell **46** and an exit end at photocell **48**.

In a preferred method of operation, when photocells **46** and **48** both detect the presence of liquid in tube **14**, the circuit **34** causes both of the counters **36** and **38** to be reset to zero. When the trailing edge of the particle sample passes the upper photocell **46**, so that the presence of air rather than liquid is detected, the particle count is permitted to begin. When the trailing edge of the liquid sample passes the lower photocell **48**, the transmission of further count pulses to the counters **36** and **38** is stopped. Thus, the count values stored in the counters **36** and **38** is related to a volume of particle carrying liquid corresponding exactly to the volume of liquid stored within the tubing **14** between the respective photocells **46** and **48**.

FIG. **4** is a circuit diagram of the circuit **34** of FIG. **3** together with components directly connected to that circuit. In this circuit, the red signals supplied through connection **30** are amplified by an amplifier **54** and supplied through a connection **56** to a sample and hold circuit **58**. From circuit **58**, the signal is connected through a connection **60** and a gang switch **62** to the X axis input of the oscilloscope **40**. Similarly, the green signal supplied through connection **32** is amplified in

an amplifier **64** and supplied through a connection **66** to a sample and hold circuit **68**. The output from the sample and hold circuit **68** is supplied through connection **70** and the gang switch **62** to the Y axis input of the oscilloscope **40**. Thus, the oscilloscope **40** may display the function of red versus green fluorescence for each particle. The sample and hold circuits **58** and **68** are pulse forming circuits or monostable multivibrators which hold the peak values of the respective signals for a predetermined period longer than the actual duration of the input signals from the photoresponsive pick-up devices **24–26**. In one preferred embodiment, the holding period is approximately forty microseconds. The maintenance of these maximum values by the sample and hold circuits **58** and **68** makes it possible to provide a display representing the combination of the maximum values for each particle as essentially a single point upon the face of the oscilloscope **40**. Another essential element for this display is a brightening signal supplied to the oscilloscope on the third input Z as described more fully below.

The amplified signal from amplifier **54** on output connection **56** is also supplied through a variable resistor **72** to an amplifier **74**. Similarly, the amplified signal from amplifier **64** is supplied through connection **66** and a variable resistor **76** to the input to amplifier **74** in common with the input through resistor **72**. Accordingly, the amplifier **74** receives and amplifies the sum of fractions of the amplified absorption and scatter signals supplied through the variable resistors **72** and **76**. The respective fractions of the signals are determined by the adjustments of the variable resistors. In the practice of the method of the present invention, variable resistors **72** and **76** are preferably adjusted so that the contribution of the signal through resistor **72** is virtually nil, the entire input to amplifier **74** being supplied through resistor **76** from the green signal amplifier. Thus, while the output of amplifier **74** is referred to below as a sum output, in the practice of the present method, from a practical standpoint it is essentially a green fluorescence signal. Furthermore, it will be understood that whenever the apparatus is constructed specifically for the practice of the method of the present invention, the variable resistor **72** and the associated connection to amplifier **74** may be deleted.

The amplified sum output from amplifier **74** is supplied through a threshold circuit **78**, and a logical AND gate circuit **80** to the counter **36**. The threshold amplifier circuit **78** operates to pass the amplified sum signal from amplifier **74** if that signal exceeds an adjustably determined threshold voltage value T2. The threshold value T2 is preferably set high enough to exclude "-noise" signals from the system and to permit any legitimate particle detection signals to come through. Accordingly, the sum circuit, including amplifier **74**, is employed to detect the presence of particles to be counted, and the resultant pulse signals are supplied to the counter **36** for individual registration and storage.

The sum signal from amplifier **74** is also supplied through a connection **98** and a threshold circuit **81** to both of the sample and hold circuits **58** and **68**. The signal supplied through threshold circuit **81** is an enabling signal which causes the sample and hold circuits **58** and **68** respectively to respond to the input signals from the absorption amplifier **54** and the scatter amplifier **64**. Thus, by appropriately setting the threshold T1 for threshold circuit **81**, the sample and hold circuits are caused to respond only to the presence of legitimate particle signals. The threshold T1 is preferably set slightly below the threshold T2 so that the sample and hold circuits **58** and **68** are always enabled whenever a pulse is passed through threshold circuit **78** to be counted in the counter **36**.

The amplified red and green signals on connections **56** and **66** are also respectively supplied through variable resistors **82** and **84** to a difference amplifier **86**. The output from amplifier **86** represents an algebraic difference between a fraction of the amplified red signal, as determined by the adjustment of resistor **82**, and a fraction of the amplified green signal, as determined by the adjustment of resistor **84**. This difference signal is supplied to two threshold circuits **88** and **90** which serve respectively as lower and upper limit circuits in passing difference signals respectively above an adjustable threshold T5 and below an adjustable threshold T6. An inverter **92** on the output of threshold circuit **90** reverses the effective operation of threshold circuit **90** from that of a lower limit to that of an upper limit circuit. The output signal from threshold amplifier **88**, and the output of threshold circuit **90** inverted by inverter **92** are supplied to a logical AND gate **94** and thus through a further AND gate **96** to the counter **38**. The AND gate **94** is a four input AND gate which responds only to the presence of input signals on all four of its inputs. The other two inputs are supplied from the sum amplifier **74** through a connection **98** and threshold circuits **100** and **102**, the output from circuit **102** being inverted by an inverter **104**. Thus, in order to obtain an output from AND gate **94**, a particular particle must produce a sum signal through amplifier **74** which is between a lower threshold limit T3, as determined at threshold circuit **100**, and an upper threshold limit T4, as determined at circuit **102**, as well as producing a difference signal which is between the limits T5 and T6. Therefore, when the signals from a particular particle fall within all of these measurement thresholds, the particle is counted and the count is registered within the counter **38**. This provides a very precise means for selecting and counting particles having particular characteristics, as previously described above in connection with FIG. **1**.

In order to be certain that the particles in the particular selected class which are counted in counter **38** are also particles which are counted in the total particle count recorded in counter **36**, the AND gate **96** must be gated open by the output signal on connection **110** from the total particle count AND gate **80**. Thus, no particle is counted in counter **38** unless it is also counted in counter **36**.

By means of a connection **106** and a switch **108**, the particle pulses received by counter **38** are also applied to control the brightening circuit Z of the cathode ray oscilloscope **40**. Thus, with switch **108** in the position shown, only the data for the particles actually counted by counter **38** is displayed, because the cathode ray oscilloscope beam is brightened so as to be visible, only for those particular particle signals. If desired, switch **108** may be shifted to the other position for connection to a conductor **110** which carries the signals at the input to counter **36**. Thus, the signals for all of the par-

ticles which are counted are then displayed by the oscilloscope **40**.

The portion of the circuit of FIG. **2** associated with the photocells **46** and **48** for automatically measuring a predetermined volume of liquid containing particles is as follows. Photocell **46** is connected through a resistor **111** to an amplifier **112** to provide an output at connection **113** in response to the detection of liquid in the pipe **14** which focuses the light upon the photocell **46**. Similarly, the presence of liquid opposite the photocell **48** provides a signal through resistor **114** to amplifier **115** to provide an output on connection **116**. The outputs at **113** and **116** are supplied as the set inputs to a flip-flop **117**. When both of these set inputs are present, the flip-flop **117** is shifted the the set state providing a logic zero output at the reset output connection **118**. That reset output is inverted in an inverter **119** and supplied through a connection **120** and an AC coupling provided by a capacitor **121** to reset both of the counters **36** and **38**. This signal also resets a flip-flop **138**, the function of which is described below. An alternative reset signal source is provided by a manual reset pushbutton **122** for use when the automatic volume feature is not employed.

In order to prevent false operation of the volume measurement apparatus in response to a mere drop of water passing through the pipe **14**, the amplifiers **112** and **115** are respectively shunted by capacitors **112A** and **115A**. These capacitors, in conjunction with the input resistors **111** and **114** provide each of the amplifiers **112** and **115** with a time delay response characteristic such that a time delay of several seconds is required during which the photocell must continuously "see" liquid in order to provide an effective output signal for changing the state of the flip-flop **117**, or for accomplishing any of the other switching functions as described below. This time delay may preferably be in the order of 5 seconds. However, as soon as a photocell "sees" air instead of liquid, the resultant drop in the signal is a sudden drop because of the presence of the diodes **111A** and **114A** respectively shunting the resistors **111** and **114**. Thus, if a mere drop of water is detected, the circuit is rapidly reset to re-commence the time delay cycle.

As soon as the flip-flop **117** is placed in the set condition, a resultant "set" output signal is supplied through a switch contact **123** and a connection **124** to the AND gate **80** to supply one of the enabling inputs to that AND gate. Subsequently, when the trailing edge of the sample of liquid passes photocell **46**, the resultant change in the photocell output is detected from connection **113** through an inverter **125**, a switch lever **126**, and a connection **127** by the AND gate **80**. This provides the final enabling signal to open gate **80** to commence the transmission of particle count signals through amplifier **78**. The switch levers **123** and **126** are ganged together and may be shifted from the automatic position shown to a manual position in which voltage conditions are obtained such that the gate **80** is continuously enabled.

The output of the inverter **125** is also supplied as one of the reset inputs to the flip-flop **117**. When the second photocell **48** detects the passage of the trailing edge of the liquid sample, the resultant signal change at connection **116** is detected through inverter **128** to supply the second reset input to flip-flop **117**, causing the flip-flop to reset. This removes the set output supplied through switch **123** and connection **124** to gate **80** and thereby disables gate **80** and stops the counters **36** and **38**. Thus, the count values stored in counters **36** and **38** are counts based exactly on a sample of particles taken from a volume of particle-containing liquid as measured by the volume stored between the photocells **46** and **48**, and only the particles within that measured sample are visible by reason of the brightening signal supplied to the oscilloscope **40** through switch **108**.

A third input is normally supplied on a continuous basis to AND gate **96** from the reset output of a flip-flop **138**. However, this signal is discontinued when the flip-flop **138** is set by a signal on the set input of that flip-flop supplied through a switch **140** from counter **36**. Switch **140** is a selector switch which may be used to select a desired output signal from counter **36** corresponding to the achievement of a particular count value in counter **36**. Generally speaking, counter **36** counts all of the particles within a selected sample, and counter **38** counts only those particles meeting particular tests. By setting the selector switch **140**, the counter **38** may be caused to stop at a particular selected total count value stored in counter **36**. Then the count stored in counter **38** represents directly the ratio between the count value recorded in counter **38** and the selected total count of particles determined b the setting of switch **140**. Preferably, the settings of the switch **140** may represent multiples of ten in the total count achieved by counter **36**. The count value stored in counter **38** after that counter is stopped then provides a direct reading of the percentage of the total particles which have the particular characteristics to be detected by the circuits feeding AND gate **94**. This is a particularly useful feature because it provides an automatic registration of a percentage value without the need for any separate calculation. Furthermore, the operation of the circuit associated with flip-flop **138** does not interfere with the further operation of the total particle counter **36**. Thus, counter **36** may continue to count and register the total number of particles within a measured sample.

In a preferred form of the invention, the selector switch **140** not only selects multiples of ten in the total count, but it is also a gang switch having another rotary switch contact (not shown) which connects enabling voltage to selected decimal point positions in the counter **38** to provide appropriate exact indications of the percentage count ultimately stored in counter **38**. The exactness of this percentage count is enhanced by reason of the control of AND gate **96** through connection **110** by the total particle count signal from gate **80**. This input to AND gate **96** provides assurance that no particle will be counted by counter **38** as a member of the special selected class of particles unless it is also recognized as a particle to be counted in the total particle count register **36**.

The apparatus of this invention may be employed to provide a permanent record of the test results. The counts stored by the counters **36** and **38** may be stored upon a suitable data record medium (not shown).

The oscilloscope **40** may be connected to register an indication of the sum and difference signals rather than

the red and green signals. This is accomplished by shifting the gang switch **62** to the lower position to provide the output of the difference amplifier **86** through connection **142** to the Y axis input of oscilloscope **40**, and to provide the sum signal output from amplifier **74** through connection **98** to the X axis oscilloscope input.

We claim:

1. A method for distinguishing white blood cells from other particles within a blood sample comprising the steps of

staining a fresh blood sample while maintaining physiological conditions of pH factor and osmolality to preserve the blood cells in the vital state by combining the blood sample with a solution of acridine orange dye having the required pH factor and osmolality, said acridine orange dye being capable of staining the nuclear material of the white cells to provide fluorescence from the stained nuclear material at a unique color different from any fluorescence from any other blood particle,

and then exposing the combination to illumination from a light source including a radiation color absorbed by the stained nuclear material,

and observing the resultant fluorescence radiation at said unique color from the stained white cell nuclei.

2. A method for making a differential blood analysis of white cells only comprising the steps of combining a fresh blood sample with an aqueous solution of acridine orange to thereby form a suspension,

said solution having a concentration of acridine orange sufficient to provide a concentration of acridine orange in said suspension in the range between 10^{-7} and 10^{-5} grams per cubic centimeter of suspension.

the pH and osmolality of said solution being at values required to maintain the pH factor and the osmolality of said suspension within the normal ranges for human blood,

mildly agitating the suspension from time to time during a period of about 10 minutes,

and then subjecting said suspension to radiation from a blue laser and distinguishing the white cells from all other blood particles based upon the detection of green fluorescence emitted from individual white cells in response to the excitation from the blue laser radiation,

detecting the differences in the magnitudes of red fluorescence emitted from individual white cells in response to excitation from the blue laser radiation, and differentially classifying the white cells based upon such detection.

3. A method as claimed in claim **2** wherein the solution of acridine orange is added in a concentration to provide a concentration of acridine orange in said suspension in the range between eight times 10^{-7} and four times 10^{-6} grams per cubic centimeter of suspension.

4. A method as claimed in claim **1** wherein

the solution of acridine orange is added in a concentration to provide a concentration of acridine orange in said suspension in the neighborhood of 10^{-6} grams per cubic centimeter of suspension.

5. A method as claimed in claim **2** wherein

said suspension is directed in a fine stream through an optical chamber in which the suspension is subjected to radiation from the blue laser,

the fine stream being so narrow as to be capable of confining the flow to substantially a single cell width such that the cells traverse the stream one at a time in single file.

6. A method as claimed in claim **5** wherein

the radiation from the blue laser is directed to the suspension stream in a uniform light field directed in a narrow beam substantially transverse to the suspension stream and having a beam dimension in the direction of the suspension stream which is in the same order of magnitude as the maximum dimension of an individual cell.

7. A method as claimed in claim **6** wherein

the step of subjecting said suspension to radiation from a blue laser is carried out by energizing an argon ion laser and directing the resulting radiation to said suspension.

8. A method as claimed in claim **6** wherein

the step of subjecting said suspension to radiation from a blue laser is carried out by energizing a helium-cadmium laser and directing the resulting radiation to said suspension.

9. A method as claimed in claim **2** wherein

the red fluorescence radiation is discriminated by an optical filter which passes wave lengths in the order of 6,500 angstrom units.

10. A method as claimed in claim **2** wherein

the differences in the magnitudes of red fluorescence emitted from individual cells are detected by converting the optical signals into electrical signals and displaying the resultant electrical signals upon the face of a cathode ray tube.

11. A method for making a differential blood analysis for white cells comprising the steps of,

combining undiluted and untreated fresh blood with an aqueous solution of acridine orange in the ratio of up to about 1 part of blood to 5 parts of a solution to thereby form a suspension.

said solution having a concentration in the neighborhood or 10^{-6} grams acridine orange per cubic centimeter of solution,

said solution containing a buffering agent in sufficient quantity to maintain the pH factor of said suspension within the normal range for human blood,

and said solution having an osmolality within the normal physiological range for human blood plasma,

and then passing said suspension through a photoanalysis apparatus and subjecting each white cell in the suspension to radiation from a blue laser,

discriminating the white cells from all other blood particles contained in the suspension by detecting fluorescent radiation emissions above a predetermined threshold value from each cell in the green portion of the visible spectrum,

and detecting the magnitudes of the fluorescent radiation emissions from each white cell in the red portion of the visible spectrum,

and classifying the cells based upon the differences in magnitudes of red fluorescence measured from the cells.

12. A method as claimed in claim **11** wherein

the green radiation signals are employed to derive a total count of the white cells from a measured volume of the suspension.

13. A method as claimed in claim **11** wherein

the magnitudes of the fluorescent radiation emissions from each cell in the red portion of the visible spectrum are detected by converting the red optical signals to electrical signals and displaying them upon the face of a cathode ray tube,

the magnitude of the red signals being indicated in terms of the cathode ray tube beam deflection on one axis,

the green optical signals being converted to electrical signals and employed to control the cathode ray tube beam deflection upon the other axis,

and the green signals being used also to provide electrical beam brightening signals to thus provide an individual display spot for each white cell.

14. A method as claimed in claim **13** wherein

a camera is placed over the face of the cathode ray tube to record the pattern of spots produced by the method.

15. A method as claimed in claim **11** wherein

a histogram plot is generated by converting the magnitudes of the fluorescent radiation emissions from the individual white cells in the red portion of the visible spectrum to electrical signals,

the frequency with which the magnitudes of the red electrical signals fall within particular narrow value limits being indicated in terms of a vertical magnitude of an individual histogram plot at a horizontal displacement corresponding to the value of the red radiation emission magnitude whose frequency is being recorded to thereby generate a histogram the shape of which is indicative of white cell blood conditions.

16. A method as claimed in claim **13** wherein

voltage thresholds are applied to select red fluorescence signals within a narrow field,

and the signals from the narrow field are individually counted for a carefully measured blood sample volume to thereby obtain a quantitative measure of those white cells having the particular characteristics exemplified by that narrow field.

17. A method as claimed in claim **13** wherein

the green fluorescence signals above a predetermined threshold are counted up to a predetermined d count value to thereby determine the passage of the number of white cells corresponding to that count value,

voltage thresholds are applied to select red fluorescence signals within a narrow field,

and red fluorescence signals from the narrow field are individually counted during the period of the green fluorescence count to thereby obtain a count ratio of those white cells having the particular characteristics exemplified by that narrow field.

18. A method as claimed in claim **17** wherein

the method is repeated with different portions of the same blood sample and with different settings of the voltage thresholds to thereby obtain a series of measurements of cells having different characteristics.

19. A method for making a count of total white cells in a blood sample comprising the steps of combining a fresh blood sample with an aqueous solution of acridine orange to thereby form a suspension,

said solution having a concentration of acridine orange sufficient to provide a concentration of acridine orange in said suspension in the neighborhood of 10^{-6} grams per cubic centimeter of suspension,

the pH and osmolality of said solution being at values required to maintain the pH factor and the osmolality of said suspension within the normal ranges for human blood,

mildly stirring the suspension from time to time during a period of about ten minutes,

and then subjecting said suspension to radiation from a blue laser and counting the cells from a measured volume of suspension based upon the detection of green fluorescence emitted from each individual cell in response to excitation from the blue laser radiation.

20. A method as claimed in claim **19** wherein

the detection of green fluorescence emitted from the cells is discriminated by an optical filter which passes radiation in the neighborhood of 5,300 angstrom units wave length.

21. A method for classifying different white cells within a blood sample consisting of the steps of

combining a fresh blood sample with a colloidal solution of a vital fluorescent dye whereby the white cells take up the dye by phagocytic activity,

and then distinguishing the different classes of white cells by measuring the magnitude of fluorescence from the individual cells in response to optical radiation to thereby detect the differences in take-up of the fluorescent dye by the phagocytic activity of the different cells.

22. A method for distinguishing white blood cells from other particles within a blood sample comprising the steps of

staining a fresh blood sample while maintaining physiological conditions of pH factor and osmolality to preserve the blood cells in the vital state by combining the blood sample with a dye which is capable of staining the nuclear material of the white cells to provide fluorescence from the stained nuclear material at a unique color different from any fluorescence from any other blood particle,

and then exposing the combination to illumination from a light source including a radiation color absorbed by the stained nuclear material,

and observing the resultant fluorescence radiation at said unique color from the stained white cell nuclei.

23. A method as claimed in claim **22** wherein

the dye is a composition which is capable of staining portions of the cytoplasmic material to fluoresce at a color different from said unique color,

and the amplitudes of cytoplasmic fluorescence are detected for individual cells and amplitude differences are used to classify the cells.

24. A method as claimed in claim **22** wherein a fresh said dye consists essentially of acridine orange.

25. A method for distinguishing reticulocytes from other red cells and from all other particles within a blood sample comprising the steps of staining a fresh

blood sample while maintaining physiological conditions of pH factor and osmolality to preserve the blood cells in the vital state by combining the blood sample with a solution of acridine orange dye having the required pH factor and osmolality,

said acridine orange dye being capable of staining the nuclear material of the white cells to provide fluorescence from the stained nuclear material at a unique green color different from any fluorescence from any other blood particle,

said acridine orange dye being capable of staining the reticulocytes included within the blood sample to provide fluorescence from the reticulocytes at a red color and having a substantially greater magnitude than any red fluorescence from the remaining red cells,

and then exposing the combination to illumination from a light source including a radiation color absorbed by the stained cellular material,

and observing and distinguishing the reticulocytes by observing and excluding the white cells based upon green fluorescence signals from the white cells and including only the remaining cells having significant red fluorescence signals.

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UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,684,377 Dated August 15, 1972

Inventor(s) LAWRENCE R. ADAMS and LOUIS A. KAMENTSKY

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 1, line 10, "ma" should read --may--.
Column 5, line 49, cancel "in" before "invention".
Column 7, line 23, "stake-up" should read --take-up--.
Column 12, line 61, "in" should read --to--.
Column 20, line 30, "b" should read --by--.
Column 21, line 60, "claim 1" should read "claim 2".
Column 23, line 48, cancel "d" before "count".
Column 24, line 61, cancel "a fresh".

Signed and sealed this 9th day of January 1973.

(SEAL)
Attest:

EDWARD M.FLETCHER,JR.
Attesting Officer

ROBERT GOTTSCHALK
Commissioner of Patents