Kirk-Othmer Encyclopedia of Chemical Technology.

AUTOMATED INSTRUMENTATION, HEMATOLOGY

Hematology analyzers provide information about blood cells and their constituents. The three basic blood cell types are erythrocytes or red blood cells, first described in 1674 by Van Leeuwenhoek using a microscope of his own design; leukocytes or white blood cells, noted by Hewson in 1773; and thrombocytes or platelets, described in 1841 by Addison, who also noted the granules present in certain of the leukocytes. Hemoglobin, the principal nonaqueous component of red blood cells, was identified by Hoppe-Seyler in 1864 when it was shown that hemoglobin is a complex of four Fe^{2+} protoporphyrins, called hemes, and a protein, or globin, consisting of two alpha and two beta chains. The absorption spectrum of the oxidized form of hemoglobin showing that hemoglobin can absorb and release oxygen was also reported (1). Hemoglobin's physiological importance gives it the status of a primary hematologic constituent.

The first hematologic cell-counting chamber, the hemocytometer, was developed in 1873. A drop of a diluted suspension of blood cells was inserted by capillary action between a glass cover slip and the finely cross-hatched surface of a glass chamber. The blood cells were allowed to settle to a common focal plane. Then, using a microscope, the red blood cells (RBC) within the cross-hatched areas were counted. Eventually, technologists used the hemocytometer to count white blood cells (WBC) and platelets (PLT) as well, first destroying the red blood cells which predominated. Hemocytometers are still used on occasion.

In 1877 Ehrlich developed a triacid stain that distinguished the nucleus and cytoplasm of cells in a stained blood film and originated the white blood cell differential count (Diff). By applying the triacid stain to a blood film slide and examining the slide under a microscope, five white cell types were distinguishable by the shapes of the nuclei and by the varying affinity of cell granules for the stain (2). The five white blood cell types are lymphocytes (LYMPHS) which have round nuclei and no granules; monocytes (MONOS) which have indented nuclei and sparse granules; neutrophils (NEUTS) which have polymorphonuclear (PMN) or segmented nuclei and granules that have affinity for neutral dyes such as picric acid-rosaniline; eosinophils (EOS) which have PMNs and granules having affinity for acidophilic dyes, such as eosin; and basophils (BASOS) which have PMNs and granules having affinity for basophilic dyes, such as fuchsin. Most clinical laboratories still routinely use Ehrlich's method for white blood cell differential counts, determining the number of each cell type in a 100 cell count, albeit using other stains.

Technologists have measured a sample's hemoglobin content in various ways. In 1878 a method for measuring hemoglobin concentration in whole blood (HGB) using visual photometry was introduced. Hemoglobin concentration may be defined as the number of grams of hemoglobin per deciliter of whole blood. Samples of blood were diluted and the color compared to that of a solution of picrocarmine. Later samples were converted to carboxyhemoglobin and the color compared to that of a carboxyhemoglobin standard or converted to acid hematin through the addition of dilute hydrochloric acid and the brown color compared to that of a standard. In 1920 a method to convert hemoglobin to cyanmethemoglobin by lysing the red cells in distilled water and adding potassium ferricyanide and potassium cyanide was introduced. The ferricyanide oxidized the heme to the Fe^{3+} form, yielding methemoglobin, and the cyanide converted methemoglobin to cyanmethemoglobin. This method was later modified by adding sodium bicarbonate, in dilute form, to reduce the turbidity of the lysed product. Hemoglobin concentration was determined by comparing cyanmethemoglobin's color to that

of a reference material or by measuring its photometric absorption at 540 nm and converting to hemoglobin concentration. Drabkin's solution, the ferricyanide, cyanide, bicarbonate mixture, was used for many years for photometric hemoglobin measurements because it effectively converted all forms of cellular hemoglobin to a single, stable form for photometric measurement. In 1966 the International Committee for Standardization of Hematology (ICSH) adopted the cyanmethemoglobin photometric method, which is related to the use of Drabkin's solution. Technologists also use physical properties of hemoglobin, such as specific gravity and refractive index, to determine concentration.

A method for determining a sample's total hemoglobin content was developed in 1929. A tube of whole blood was centrifuged, packing the red cells at the bottom of the tube, and the percentage of total sample volume occupied by the RBC was determined by dividing the height of the packed cells by the total height of the sample. This percentage was called packed cell volume (PCV) or hematocrit (HCT). In combination with RBC measurements, the hematocrit provided a sample's mean red blood cell volume (MCV) (3).

$$MCV = (HCT/RBC) \times 10$$

Two additional parameters, the mean cellular hemoglobin concentration (MCHC) and the mean cellular hemoglobin (MCH), can be obtained.

$$MCHC = (HGB/HCT) \times 100$$

$$MCH = (HGB/RBC) \times 10$$

By 1935 a technologist could perform a complete blood count (CBC) consisting of: WBC in $10^3/\mu$L, RBC in $10^6/\mu$L, HGB in g/dL, HCT in %, MCV in fL (10^{-15} L), MCH in pg, MCHC in g/dL, and PLT in $10^3/\mu$L; and a complete white blood cell differential count, consisting of all white blood cell types, ie, the five normal types plus any other cells not normally found in peripheral blood.

The first commercially successful automated blood cell counter, the Model A Coulter Counter, was introduced in 1956. The Model A counted cells by using electrical impedance properties, and the Coulter Counter quickly became the instrument of choice for counting red and white blood cells. When it was later demonstrated that cell volume was roughly proportional to the electrical impedance signal amplitude, the Coulter Counter was modified to provide MCV as the mean of the individually measured red cell volumes. Impedance counters calculated HCT as (RBC × MCV)/10 and, by the early 1970s, these instruments also counted platelets. By the mid-1970s, impedance counters combined with photometric hemoglobinometers to produce CBCs.

In 1965 Technicon introduced the SMA 4/A Autocounter, which counted red and white cells by using light-scattering properties and determined a sample's HCT by measuring electrical conductivity. The instrument included a photometer for automated HGB measurements. The Autocounter was the basis for the first fully automated CBC analyzer including PLT, the Hemalog 8, introduced in 1970. The first commercial automated five-part Diff analyzer, the Hemalog D, introduced in 1971 by Technicon Instruments Corporation, produced five-part white blood cell differential counts by combining measurements of the light-scattering and cytochemical properties of white cells. By 1975 Geometric Data, Corning Medical and Scientific, Abbott Laboratories, and Perkin-Elmer each had instruments on the market. The latter instruments were automated slide readers equipped with computerized image analyzers (4). Commercial success was limited, however, because these instruments were only slightly faster than manual differential counting methods. Additionally, they were expensive to operate and suffered from questionable quality of the prepared blood film slides.

The first automated CBC/five-part Diff analyzer was introduced in 1981. This instrument used modified Hemalog D 90 technology as well as other light-scattering measurements and photometric hemoglobinometry. It was followed by an improved version, the H*1 (Technicon), that incorporated a novel method for measuring

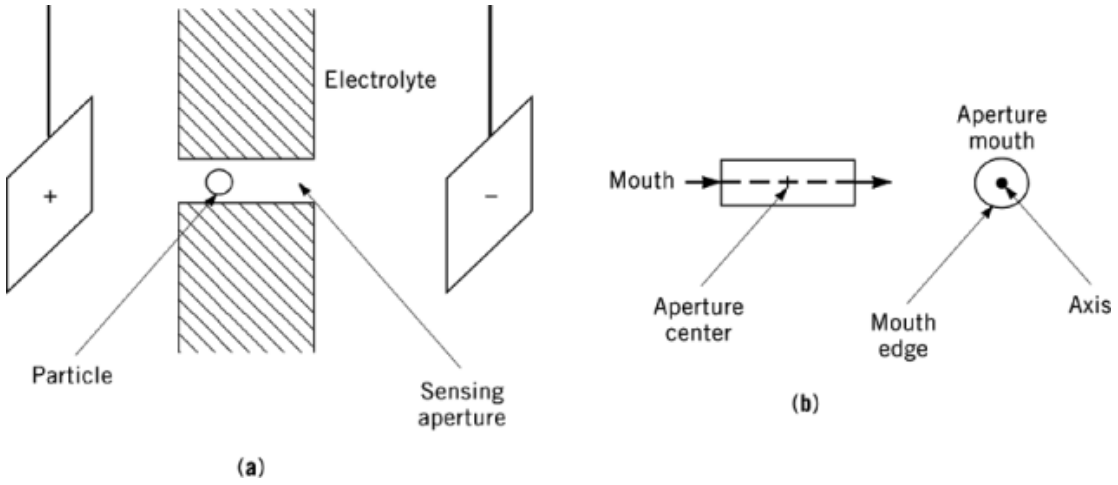

Fig. 1. (**a**) Schematic diagram of the electrical impedance method for counting and sizing blood cells. The electrodes are represented as charged rectangles. As particles flow through an aperture, the impedance and, hence, the flow of current are modified; (**b**) schematic diagram of the sensing aperture of a flow cytometer.

certain red blood cell parameters. Then in 1989 TOA introduced the NE-8000, which produces five-part differentials by combining impedance technology with cytochemistry; Unipath introduced the Cell-Dyn 3000, which uses light scattering only to produce five-part differentials; and Coulter introduced the Coulter STKS, which performs five-part differential counts by using impedance and light scattering.

1. Aperture Impedance Instruments

The aperture impedance principle of blood cell counting and sizing, also called the Coulter principle (5), exploits the high electrical resistivity of blood cell membranes. Red blood cells, white blood cells, and blood platelets can all be counted. In the aperture impedance method, blood cells are first diluted and suspended in an electrolytic medium, then drawn through a narrow orifice (aperture) separating two electrodes (Fig. 1). In the simplest form of the method, a d-c current flows between the electrodes, which are held at different electrical potentials. The resistive cells reduce the current as the cells pass through the aperture, and the current drop is sensed as a change in the aperture resistance.

Aperture impedance counters are flow cytometers, as are all current automated cell counters. Flow cytometers measure cells as the cells flow hydraulically, one at a time, through sensing zones. All flow cytometers, including aperture impedance counters, count fewer cells than actually pass through their sensing zones (6–11). When two or more cells are in a sensing zone simultaneously, by coincidence, the counter senses only one cell. A number of approaches may be taken to correct for count loss resulting from coincidence. The simplest is to dilute the sample to the point where the probability of coincidence is acceptably small (12, 13). However, because this approach typically requires dilution factors of a million or more, it introduces dilution errors. Also, because dilution significantly reduces the number of cells counted, this approach also increases sampling error. An alternative approach is to model the probability of coincidence as a function of either sample concentration or the fraction of time the sensing zone is occupied by passing cells (dead time) (6–11). The model produces a coincidence-correction factor which is applied to the count. Still another approach is to reduce the size of the sensing zone, which reduces the probability of coincidence (7). In practice, commercial counters combine the latter two approaches.

Flow cytometer cell counts are much more precise and more accurate than hemocytometer counts. Hemocytometer cell counts are subject both to distributional (13) and sampling (14–16) errors. The distribution of cells across the surface of a hemocytometer is sensitive to the technique used to charge the hemocytometer, and nonuniform cell distribution causes counting errors. In contrast, flow cytometer counts are free of distributional errors. Statistically, count precision improves as the square root of the number of cells counted increases. Flow cytometer counts usually involve 100 times as many cells per sample as hemocytometer counts. Therefore, flow cytometry sampling imprecision is one-tenth that of hemocytometry.

Aperture impedance counters provide cell volume information as well as cell counts. The relationship between aperture resistance change and cell volume may be expressed as (17–20):

$$\frac{DR}{R} = f\,\frac{v}{V}$$

where DR = change in resistance resulting from the presence of a cell in the aperture, R = aperture resistance of the electrolyte alone, f = function of particle shape and orientation, v = cell volume, and V = effective aperture volume. Aperture impedance counters provide mean red cell volume (MCV) and mean platelet volume (MPV), both in femtoliters, as:

$$MCV = \frac{\sum red\ cell\ volumes}{RBC}$$

$$MPV = \frac{\sum platelet\ volume}{PLT}$$

Aperture impedance and most other automated counters measure MCV and RBC independently, in contrast to the manual methods where MCV and MCH accuracies depend on hemocytometer red cell count accuracy.

Automated counters can also provide cell volume distribution information because they measure volume on a cell-by-cell basis. Manual methods measure MCV indirectly. Automated counters provide red cell volume distribution width (RDW) and platelet volume distribution width (PDW) where RDW is the standard deviation of red cell volumes/MCV and PDW is the standard deviation of platelet volumes. RDW measured as a percentage and PDW in units of femtoliters have descriptive value. For example, a high RDW value suggests the presence of microcytes (small red cells) and/or macrocytes (large red cells). Microcytes may be associated with red blood cell iron deficiency and macrocytes with vitamin B-12 or folate deficiency. Automated counters also group cell volumes by frequency to form cell volume histograms. The histograms provide integrated views of cell volume distribution (Fig. 2).

Several factors affect aperture impedance cell volume measurements. DR/R depends on *f,* the shape and orientation of the cells passing through the aperture. For spherical objects $f = 1.5$, whereas for needlelike objects oriented along the cylindrical axis of the aperture, $f = 1$ (20–22). Additionally, as needle-shaped objects tumble, the f values change. Red cells are especially subject to shape and orientation effects in apertures because these cells are highly deformable and their shape in the aperture depends on the shear forces therein. Photographs show that as red cells pass through apertures at typical flow rates, they deform from their native biconcave disk shape to one approximating an oblate ellipsoid (cigar-shape) (22). If all red cells deformed to the same extent, deformability could be factored into *f.* However, red cell deformability depends on cellular hemoglobin concentration. High cellular hemoglobin concentration reduces red cell deformability thereby reducing elongation from shear forces. Less deformed red cells have higher f values than normally deformed cells. Therefore, aperture impedance counters often assign erroneously high cell volume values to red cells having high cellular hemoglobin concentration, and erroneously low cell volume values to cells where the cellular hemoglobin concentration is low. A fraction of the red cells may tumble about their long (deformation)

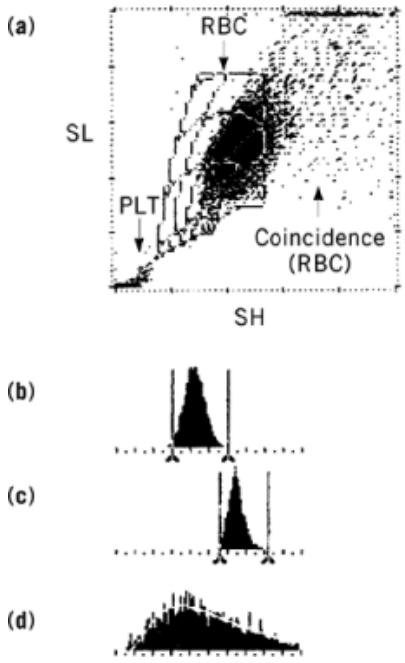

Fig. 2. Histograms representing (**a**), the frequency of occurrence of platelets (PLT) and red blood cells (RBC) and illustrating coincidence where SL is low angle scatter, 2°C–3° and SH is high angle scatter, 5°C–15° (**b**), the volume of RBC, from 0 to 200 fL; (**c**), the hemoglobin (HGB) concentration from 0 to 50 g/dL; and (**d**), the platelet volume from 0 to 20 fL.

axis as they pass through the aperture along the aperture wall (21–23), because of discontinuous changes in hydrodynamic forces near the wall. As the cells tumble, f increases and skews MCV upward.

Aperture impedance measurements of cell volume are affected by variations in the electric field strength that occur in the region of the aperture. The field is strongest at the edge of the aperture mouth and weakens as the aperture axis is approached (21, 22) (Fig. 1**b**). The field also weakens from the aperture entrance to the center of the aperture. Therefore, DR/R and computed cell volumes are greater for cells passing along the aperture wall than for cells passing along the aperture axis.

A number of investigators (24–26) demonstrated that aperture impedance cell volume measurements could be improved by focusing or restricting cell flow to the aperture axis, thus reducing variations resulting from inhomogeneous electric and hydrodynamic fields. Counters that caused the cell suspensions and electrolyte solutions to flow concentrically through the apertures were designed. The electrolytes sheathed the cell suspensions, narrowing them and centering the cells on the aperture axis.

Aperture impedance measurements of cell volume must take into account the osmolality and pH of the medium. A hypotonic medium causes cells to swell; a hypertonic medium causes them to shrink. Some manufacturers of aperture impedance counters deliberately provide hypertonic electrolytic media for red blood cell measurements. The shrunken red cells not only become more nearly spherical and thus less affected by orientation, but also less deformable than cells in isotonic media and thus less affected by differences in hemoglobin content.

The basic aperture impedance method can produce three-part white cell differential counts. Impedance counters can distinguish three white cell types by size: the LYMPHS, mid-range cells including MONOS and BASOS, and granulocytes including NEUTS and EOS.

A refined version of the aperture impedance method which combines radio frequency (r-f) aperture currents and d-c currents, can produce four-part differentials. R-f aperture impedance is sensitive to the internal structure of white cells (27). R-f aperture impedance measurements distinguish BASOS from LYMPHS by virtue of BASOS lower nucleus-to-cytoplasm ratio. Therefore, the combined d-c/r-f aperture impedance counter distinguishes LYMPHS, MONOS, BASOS, and NEUTS + EOS. To distinguish EOS from NEUTS, and thereby produce five-part differentials, requires the use of light-scattering measurements.

In summation, aperture impedance counters provide information on WBC, RBC, HCT, MCV, PLT, MPV, RDW, PDW, and three-part Diff, for d-c current only, or four-part Diff using a d-c/r-f combination.

2. Light-Scattering Instruments

The light-scattering principle of cell counting is based on the observation that microscopic particles, such as blood cells, scatter into small (0–15°) angles, most of the visible light incident upon them (28). This principle is used to count red blood cells, white blood cells, and platelets. In the basic form of the light-scattering method, a dilute suspension of cells and a sheathing fluid having a matching refractive index flow concentrically through an optical flow cell onto which light is focused. The cells scatter the incident light as they pass through the flow cell, and the light scattered into a small-angle interval, typically 1–3 degrees, is sensed by an optical detector. The purpose of the sheath is to narrow the cell stream and reduce the cell passage to single file while centering the stream in the flow cell. The unscattered component of focused light is much larger than the scattered component and must be blocked from the detector so that it does not swamp the desired small-angle scatter signal (Fig. 3).

The basic single-angle interval light-scattering method cannot accurately measure individual red blood cell or platelet volumes, but it can provide MCV and MPV. Red cells are bi-concave disks, and platelets are rod to disk shaped. Scattering intensities depend on the orientation in the flow cell. Because the cells can interrupt the optical path in random orientations, individual scattering intensities are not proportional to cell volume. However, because thousands of cells of each type pass through the flow cell, the effects of orientation can be averaged. To a first approximation HCT and platelet crit (PCT), the percentage of blood sample volume occupied by platelets, is proportional to the sums of the scattering intensities of the red cells and platelets, respectively. MCV can be computed from HCT and RBC, whereas MPV can be computed from PCT and PLT. The accuracy of MCV determined by this method is tied to the RBC accuracy, as is the case for the manual MCV method. Ortho Instruments Corporation's ELT-8 uses these counting and sizing methods.

Light-scattering measurements of sphered cells are not subject to orientation effects, and a method for the rapid sphering and fixing of red cells for the purpose of measuring them in a light-scattering flow cytometer has been developed (29). The reagents used to lyse red blood cells, typically surfactants (qv), can also be used at lower concentrations to sphere them. In fact, the sphering process is part of the overall process of hemolysis (30). The red cell's sphered state is the last intact state before it is hemolyzed. Hemolysis is, however, undesirable because, depending on the extent of hemolysis, red cells are reduced in size or totally destroyed. The sphering agent concentration should not be too high, because it can hemolyze the red cells in samples having low red blood cell concentrations. However, the concentration of sphering agent must be high enough to sphere all the red cells in samples having high red blood cell concentrations. Fixing the cells after they are sphered prevents hemolysis in samples having low RBC values, yet permits concentrations of sphering agents that are high enough to sphere all red cells in concentrated red cell suspensions.

A single reagent capable of rapidly sphering and fixing all the red blood cells in a sample over a wide range of red blood cell concentrations has been developed. The reagent composition ensures that red cells are not fixed before they are sphered, and that the cells do not lyse before they are fixed. The sphering and fixing reagent contains sodium dodecyl sulfate (SDS) [151-21-3], a buffer, and gluteraldehyde [111-30-8], $C_5H_8O_2$. SDS is the sphering agent, the buffer provides an isotonic medium in which to isovolumetrically sphere red

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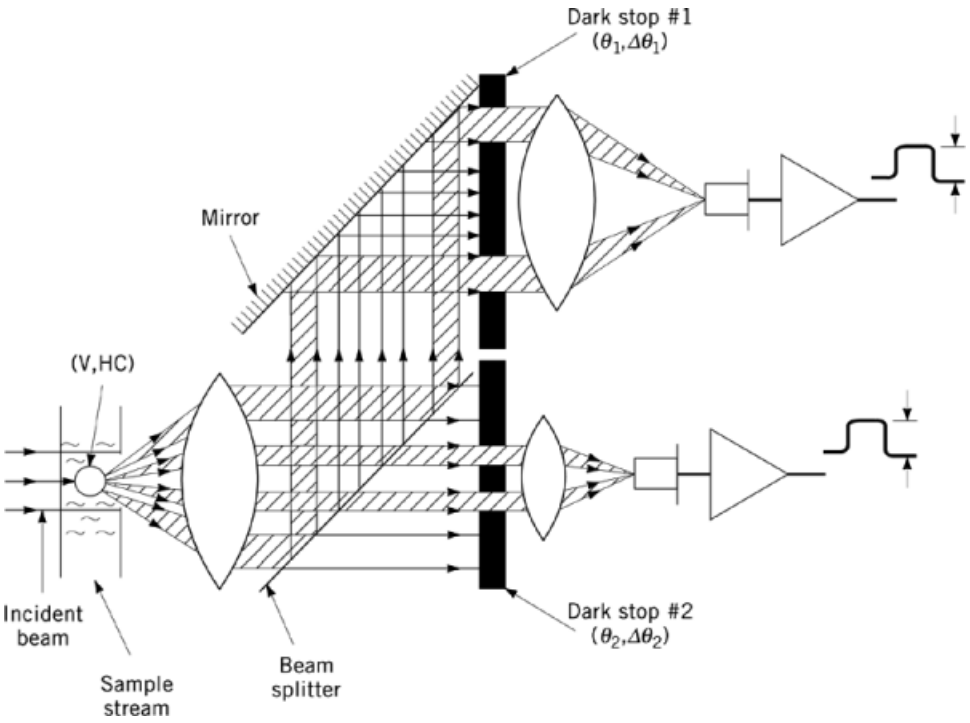

Fig. 3. Schematic diagram of a dark field system for measuring the light scattering by a spherical red blood cell where V=volume of sample and HC=hemoglobin concentration. Optical flow cell system having double-angular interval detection at angles θ_1 and θ_2.

cells, and gluteraldehyde is the fixing agent. The Technicon H*6000 Hematology Analyzer incorporated this method in order to measure red cell volume on a cell-by-cell basis. In contrast to the light-scattering method for sphered and fixed red cells, the aperture impedance method for determining the MCV of unsphered red cells is subject to orientational effects.

Light-scattering measurements of red cell volume are accurate only if the measurements account for the effects of cell refractive index on scattering intensity. Basic single-angle interval light-scattering measurements of red cell volume, even for sphered and fixed red cells, are only accurate to within approximately 10%. Mie Scattering Theory predicts that the angular scattering intensity pattern for sphered red cells depends on cell refractive index as well as cell size (31, 32). Red cell refractive index is linearly related to cellular hemoglobin concentration (33). Therefore, two normal red cells of the same volume but of different cellular hemoglobin concentration scatter light differently. A sphered cell's volume and the hemoglobin concentration can both be accurately determined from Mie Scattering Theory calculations by making simultaneous measurements of light-scatter intensity over two suitably chosen angle intervals (34). Two suitable angle intervals for red cells are 2–3 degrees, known as low angle scatter (SL), and 5–15 degrees, high angle scatter (SH) (Fig. 2). In contrast to the aperture impedance method, this method for determining red cell volume does not suffer from inaccuracies as a result of cellular hemoglobin concentration variability.

In principle, the two-angle interval method can produce all CBC parameters within a single measurement channel, uniquely providing cell-by-cell hemoglobin concentration. The mean of the concentrations provides an alternative (and direct) measurement of MCHC. The method also provides an alternative HGB measurement, because HGB may be set equal to (RBC $\times$ MCV $\times$ MCHC)/1000. This method, like the basic light-scattering

method, uses the same flow cell to measure platelets and red cells with the result that the method is capable of providing the CBC parameters RBC, HGB, HCT, MCV, MCHC, MCH, and PLT. The method can also count a sample's white blood cells if the sample's red blood cells have been lysed.

The distribution of cellular hemoglobin concentrations within a blood sample, also known as hemoglobin distribution width (HDW), can be provided. HDW is the standard deviation of cellular hemoglobin concentrations measured in units of g/dL. HDW has descriptive value: a high HDW value suggests the presence of abnormally high numbers of hyperchromic and/or hypochromic red cells, ie, either high or low cellular hemoglobin concentration cells. Hypochromic cells are usually associated with anemia, whereas hyperchromic cells may be associated with hereditary spherocytosis or spherical blood cells, a condition in which the survival rate of the spherocytes is decreased. Instruments using the two-angle interval scatter method can display cellular hemoglobin concentration histograms which provide integrated views of cellular hemoglobin concentration distributions (Fig. 2).

In summation, the two-angle interval scattering method can provide CBC, RDW, PCT, and HDW. This two-angle interval method has been incorporated into the Technicon H*1 system which provides RBC, HCT, MCV, PLT, MPV, RDW, PDW, PCT, and HDW.

The single-angle interval light-scattering method counts white cells but cannot produce five-part Diffs. White cell light-scattering intensity depends on cell size, cell refractive index, and internal cell structure. White cell size ranges overlap and the cells differ in refractive index. Scattering measurements can distinguish among cell types based on differences in internal structure alone, but only if the cell types have the same overall size and refractive index.

Light-scattering measurements made over two suitably chosen angle intervals can combine with depolarized light-scattering measurements to provide five-part Diffs (35). Unipath's Cell-Dyn 3000 instrument measures white cell light scatter at 1–3 degrees and at 3–11 degrees in order to resolve LYMPHS, BASOS, MONOS, and NEUTS + EOS. The two-angle interval light-scattering method distinguishes the cell types based on size and refractive index and is similar to the Technicon H*1 RBC/PLT method. The Cell-Dyn 3000 instrument uses the scattering behavior of EOS granules to distinguish EOS from NEUTS. EOS granules are refractile, numerous, large, and closely packed. Light incident on the granules is scattered a number of times, from granule to granule, before reaching the detectors. If the incident light is polarized, multiple scattering depolarizes the light scattered onto the detectors. The other white cell types scatter polarized incident light once, but do not depolarize it. Depolarization as a result of multiple scattering is enhanced at high scattering angles. Thus the Cell-Dyn 3000 measures the depolarized and polarized components of light scattered at 90 degrees to incidence by white cells and plots depolarized scattering intensity vs polarized scattering intensity for each cell. A distinct EOS cluster forms on the plot.

Basic light-scattering measurements can be combined with other measurements to provide five-part Diffs. Coulter's STKS combines d-c/r-f aperture impedance and light-scattering measurements to provide five-part Diffs. The STKS lyses a sample's red cells, then shrinks the white cells by suspending them in a hypertonic medium. The system distinguishes among the shrunken cells based on cell size differences and internal structure differences related to r-f impedance and light-scattering characteristics. D-c/r-f measurements distinguish among LYMPHS, BASOS, MONOS, and NEUTS + EOS based on cell size and r-f impedance. Small-angle scattering measurements resolve EOS and NEUTS based on EOS granules scattering behavior.

3. Cytochemistry

Cytochemical techniques can be combined with light-scattering and absorption measurements to provide five-part Diffs. Cytochemistry concerns the chemical reactions of cell components. The reactions for automated white blood cell differential analysis include those that bind chromophores to the granules of white cell types, based on the presence of various substrates and enzymes in the granules. These reactions yield products suitable

for light-scattering and optical absorption measurements. Other reactions exploit the differential resistance of white cell types to cytoplasmic stripping by the lysing action of surfactants. The reaction products are suitable for light-scattering and aperture impedance measurements. Optical absorption measurements made on intact cells in suspension are different from those made on dissolved hemoglobin. Measurements made on intact cells involve reduced overall scattering intensity and do not obey Beer's Law; measurements made on solutions involve dissolved chromophores and do obey Beer's law (see Spectroscopy).

Although both the manual and light-scatter—cytochemistry methods of counting white cell differentials involve the staining of white cells, the staining and measurement techniques are different (36). Classic stains used in manual differential analyses, such as Wright's Stain and Giemsa Stain, consist mainly of azure dyes, which are basic, and eosin dyes, which are acidic. The DNA of white cell nuclei are azurophilic (basophilic) and are stained blue to magenta by the azure dyes, as are the granules of NEUTS and the DNA contained in the cytoplasm of LYMPHS and MONOS. The heparin in BASOS granules is also basophilic. The acidophilic (eosinophilic) proteins in EOS granules and nonazurophilic NEUT granules, however, are stained red by eosin. The white blood cell types are not distinguishable by azure-eosin staining specificity and intensity alone. In order to distinguish among the white blood cell types, a visual examination of differences in their size, shape, granular content, and nuclear structure is also necessary.

The Technicon Hemalog D was the first hematology analyzer to combine cytochemistry with light-scattering—optical absorption measurements to produce five-part Diffs. This instrument used cell-specific staining properties of the leukocytes as well as their light-scattering behavior to distinguish among the five normal leukocytes types (37, 38). BASOS are unique in that the heparin in their granules reacts with alcian blue to precipitate a blue-green dye in the granules. MONOS alone contain lipase, an enzyme catalyzing the reactions of esters, and the substrates α-naphthyl butyrate and basic fuchsin combine, in the presence of lipase and a diazonium salt, to precipitate a red-brown azo dye. Only EOS are peroxidase-active below pH 3. Between pH 3 and pH 5, NEUTS are also peroxidase-active, though not as strongly as EOS, as are MONOS weakly peroxidase-active in this pH range. Therefore, EOS, NEUTS, and LYMPHS + BASOS + MONOS are distinguishable by peroxidase staining alone. By combining the three staining techniques, the Hemalog D could have distinguished among the five normal white cell types based on stain absorption alone. However, the Hemalog D achieved improved resolution of LYMPHS and MONOS by adjusting peroxidase staining conditions and measuring white cell light scattering as well as absorption in the peroxidase channel.

In order to use the leukocytes' enzymatic properties, the Hemalog D carried out reactions under specific conditions. Because enzymes are easily damaged or released from cells, the cells were fixed to stop metabolism and to immobilize the enzymes without significantly changing their catalytic activity. Fixation was controlled so that plasma proteins did not precipitate and generate spurious particle counts, clogging the system. Red cells were lysed at elevated temperatures to destroy red cell and serum catalase and hemoglobin pseudoperoxidase activity, which would otherwise have completely consumed the peroxidase reaction substrates. Then plasma esterase inhibitor was inactivated by adding concentrated formaldehyde and ethylene glycol, in which the inhibitor is labile. The esterase inhibitor would otherwise have stopped the MONO reaction. In order to specifically bind alcian blue to heparin and not all nuclei, the BASO reaction was carried out at pH 2.2. The three staining reactions are

(1) Peroxidase reaction for NEUTS, EOS, (MONOS)

$$4\text{-chloro-}1\text{-naphthol} + H_2O_2 \xrightarrow[\text{peroxidase}]{} \text{dark precipitate}$$

(2) BASO reaction

$$\text{alcian blue} + \text{heparin (in BASO granules)} \longrightarrow \text{blue-green dye}$$

(3) MONO reaction

$$\alpha-\text{naphthyl butyrate} + \text{basic fuchsin} \xrightarrow[\text{lipase}]{\text{MONOS}} \text{butyric acid} + \alpha-\text{naphthol}$$

$$\alpha-\text{naphthol} + \text{diazonium salt} \longrightarrow \text{red}-\text{brown azo dye}$$

The system measured small-angle light-scattering intensity and/or cell stain absorption in each of three reaction channels. In the MONOS channel, an absorption detector registered as MONOS those white cells that absorbed light at the MONOS stain-absorption wavelength as the cells passed through a flow cell. In the BASOS channel, white cells interrupted a broad wavelength band of focused light as they passed through a flow cell. Two scattering detectors, one with a green filter, the other with a red filter, registered scattering intensity. The analyzer counted as BASOS those cells that scattered less green light than red light. In the peroxidase channel, white cells scattered and absorbed light differentially, depending on their peroxidase activity, as they passed through a flow cell. The analyzer plotted small-angle light-scattering intensity versus absorption, for each cell. The plotted points formed clusters by cell type. Cluster-analysis algorithms distinguished among LYMPHS + BASOS, NEUTS, EOS, and, to a limited extent, MONOS. The Hemalog D also used the sum of the small-angle scatter signals in the peroxidase channel to provide WBC.

The Technicon H*6000 system used a different combination of cytochemistry and light-scattering—absorption measurements to produce five-part Diffs. The system retained the Hemalog D peroxidase channel but eliminated the MONO channel, thus reducing the number of system flow cells by one. The system relied on modified staining conditions and peroxidase channel cluster analysis to distinguish MONOS as well as LYMPHS + BASOS, NEUTS, and EOS (39). A series of reagents were developed that better fix monocytes, as well as the other white cell types, than did the Hemalog D reagents. The well-fixed cells are better able to retain peroxidase stain, stain more uniformly, and have narrower size distributions than the cells fixed by Hemalog D reagents. The resultant peroxidase channel clusters are tighter and thus easier to distinguish.

The Technicon H*1 and H*2 systems use a different method to distinguish BASOS, exploiting the BASOS resistance to cytoplasmic stripping. The BASOS reagent contains surfactant and phthalic acid plus hydrochloric acid. The surfactant lyses the red cells. Additionally, surfactant and phthalic acid, at low pH, strip the cytoplasm from all white cell types except BASOS. The reaction mixture then contains intact BASOS and the nuclei of the other white cell types. BASOS and nuclei pass through the same flow cell as the one used for RBC/PLT measurements. Intact BASOS and nuclei scatter light into the SL and SH detectors. BASOS scatter much more light into the SL detector than do nuclei because the intact BASOS are much larger. Thus the analyzer distinguishes BASOS by the high SL scattering intensity.

The H*1 and H*2 method of performing five-part Diffs is superior to the H*6000 method because a single stain is used instead of two; the same flow cell is used for BASOS measurements as for the RBC/PLT ones, thus reducing the number of flow cells from three to two; and the BASO channel provides more information about white cells because the BASO channel produces the lobularity index (LI), a measure of granulocyte maturity, in addition to producing a percentage of BASO and absolute BASO count.

The H*1 and H*2 systems plot SL intensity versus SH intensity for the nuclei. Two clusters form on the BASOS cytogram as shown in Figure 4. The left-hand cluster is comprised of LYMPHS and MONOS, which are both mononuclear (MN), or nonsegmented. The right-hand cluster is comprised of NEUTS and EOS, which are polymorphonuclear (PMN), or segmented. SH intensity is sensitive to refractive index and number of nuclear segments. The higher the refractive index or the greater the number of nuclear segments, the higher the SH intensity. Mature granulocytes have more nuclear segments than younger granulocytes. In addition, the more mature nuclei have higher refractive indexes than the less mature ones, because the more mature ones are denser. Therefore, the more mature segmented nuclei appear further to the right on the BASO cytogram than

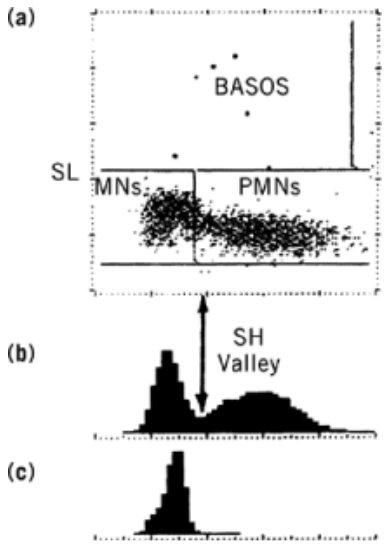

Fig. 4. (**a**), BASOS cytogram showing intensities for BASOS, mononuclear (MNs), and polymorphonuclear (PMNs) cells; (**b**), intensity at SH, high angle scatter; (**c**), intensity at SL, low angle scatter.

the less mature segmented nuclei. Technicon H*1 and H*2 systems define the lobularity index (LI) as the ratio of the PMN histogram mode channel to the MN histogram mode channel (Fig. 4).

The TOA NE-8000 system combines d-c/r-f aperture impedance measurements with cytochemistry to produce five-part Diffs. The d-c/r-f impedance measurements are used to distinguish LYMPHS, mid-range cells, and granulocytes. The NE-8000 system lyses all white cell membranes except those of BASOS and identifies the BASOS by their higher d-c aperture impedance. Then, in a separate reaction, the NE-8000 lyses all white cell membranes except those of EOS and identifies EOS by their higher d-c aperture impedance.

Various method combinations can be used to produce five-part Diffs including d-c/r-f aperture impedance plus light scattering; two-angle interval light-scattering plus depolarized light scattering; light scattering plus cytochemistry; and d-c/r-f aperture impedance plus cytochemistry. Flow cytometric methods produce five-part Diffs that are generally more precise and accurate than manual Diffs. However, manual methods can provide more information. White cell differential counts based on microscopic examination of blood smears are subject to sampling errors (14–16). Manual white cell differential counts are usually based on 100 cells. Therefore, for basophils which comprise 0–2% of the differential count, and for eosinophils (1–4%), and even for monocytes (2–8%), statistical sampling error is large. Flow cytometer differential counts, which are typically based on 5,000–10,000 cells, have much smaller sampling errors for these white cell types. Also, flow cytometers provide both direct absolute counts of each white cell type and differential counts, whereas manual methods provide only indirect absolute counts as the product of the differential count and WBC. Manual white cell differential counts are subject to distributional errors (40–43). Drawing a film of blood across the surface of a glass slide tends to force the larger, less dense blood cells to the edges of the slide. Uneven cell distribution across the surface of the slide can cause counting errors. Flow cytometric differential counts are not subject to distributional error. On the other hand, a technologist performing manual white blood cell differential counts can identify abnormal cell types that the automated analyzers cannot identify.

4. Photometric Hemoglobinometry

Most automated hemoglobinometry methods are derivatives of the manual ICSH reference method (44). The manual method follows three steps: (*1*) The blood sample is diluted in a reagent containing Triton X-100, a nonionic surfactant. The surfactant lyses the RBC membrane, releasing hemoglobin into solution. (*2*) Ferricyanide present in the diluent diffuses into the hemoglobin molecule and oxidizes heme from the Fe^{2+} to the Fe^{3+} form, yielding methemoglobin. (*3*) Cyanide diffuses into methemoglobin's interior and reacts with heme to yield cyanmethemoglobin (HiCN), which has a characteristic absorption spectrum that is measured spectrophotometrically.

The Technicon H*1 HGB automated method follows four steps: (*1*) The blood sample is diluted in a reagent containing lauryldimethylamine *N*-oxide (LO), an ionic surfactant and more powerful lytic agent than Triton X-100.The RBC membrane is lysed as is Step 1, above. (*2*) The combined action of surfactant and a pH 11.3 releases hemes from globin as a mixture of ferrous (Fe^{2+}) and ferric (Fe^{3+}) hemes. (*3*) Hemes are air-oxidized to ferric (Fe^{3+}) form. (*4*) The ferric hemes are ligated by cyanide and micellized by LO, forming reaction products with characteristic absorption spectra that are measured spectrophotometrically.

The automated method differs from the ICSH method chiefly in that oxidation and ligation of heme iron occur after the hemes have been released from globin. Therefore, ferricyanide and cyanide need not diffuse into the hemoglobin and methemoglobin, respectively. Because diffusion is rate-limiting in this reaction sequence, the overall reaction time is reduced from approximately three minutes for the manual method to 3–15 seconds for the automated method. Reaction sequences in the Coulter S + II and the Technicon H*1 and H*2 are similar. Moreover, similar reactions are used in the other Coulter systems and in the TOA and Unipath instruments.

5. Summary

All automated hematology analyzers are comprised of flow cytometers, which count and size red cells, white cells, and platelets, and photometric hemoglobinometers, which measure hemoglobin concentration. All analyzers produce CBCs and most also produce MPV, RDW, and PDW. Automated CBC analyzers use either the basic aperture impedance method or one of the light-scattering methods to count and size cells. Automated analyzers that also produce five-part Diffs use some combination of d-c/r-f aperture impedance, light scattering, and cytochemistry to do so. One disadvantage of automated systems is the inability to identify abnormal cell types.

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