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OPTICAL PLATELET ANALYSIS

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Platelets or thrombocytes are disc-shaped cytoplasmic fragments, normally present in the peripheral blood. Circulating platelets are produced from megakaryocytes primarily in the bone marrow. Mature platelets, although small in size, normally between 2 and 20 fL, as compared to erythrocytes or leukocytes, play an important role in both primary hemostasis, along with the coagulation factors, and body's inflammatory response.

Platelet associated parameters

Quantitative and qualitative platelet disorders are common and the platelet count is vital in assessing the risk of a particular patient developing spontaneous bleeding. Blood platelet concentration (PLT) is a basic parameter of the complete blood count (CBC). Platelet indices (PI) — plateletcrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW) and platelet-large cell ratio (P-LCR) — are a group of derived platelet parameters obtained as a part of the automatic CBC. Mean platelet volume (MPV) is calculated by dividing the plateletcrit (PCT), analogous to the red cell hematocrit, by the number of platelets (1). The MPV, measured using automated blood analyzers, reflects the average size of platelets in circulation. It is known to indicate accelerated platelet synthesis by bone marrow in response to increased peripheral platelet destruction (2). PDW is the width of the platelet size distribution curve (3). Platelet-large cell ratio (P-LCR) is defined as the percentage of platelets that exceed the normal value of platelet volume of 12 fL in the total platelet count. It is calculated in automated blood analyzers using the formula:

P-LCR = P-LCC/PLT, where P-LCC is the platelet large cell count. Platelet size has been shown to reflect platelet activity; therefore MPV (=Mean Platelet Volume) and P-LCR are a simple and easy method of indirect assessment of platelet stimulation.

Platelet count methods

The four main procedures for platelet counting are: manual – phase contrast microscopy, impedance, optical light scatter / fluorescence and flow cytometry immunological counting.

Until recently, the manual counting method was the reference against which all automated methods were compared. Although time-consuming, it is still performed in the routine laboratory in the event of a low platelet count or if atypical platelets are present in the sample. The introduction of automated full blood counters resulted in a dramatic improvement in precision and reduced significantly the time required for analysis, when compared to a manual method. Still, automatic counters have their limitations and today's reference method for platelet count is a combination of fluorescence flow cytometry and aperture-impedance count (4).

In impedance platelet counting method, cells are regarded as completely nonconductive resistivity particles. When a blood cell suspended in electrolyte solution passes through the sensing zone of an aperture, a detectable change in electrical impedance proportional to the cell volume is observed. Each individual cell gives an impedance signal, which is proportional to the volume of the cell detected. Using this relationship between the impedance signal and

size of the cell / particle, they are further discriminated in to two cellular categories:

- Small cells – 2 to 30 fL – relating to platelets
- Larger cells – 30 to 200 fL – relating to red blood cell (RBCs) counting range.

A major disadvantage of the electrical impedance method in counting platelets is the difficulty in distinguishing large platelets from extremely microcytic or fragmented red cells, fragmented enucleated white blood cells and other similar sized particles, resulting in false increase in the reported platelet count. The difficulty is due to the discriminator used based on size to segregate platelets from RBCs. Conversely, for samples containing large platelets as well as platelet clumps with > 30 fL size (as observed in cases with EDTA-dependent- agglutinins) a false decrease will be reported. Finally, significant variance in reproducibility of results may be observed between different analyzers due to differences in analytical techniques used by different manufacturers.

More recently, optical light scattering methods have been introduced for counting platelets. The cells in a suitable diluent pass through a narrow beam of light (i.e., helium-neon laser). The illumination and light scatter by each cell is measured at a single angle (one-dimensional) or at two angles (two-dimensional) of light scattering. The volume and refractive index of individual platelets are simultaneously determined on a cell-by-cell basis. The two scatter measurements are converted to volume (platelet size) and refractive index (platelet density) values using the Mie theory of light scattering for homogenous spheres. Integrated analysis is then used to distinguish platelets, large platelets, red cells, cell fragments and debris (5). Platelets are identified within the map on the platelet scatter cytogram based on their volume and refractive index (1.35–1.40).

In optical fluorescent platelet count a polymethine dye is used to stain the RNA/DNA of reticulated cells and platelet membrane and granules. Within the flow cell, each single cell is passed through the light beam of a semiconductor diode laser. This technology allows the simultaneous counting of the red cell reticulocytes, erythrocytes and fluorescent platelets. The fluorescence intensity of each cell is analyzed, which allows the separation of platelets from red cells and reticulocytes.

Optical platelet detection on HORIBA analyzers

On PLTOx mode of Yumizen analyzers, two measuring methods, impedance and light extinction (absorbency), are combined in order to obtain the most reliable result (Figure 1).

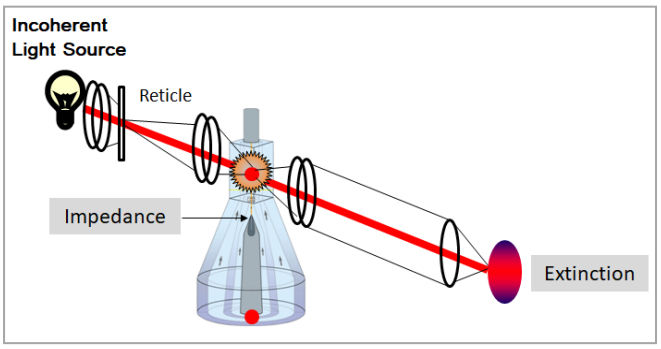

Figure 1: HORIBA Medical PLT Ox measuring method using an incoherent light source

The measurement principle is similar to the LMNE one. After sample dilution, the solution passes through the aperture of the LMNE flowcell and the optical window. Each cell is measured both in resistivity (volume) and optical extinction (cytochemistry). Platelet density or refractive index of cell determines the degree of light scattering and therefore, absorbency or optical extinction. From these measurements, a matrix is drawn up with volumes on the X-axis and optical absorbency on the Y-axis (Figure 2).

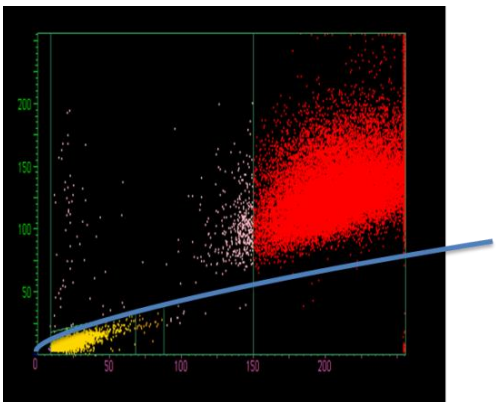

Figure 2: The displayed matrix for the PLT/RBC differentiation based on optical measurement

Since the impedance and optical measurements of each cell are done with a delay, there is an associating algorithm fusing both measures. This allows a matrix representation similar to a scattergram. Matrix representation allows a better classification when populations overlap on one or both measurements. Extinction light is sensitive to refractive index, allowing differentiating for the same size, a PLT from a RBC (Figure 2). At the same time, the instrument runs an RBC/PLT Low-Value cycle.

The PLTOPT parameter corresponds to the percentage of platelets relative to the RBC identified on the matrix, multiplied by the count of RBC identified in the measuring chamber. There is no need to calibrate this parameter individually since it relies on the calibration of the RBC.

The incoherent light source technology

The HORIBA measurement technology for platelet extinction is made using an incoherent light source (extended source). The high numeric aperture of the light beam allows the observation of cells from different angles revealing the morphology of the different cell compartments on the one hand, and intra-cytoplasmic spectroscopic characteristics on the other hand.

Contrary to a laser monochromatic light generating a perfectly parallel beam and consequently allowing a vision of the cell from one dimension, the measurement made using an incoherent light source is less vulnerable to cell anisotropy and its position or orientation in the beam, and gives information depending on the intrinsic characteristics of the cell (Figure 3).

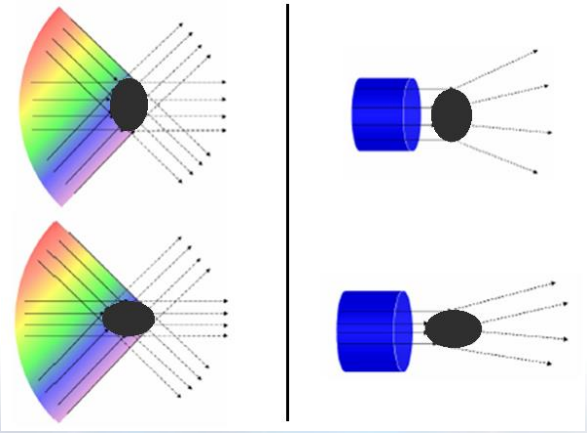

Figure 3: Spectral light scattering (left) compared to laser generated scattering (right)

With a spectral light source like Laser monochromatic light, the signal related to the characteristic of the cell is highly dependent on cell passage position and orientation at the measuring point and needs to be centered on the axis. Otherwise, the light response may be distorted and will give different results, as demonstrated in Figure 4.

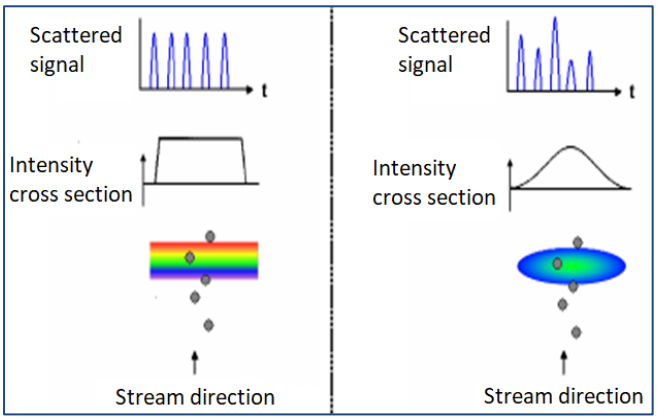

Figure 4: Spectral light (left) scattering compared to laser generated scattering (right)

When any light falls on a cell, the light is diffracted by the cell in a specific way. The light scattering properties are related to cell morphology. The numerical aperture (NA) of an optical system is a measure for its angular acceptance for incoming light. In PLT study with laser based technology, the NA is smaller and corresponds to about 11% of the total diffracted light. With HORIBA incoherent light technology, the greater NA gives a diffracted light corresponding to about 88% of the total diffracted light (figure 5); the analysis is therefore much better and accurate.

Immunological platelet count

The principle of immunological counting consists in labelling EDTA-anticoagulated blood with a suitable antiplatelet monoclonal antibody which has been conjugated with a fluorophore (i.e. fluorescein isothiocyanate FITC). A number of such antibodies have been described, including anti-CD42a; anti-CD41b; anti-CD61; and anti-CD41, anti-CD42, and anti-CD61.

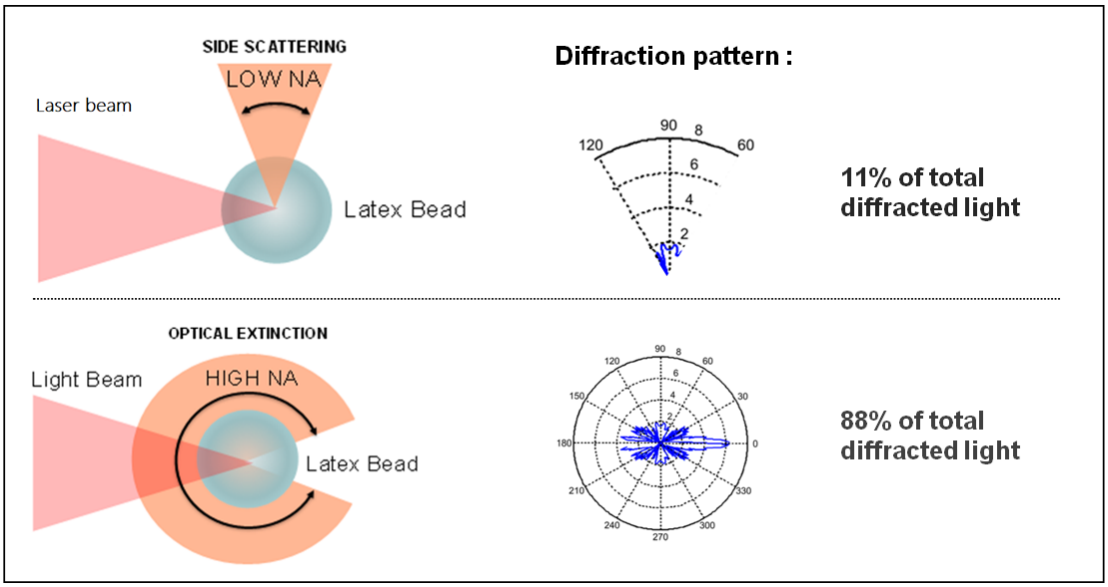

Figure 5: Laser and incoherent light beam technologies and their corresponding numerical apertures (NA) and diffraction patterns

The sample is then analyzed using a flow cytometer. The method derives the platelet count from the ratio of fluorescent platelets to red cells within the sample. Immunological platelet counting performed with a flow cytometer is accurate and reliable; however, this technique would have a significant cost if applied to all the samples in a fully automated blood cell counter.

The reference platelet count method

For the assignment of values of hematology analyzer calibration and control materials, the International Council for Standardization in Haematology (ICSH) and the International Society of Laboratory Hematology (ISLH) recommend the counting of specifically labeled platelets relative to the RBCs with a fluorescence flow cytometer, together with an accurate RBC count determined with a semiautomated, single-channel aperture-impedance counter as a reference method for the enumeration of platelets.

Therefore, several methods and technologies are combined to achieve the most reliable result. Fresh EDTA-anticoagulated venous blood specimens are measured within 4 hours of the draw. The specimen is prediluted and the platelets labeled with two monoclonal antibodies specific to a cluster of differentiation (CD) common to all platelets. At least 50,000 events with a minimum of 1,000 platelet events are counted with a flow cytometer to determine the RBC/platelet ratio. The platelet count is then calculated from this ratio and the RBC concentration of the original blood specimen (6).

Inference

Platelets are complex blood components that have varied functions. Their number, shape, volume, and derived associated platelet indices are markers of different pathological states (7, 8). Platelet counting technologies have evolved over time and the current reference counting method combines several technologies. Given the price and limited access to these technologies, the reference counting method cannot be applied to all the samples in routine analysis.

With its unique incoherent light technology combined to impedance count and clear matrix representation of RBC/PLT ratio, HORIBA analyzers provide an optimal solution, as close as possible to the reference method, to this multifaceted routine analysis.

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