LEARNING GUIDE

PLATELET COUNTING

CORELABORATORY.ABBOTT/HEMATOLOGY

Abbott

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SECTION 1

THE DISCOVERY OF PLATELETS PLATELETS FUNCTION

INTRODUCTION

Platelets are the smallest cell components occurring in blood **(Figure 1)**. They are produced in the bone marrow by cytoplasmic fragmentation of megakaryocytes and hence platelets are non-nucleated. Despite their small sizes, platelets are of vital importance, since they play a key role in the hemostatic system that protects the host from excessive blood loss after vascular injuries. The lack of a nucleus and their small size represent major technical challenges for the hematology laboratory in platelet counting in terms of precision and accuracy, particularly in conditions where blood may contain interfering substances.

In the healthy population, blood platelet levels range between 150 x 10^9/L and 400 x 10^9/L and this concentration is more than sufficient to support adequate coagulation, even after trauma. However, if the platelet count is decreased, excessive bleeding might occur after minor trauma. At very low platelet concentrations, spontaneous bleeding might occur, which could even be life-threatening to a patient.

Inverse relationship exists between platelet count and bleeding: the risk of bleeding increases as platelet count decreases below a certain threshold. This implies that the hematology laboratory needs to be able to report accurate platelet values, especially in patients with low platelet counts (thrombocytopenia), in order to enable the clinician making a good estimation of the bleeding risk and making a well-considered decision on giving platelet transfusion or not. Due to the nature of platelets and limitations in technology, it is not always easy to enumerate platelets precisely and accurately. This is especially true when platelet counts drop to low (< 50 x 10^9/L) and very low (< 20 x 10^9/L) values.

A further complication is that in some disease states, non-platelet particles may interfere in the platelet count.[1] The degree of interference is mainly a consequence of the technology used by the hematology analyzer, as will be illustrated in later chapters. It means that clinical laboratory professionals should be aware of the technological limitations of their hematology analyzer in order to recognize potentially unreliable platelet results. They should develop procedures for alternative counting methods in order to provide clinicians with platelet counts of sufficient reliability for basing diagnostic and therapeutic decisions on, especially when dealing with severely thrombocytopenic patients.

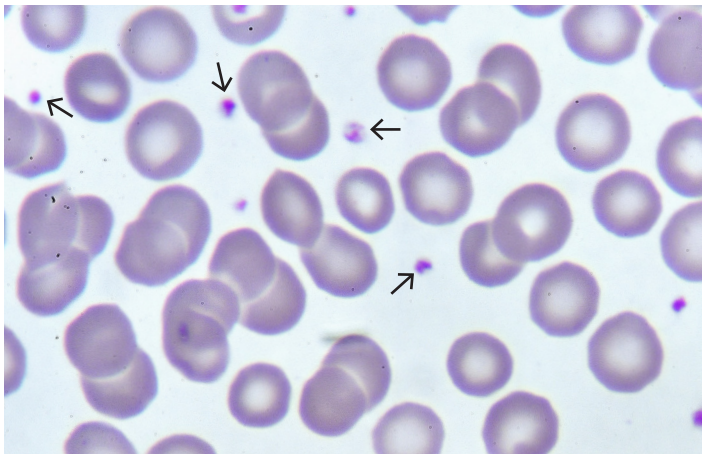

Figure 1. Normal Platelet Morphology from a Blood Smear. Platelets in circulation are anucleate discoid cells.

THE DISCOVERY OF PLATELETS

The honor of first describing platelets and their association with hemostasis belongs to Max Schultze (1865, **Figure 2**) and Guilius Bizazarro (1882) respectively. Their contibutions to the discovery of platelets have been expertly reviewed by Brewer (2006). For his part, Schultze was able to describe the morphology of platelets and noted "Because of their pallor and very small size, which is 6-8 times smaller than the red cells, the individual spherules can only be recognized with a good strong lens." [2] Bizazerro was able to build on the morphological observations of Schultze, nothing, "in addition to the red and white blood corpuscles, a third sort of morphological element circulates in the blood vessels. In form, they are very thin platelets, disc shaped, with parallel surfaces or rarely lens-shaped structures, round or oval and with a diameter of the red cells." [2] More importanly, however, Bizazerro was able to observe that "Whereas under normal conditions they (the platelets) float isolated in the plasma, when subject to an influence that leads to thrombosis, they adhere one to another to form a plug. The blood platelets, free in the blood stream and being hurried along are held up by other platelets that they come into contact with as they become stickier than they are under normal conditions." [2]

Figure 2. Max Schultze (1825-1874)

PLATELET FUNCTION

Platelets themselves play a key role in primary hemostasis. Studies of their function at the cellular and molecular level in health and disease have been exhaustive and an extensive review of the literature on the subject is beyond the scope of this monograph. However, at the basic level, it is worth noting that the process, by which platelets initiate and propagate the formation of a primary clot, follows distinct stages.

The initial stage is known as platelet adhesion. In this stage, the platelets come into contact with the negatively charged surfaces of the exposed endothelium of damaged tissues and adhere to them. This process is principally mediated through interactions between platelet surface glycoproteins (particularly GPlb), Von Williebrand's factor and collagen fibers of the sub-endothelium.[3]

The next stage is platelet activation. In this stage, platelets stuck to the collagen undergo a series of morphological and biochemical changes. Mediated through cleavage of membrane phospholipids, biochemical signals begin a sequence of changes during which intracellular calcium levels rise and the glycoprotein llb/llla complex is expressed on the surface of the platelet. These biochemical changes are associated with distinct morphological changes. Rather than remaining discoid, the activated platelets become more spherical with distinct irregular pseudopods being projected from their surface. Once activated, the platelet membranes begin to form a template on which the blood coagulation process is enhanced. The process of platelet activation also initiates release of powerful secondary aggregating agents from the platelet granules.

The release of platelet agonists like ADP and Thromboxane A2 causes propagation of the final process, which is called platelet aggregation. During aggregation, the exposed glycoprotein llb/llla complex on the extended pseudopods causes platelets to bind together in aggregates to reinforce the primary platelet/fibrin clot **(Figure 3)**.

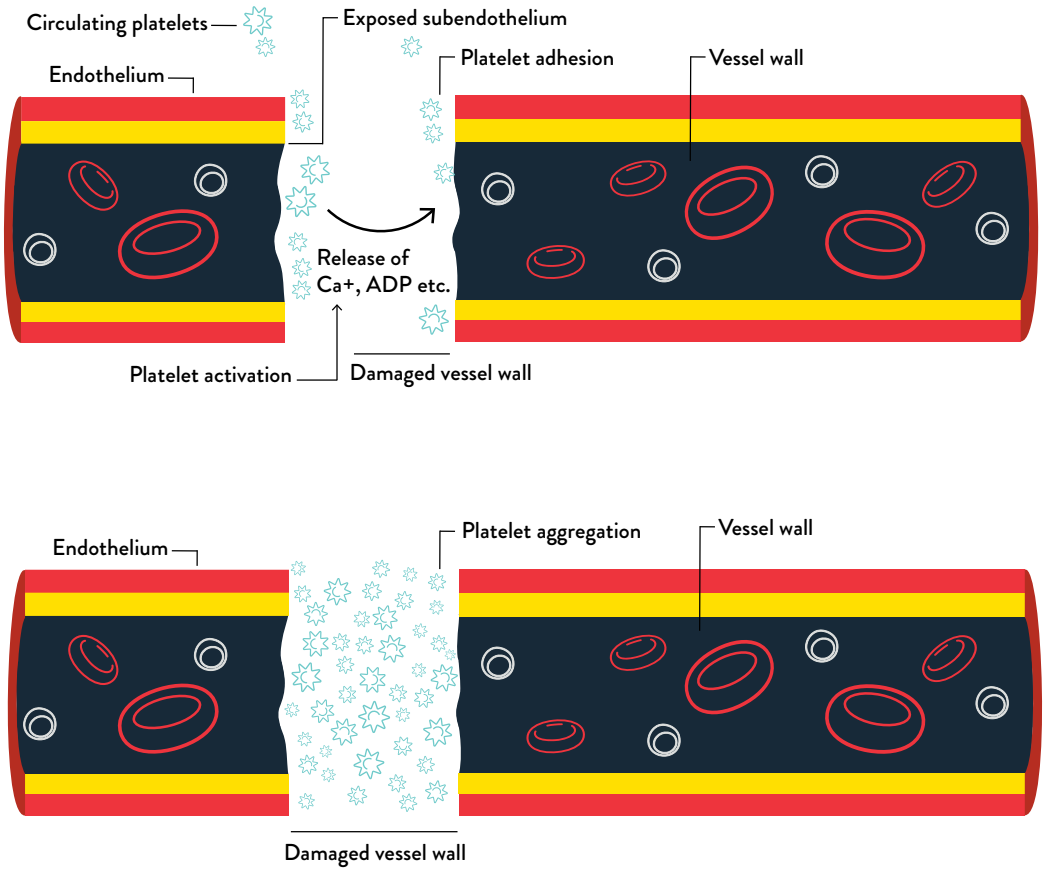

Figure 3. Platelet adhesion, activation and aggregation.

THE NEED TO COUNT PLATELETS

The platelet count has become an accepted part of the automated blood count. The clinical value of platelet counting is a critical aspect of patient diagnosis and treatment monitoring, and in routine wellness check and pre-surgical screening **(Figure 4)**. Reported reference ranges for the platelet count in normal subjects typically fall in the range of 150 x 10^9/L to 400 x 10^9/L. Demonstration of thrombocytopenia (low platelet count) or thrombocytosis (high platelet count) are valuable findings in the context of disease diagnosis.

SCREENING CBC
Pre-operative screening, general wellness

DISEASE MONITORING
To determine trend in patients with abnormal platelet count that are secondary to disease processes

THERAPEUTIC MONITORING
Increase or decrease in platelet count

PLATELET TRANSFUSION
Assessing severity of thrombocytopenia, risk of severe bleeding and need for platelet transfusion

Figure 4. Platelet count uses.

QUIZ QUESTIONS

1. Name two investigators credited with the discovery of platelets:

   _____

   _____

2. Which platelet function is associated with the initial stage of platelet adhesion?

   A. Rising levels of intracellular calcium

   B. Expression of the GPIIb/IIIa receptor

   C. Release of ADP

   D. Adherence to negatively charged surfaces on the damage endothelium

3. Which platelet glycoprotein functions in binding platelets to each other during platelet aggregation?

   A. GPIIb/IIIA

   B. GPIb

   C. GPIa/IIa

   D. GPV

SECTION 2

MEGAKARYOPOIESIS AND THROMBOPOIESIS

Under the control of various growth factors and cytokines, of which thrombopoietin (TPO) is the most important,[5,6] the pluripotent stem cell differentiates into a megakaryoblast by a number of sequential steps. Intermediate stages are the myeloid stem cell and the committed progenitor cell of the megakaryocytic cell line. Once the megakaryoblast stage is reached, the cell loses its proliferative capacity and starts its maturation process. The process of megakaryocyte development and maturation is called **MEGAKARYOPOIESIS**.[4] Megakaryopoiesis is a complex, stepwise process that takes place largely in the bone marrow. At the apex of the hierarchy, hematopoietic stem cells undergo a number of lineage commitment decisions that ultimately lead to the production of polyploid megakaryocytes. Megakaryopoiesis has a unique way of maturation that does not occur in other cell lineages - endomitosis: the cell multiplies its nuclear material within the same cell. In other words, endomitosis is nuclear division without cell division. Eventually maturation results in a mature megakaryocyte, which possesses multiple DNA copies. The nuclear ploidy of a megakaryocyte is normally between 8N and 64N, while higher and lower ploidy may occur in pathological conditions.

The final stage of a series of events that commences with the generation of the pluripotent hematopoietic stem cell in the bone marrow is called **THROMBOPOIESIS**. It mainly, but not exclusively, occurs through the organization of cytoplasmic extensions (proplatelets) that fragment and are released as platelets into the bloodstream[4] **(Figure 5)**. Depending on the physiological need of new platelets, endomitosis stops and the formation of platelets commences. It starts with the intracytoplasmic formation of channel-like structures composed of lipids, called the membrane demarcation system. These lipids later assemble into membrane bilayers and form the cell membranes of pro-platelets when the megakaryocyte cytoplasm starts to disintegrate. Eventually, megakaryocytes form pseudopodia-like extensions protruding into sinuses and release platelets into the bone marrow, from where they rapidly migrate into peripheral blood. A healthy adult produces approximately 1–2 million platelets per second.

Cell volume of megakaryocytes expands in synchronization with nuclear ploidy. Ploidy eventually determines the number of platelets that a megakaryocyte will produce.[5,6] One single mature megakaryocyte can generate up to 5000 platelets. When platelets are released from the megakaryocyte cytoplasm, they still contain small amounts of nucleic acids. The latter portion represents the youngest platelets in the circulation and is named reticulated platelets (retPLT), analogous to reticulocytes in erythropoiesis.

It is not precisely known how thrombopoiesis is regulated in humans, but it is generally assumed that TPO plays a role here. In steady-state conditions, the production of platelets is aimed at keeping the total circulating platelet mass (Platelet number x Mean Platelet Volume, also called plateletcrit) constant.[5] During stress, platelets are released from megakaryocytes at an earlier stage, which results in larger platelets.

Each individual has their own set point for platelet count and platelet volume; most likely these are under genetic control and platelet count seems to be tightly regulated under normal conditions.[7] As a consequence, intra-individual variations in platelet count are quite small in comparison with the population reference ranges. In the normal population, platelet count is inversely correlated with mean platelet volume, and consequently the total circulating platelet mass is more or less constant among individuals.[8]

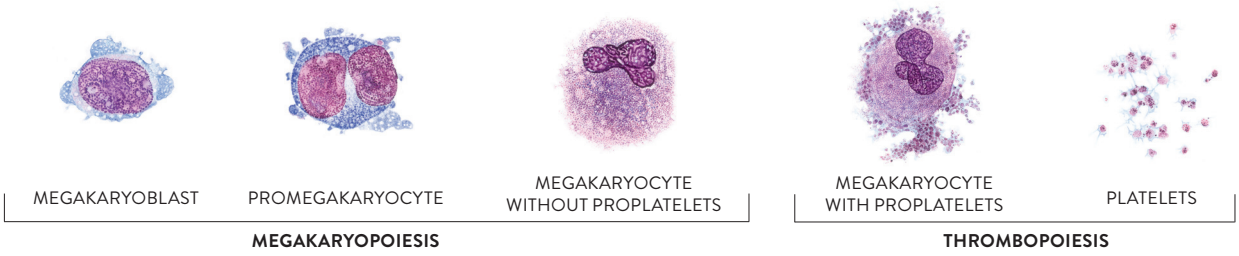

Figure 5. Megakaryocytes are derived from pluripotential hemopoetic stem cells that have undergone expansion, differentiation and maturation under the control of the glycoprotein hormone thrombopoietin and other growth factors. Platelets are formed from the cytoplasmic budding of megakarocytes.

QUIZ QUESTIONS

1. One single mature megakaryocyte can produce up to 5000 platelets.

   A. True

   B. False

2. The youngest platelets in the circulation are called _____.

3. Which of the following molecules is responsible for regulating thrombopoiesis in humans?

   A. ADP

   B. TPO

   C. MPO

   D. TXA2

SECTION 3

PRE-ANALYTICAL FACTORS OF PLATELET COUNTING

CHOICE OF ANTICOAGULANT

Like all blood cells, platelets are analyzed in blood that is made non-clottable with an anticoagulant. In regular laboratory practice the recommended anticoagulant for hematologic investigations is the dipotassium salt of EDTA (K_2 -EDTA), as this causes the least changes in blood cells.[9] It is known that the platelet count remains stable in EDTA blood, but mean platelet volume (MPV) does not.[10,11] In general, MPV increases over time after blood draw, although this may depend on the technology used in the analyzer.[12] Recently it was found that even the brand of K_2-EDTA is a factor that can cause clinically significant pre-analytical variation.[10] Furthermore, it is important to realize that the quality of blood collection tubes is not always in agreement with specified criteria.[14]

Anticoagulants other than EDTA are not recommended for cellular analysis, however citrate-based anticoagulants may be a preferred option for special studies on MPV, because citrate does not affect MPV after blood draw.[15,16]

EDTA-INDUCED PSEUDOTHROMBOCYTOPENIA

Spuriously low platelet counts due to EDTA-induced platelet agglutination is frequently observed in any medical laboratory; the incidence ranges from 0.07–0.20 % in unselected donors to as high as 2.0 % in hospitalized patients (1, 14–16). There is no apparent association with disease, although some reports suggest that this phenomenon is more frequent in autoimmune diseases and hepatitis A virus infection.[20]

Classically, EDTA-induced platelet agglutination is caused by auto-antibodies, which normally do not react with platelets. When blood comes in contact with EDTA, a chemical that binds calcium ions, the spatial structure of the platelet surface proteins is altered. As a result, these surface proteins now become accessible to the auto antibodies causing the platelets to stick together in large agglutinates, thereby reducing the number of free platelets in the blood sample (pseudothrombocytopenia). EDTA agglutination is purely an in vitro phenomenon, and the antibodies have no in vivo effect.[21–23] Some authors have reported that EDTA-induced thrombocytopenia may also be dependent on temperature and time.[24]

Recently it was recognized that EDTA-thrombocytopenia is not limited to auto-antibodies, as several cases have been reported where therapeutic monoclonal anti-platelet antibodies were involved.[25–27] In addition, it was postulated that not only EDTA, but also hirudin caused anticoagulant-induced pseudothrombocytopenia.[28]

A closely related phenomenon, supposedly due to the same mechanism but much less frequent, is formation of platelet-neutrophil agglutinates or platelet satellitism.[1, 29–31] Even rarer is satellitism of platelets around malignant lymphoma cells.[32] This can lead to the reduction in platelets counts.

Alternatively, anticoagulant mixtures containing calcium or magnesium have been demonstrated to effectively prevent EDTA-induced platelet agglutination.[35,36] This means that it is no longer necessary to collect another blood sample in an alternative anticoagulant, as is still common practice in many laboratories.

OTHER PRE-ANALYTICAL FACTORS

Apart from platelet agglutination, poor blood collection technique is another frequent source of falsely low platelet counts, irrespective of the measuring technology. After prolonged stasis or when blood is not properly mixed with anticoagulant during specimen drawing, the blood coagulation system can become activated and small clots may form, consequently resulting in spuriously low platelet counts.[37]

QUIZ QUESTIONS

1. Choose the correct statement below concerning MPV.

   A. The MPV decreases over time in blood collected in K2-EDTA.

   B. The MPV increases over time in blood collected in K2-EDTA.

   C. The MPV remains stable over time in blood collected in K2-EDTA.

   D. The MPV initially increases then decreases over time in blood collected in K2-EDTA.

2. The presence of autoantibodies in a blood sample has an in vivo or in vitro effect on platelet counting.

   A. in vivo.

   B. in vitro.

SECTION 4

PLATELET COUNTING METHODS

MANUAL / MICROSCOPY

INDIRECT METHODS

Probably the first method for quantifying platelets in blood was the Fonio technique, developed by a Swiss physician in the 1940's. It is performed in a thin blood smear that is stained as for white blood cell differentiation. Using a microscope with high magnification, the number of platelets is counted in relation to the red blood cells. In a separate assay, the red blood cell concentration is quantitatively determined and finally, the platelet concentration is calculated from the platelet/red cell blood ratio. The advantage of this method is that platelets can be clearly distinguished from other small particles by their specific morphological characteristics. Disadvantages are that the method is highly imprecise and time consuming. Some laboratories continue to use this method as an approximate estimation for verifying automated platelets counts.

DIRECT METHODS

Several years after Fonio's description, Feissly and Ludin suggested the use of phase-contrast microscopy for platelet counting. This method was improved by Brecher and Cronkite and was later adopted as the international reference method for platelet counting. A small amount of diluted, hemolyzed blood is brought into a calibrated glass hemocytometer (counting chamber) with an engraved network of known dimensions, which enables counting platelets in an exactly defined volume of fluid **(Figure 6)**.

In skilled hands phase-contrast microscopy is accurate enough for clinical purposes, but the method has two major disadvantages: high imprecision and potential interference by particles other than platelets. The high imprecision is a direct consequence of the relative low number of platelets counted in the hemocytometer, particularly in thrombocytopenia. Coefficients of variation of 20–40 % are not uncommon at all. Interference may occur in conditions where small-sized cellular fragments of non-platelet origin circulate in blood. These may be very difficult to distinguish from platelets. Leukocyte cytoplasmic fragments in some forms of leukemia are a good example of this interference. In addition, microscopic platelet counting is time-consuming and requires a high level of technical expertise, which is disappearing from many clinical laboratories after the widespread introduction of automated platelet counting in hematology analyzers. In 2001, phase-contrast microscopy was replaced as the international reference method and has become obsolete since.

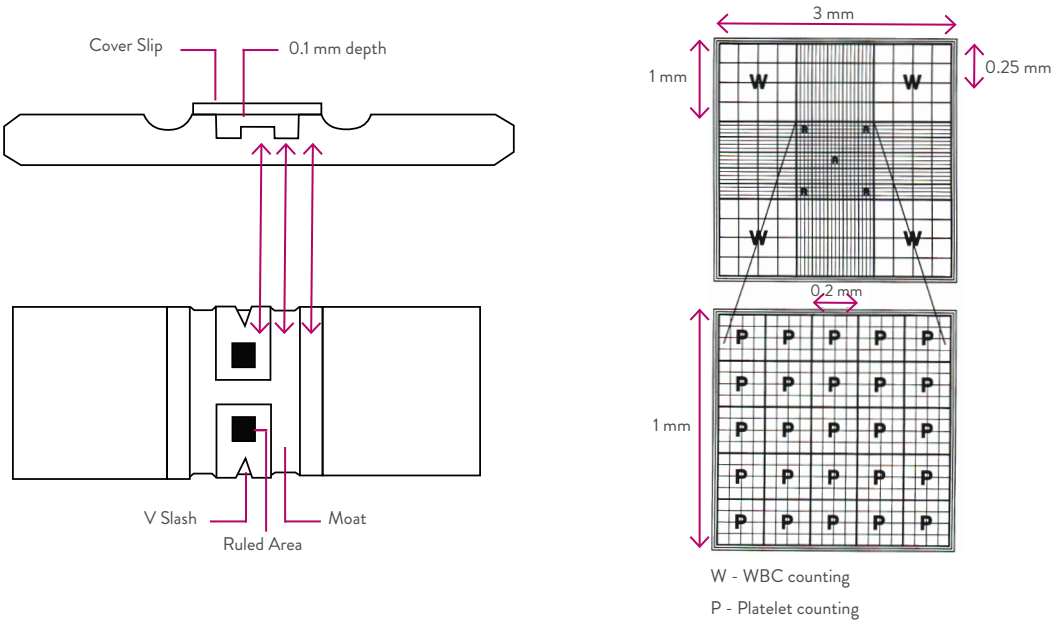

Figure 6. Phase contrast microscopy using hemocytometer

IMPEDANCE

The principle of impedance counting, also known as the Coulter principle after its inventor Wallace Coulter, is the passage of cells suspended in a known dilution through a small orifice. The electrolyte-containing diluent serves as a conductor of a constant electrical current between two electrodes. Cells are poor electrical conductors and as they pass through the orifice, they impede the passage of current, which is detected as an increase in electrical resistance. Each cell will cause a resistance pulse, thus allowing cell counting. Furthermore, the magnitude of the resistance peak is directly related to cell volume.[38] Impedance counting is schematically illustrated in **Figure 7.1.**

Impedance platelet counting is typically performed in the presence of red blood cells (RBC), which are counted simultaneously. Platelets are differentiated from RBCs based on histogram analysis of the accumulated resistance events, meaning their size. Thresholds are used to find optimal separation between the two cell populations. Over years of development, technical nuances have been introduced into impedance counting for improving accuracy. One of the known limitations of impedance counting is the potential for a phenomenon called "recirculation" that can cause falsely increased cell counts. This phenomenon (shown in **Figure 7.2** occurs when cells that have traversed the orifice become caught in eddy currents behind the orifice. These cells recirculate in and around the detection zone and can be recounted, which obviously results in spuriously higher counts.

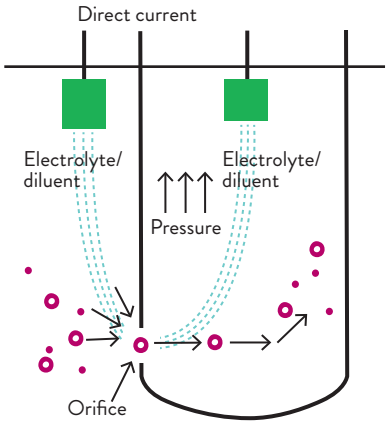

Figure 7.1. Principle of cell counting using impedance technology. A vacuum draws the cells suspended in conductive diluent from left to right through the orifice. Passage of each cell is registered as a peak in electrical resistance between the two electrodes.

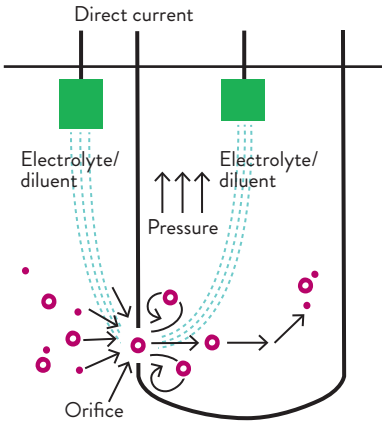

Figure 7.2. Recirculation of cells trapped in the eddy current just behind the orifice, giving rise to false recounts.

Various approaches to resolve this artifact have been applied. Some instrument manufacturers use lateral flow of reagents to sweep already counted cells away from the detection zone. Other manufacturers use a plate close to the orifice, which ensures that any cell recirculation takes place away from the detection zone. These devices are called after their inventor, von Behren's plates **(Figure 7.3)**. Another alternative is the use of hydrodynamic focusing. This technique employs a sheath of fast moving fluid that guides and confines the cell suspension, ensuring that during analysis the cells are continuously propelled forwards through and beyond the orifice and therefore away from the impedance detection zone **(Figure 7.4)**. One further advantage of using hydrodynamic focusing is that it focuses the cells on the very center of the orifice Instruments that do not use hydrodynamic focusing are prone to what is known as the "edge effect". This phenomenon implies that cells flowing through a simple bulk flow transducer may traverse the orifice at its center, but may also pass at the periphery of the orifice. The consequence then is an irregular impedance profile, resulting in false estimates of cell size **(Figure 7.5)**. Although electronic and algorithmic editing of the pulses can correct this phenomenon to some extent, hydrodynamic focusing is the preferred means of resolving edge effects.

Impedance analysis has some benefits. The method has historically been widely accepted and from an economic perspective, impedance detectors can be cheaply manufactured. The disadvantage of impedance analysis is that the discrimination between platelet and non-platelet events is purely based on size. Although normal sized platelets and normal sized RBCs show little overlap, this may not be true in cases of pathology. Specific examples of poor impedance separation are shown in **Figures 7.6B** and **7.6C**. In some impedance analyzers attempts have been made to optimize separation between platelets and RBCs, for example by using dynamic thresholding to find valleys between the two cell populations. Alternatively, software algorithms have been developed that improve the accuracy of platelet counts in comparison with the use of fixed thresholds.

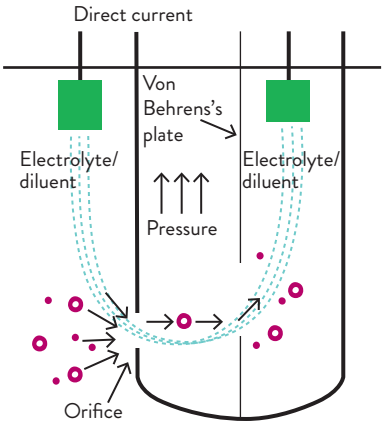

Figure 7.3. A von Behren's plate prevents recirculation in the zone behind the orifice.

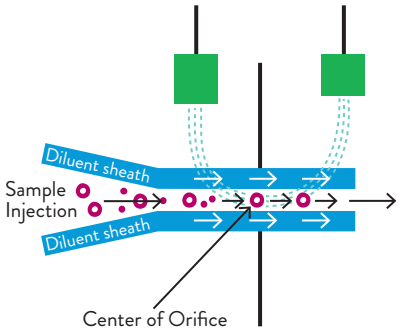

Figure 7.4. Hydrodynamic focusing prevents recirculation as well as the edge effect.

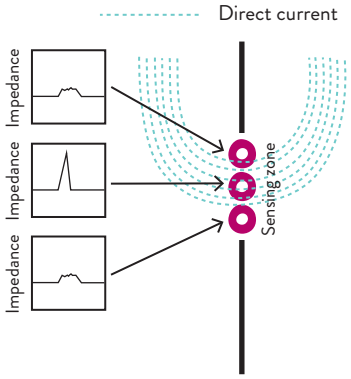

Figure 7.5. The "edge" effect causes cells flowing non-centrally through the orifice, resulting in irregular impedance signals that no longer represent the volume of the cells.

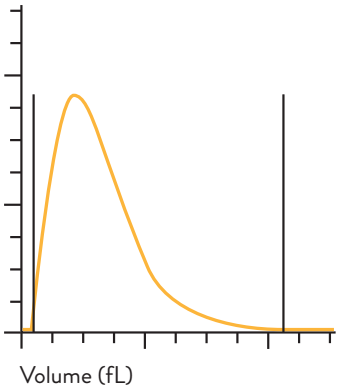

Figure 7.6A

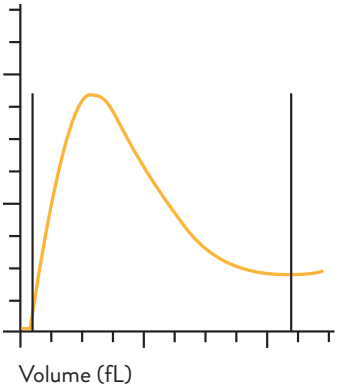

Figure 7.6B

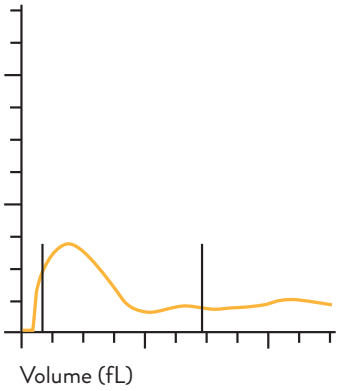

Figure 7.6C

Figure 7.6. Histograms of CELL-DYN Sapphire platelet impedance measurements. 7.6A: (left) normal platelet count and normal MCV; no overlap with RBC. 7.6B: (center) normal platelet count and low MCV; clear overlap between platelets and microcytic RBC. 7.6C: (right) very low platelet count; the separation between platelets and non-platelets is difficult to define.

OPTICAL

Optical platelet counting is based on the light scatter properties of blood cells and this technology is being used by several manufacturers. Using flow cytometry, either two angles of light scatter are measured or single-angle light scatter in combination with fluorescence that is generated by a dye binding to platelets is measured. The advantage of a two-dimensional approach is that the resolution between platelets and non-platelet particles is not based on size alone and therefore is more specific.[40]

In general, the low-angle scatter signal represents volume, whereas the higher-angle scatter is derived from the cellular density. In the case of platelets, higher-angle scatter mainly represents granulation. In CELL-DYN Ruby, 0° and 10° light scatter are used, resulting in excellent separation between platelets and RBC **(Figure 8.1)**. CELL-DYN Sapphire utilizes 7° and 90° scatter and although the scatterplot looks somewhat different, it allows accurate and specific delineation of the platelet cluster **(Figure 8.2)**.

The precision of the CELL-DYN Sapphire is high because the instrument is able to monitor the cell count as platelet events are being accumulated. If the analyzer detects a reduced platelet count, then it automatically extends the platelet data acquisition time, thereby increasing the number of platelet events to be counted. This eventually results in improved counting statistics.[41] Also the fluorescent method can reach very good precision.[42] However, this method seems to suffer from systematic bias when compared with the immunological international reference method.[42,43]

Despite two-dimensional optical counting being significantly less prone to interference by non-platelet particles than impedance technology, there are still rare conditions where optical methods are sensitive to interference.

A newer version of the optical method for counting platelets is the Advanced MAPSS technology. This technology utilizes 5 scatter signals (ALL, PSS and 3 IAS signals) to differentiate platelets from red blood cells based on size and internal complexity of the cells. These multiple angles of scatter measurement enable better separation of RBC from platelets, even in the presence of very small RBC or RBC fragments **(Figure 8.3)**.

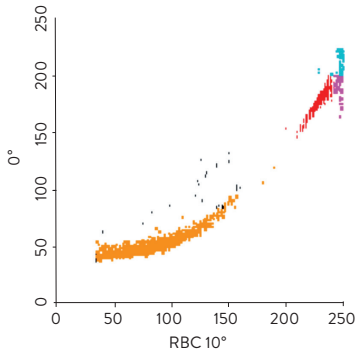

Figure 8.1. Optical platelet scatterplot of CELL-DYN Ruby, displaying 0° against 10° light scatter. The platelets (yellow dots) are well separated from non-platelet particles (black dots) and from the red blood cells (red dots).

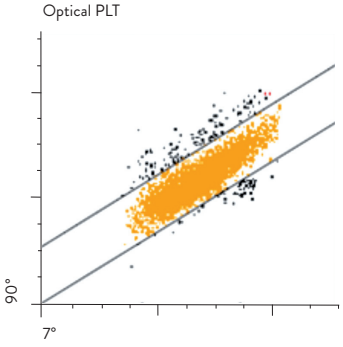

Figure 8.2. Optical platelet scatterplot of CELL-DYN Sapphire showing 90° against 7° light scatter. The two lines are dynamic thresholds for separating platelets (yellow dots) from non-platelet particles (black dots), which can be located both above and below the platelet cluster.

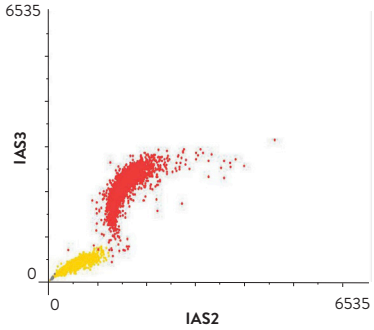

Figure 8.3. Intermediate angles of light scatter plot. This scatterplot shows the separation of the PLT and the RBC populations based on two angles of intermediate light scatter (IAS2 vs. IAS3). RBC (red) smaller in size are positioned near the platelet cluster (yellow); however, differences in the internal complexity of RBC and PLT enables differentiation between them. This suggests that the platelet count is not affected by the presence of microcytic RBC. In patients with normocytic RBC, there is a wider separation between the RBC and PLT populations due to differences in both size and internal complexity.

IMMUNOLOGICAL

The most reliable technology for measuring platelets is based on monoclonal antibodies to platelet-specific surface antigens. The international reference method employs dual-color immunofluorescence flow cytometry using a mixture of two different antibodies, CD41 and CD61.[44] Platelets are identified by their reaction with these antibodies and the platelet/erythrocyte ratio is determined by selective gating. Subsequently, erythrocytes are counted in a separate hematology analyzer, which eventually allows calculation of the platelet concentration. This ICSH reference method is a two-platform technique that cannot be fully automated.[44] This fact and the requirement for an experienced flow cytometrist render it difficult to perform the reference method on a routine basis in the hematology laboratory.

The CELL-DYN Sapphire offers a variant of the ICSH reference method. It is a fully automated, single-color flow cytometric technique using CD61 monoclonal antibodies that can easily be run in a routine setting by staff that is not experienced in flow cytometry. It is essentially identical to the method that was developed for the CELL-DYN 4000 back in the 1990's.[45,46]

In short, the analyzer dispenses a small aliquot of blood into a tube that contains lyophilized FITC-labeled CD61 monoclonal antibodies. When the reaction mixture incubates, the CD61 monoclonal antibodies bind to specific epitopes on the platelet surface membrane. Subsequently, the mixture is further diluted and then passed through the optical flow cell of the analyzer. Two angles of light scatter are measured (7° and 90°), along with the FL1 fluorescence signal that comes from cell-bound FITC, representing CD61. Through knowledge of the dilution used, the flow rate and the duration of analysis, the instrument is able to directly calculate the concentration of platelets, the CD61 positive events. CD61 negative, non-platelet events are automatically excluded from the analysis **(Figure 9.1)**.

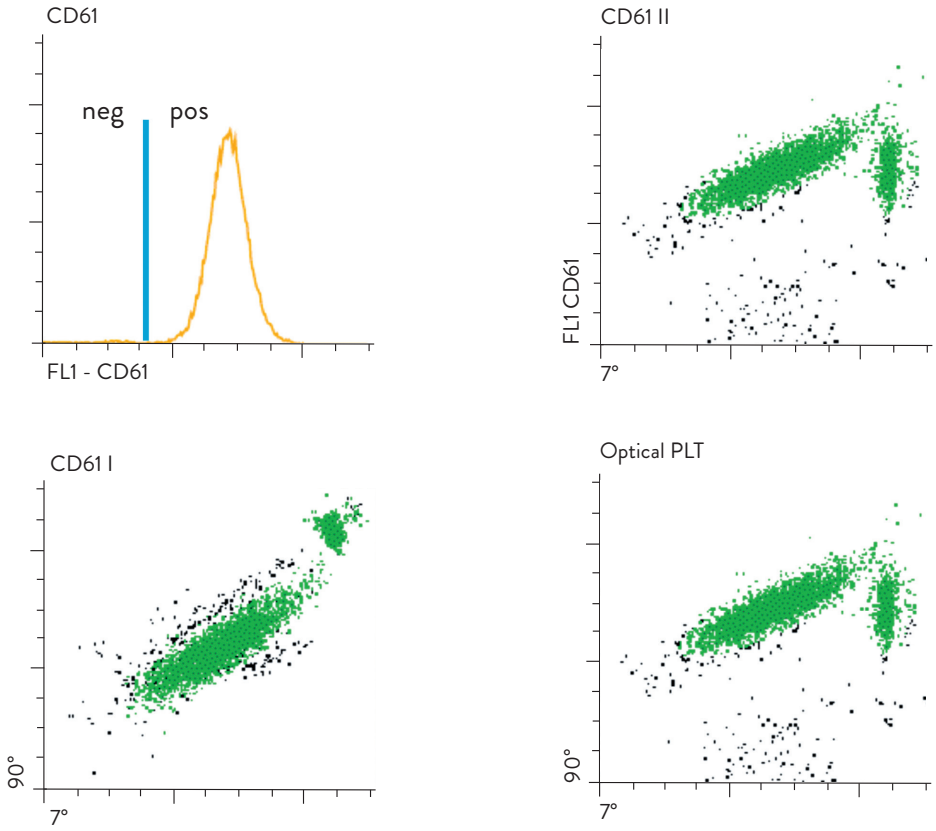

Figure 9.1. The CELL-DYN Sapphire CD61 immunoplatelet method. Upper left: histogram of FL1 fluorescence for defining CD61-positive events as platelets. Upper right: FL1 against 7° scatterplot showing platelets (large green cluster) as well as platelet-erythrocyte coincidence events (small cluster on the right). Lower left: 90° against 7° scatterplot in which all CD61-positive events are colored green and non-platelet events colored black. In this plot the dynamic thresholds are identical with those in the Optical PLT scatterplot (lower right).

Several studies have demonstrated very close correlation between the CD61 immuno-platelet method and the ICSH reference method. [47,48] Moreover, it has been shown that the precision of the Sapphire CD61 method is excellent, possibly even better than the CD41/CD61 ICSH reference method.[47] In severe thrombocytopenia with platelet counts ranging between 5 x 10^9/L and 10 x 10^9/L, the coefficients of variation of the CD61 assay were found to be 1.6–2.3 % only, whereas the corresponding data of the ICSH reference method were 3.8–5.6 %.[41] Some authors recommend the CD61 immunoplatelet assay as the method of choice for samples with low platelet count and from neonates.[49]

QUIZ QUESTIONS

1. Name the platelet counting method that is associated with discrimination between platelet and non-platelet events based solely on size:

   _____

2. In optical platelet counting using a two-dimension approach, resolution between platelets and non-platelet particles is more specific since it is not based on size alone.

   A. True

   B. False

3. Which of the following is the ICSH (International Committee for Standardization in Haematology) reference method for platelet counting?

   A. Impedance

   B. Optical

   C. Immunological

   D. None of the above

SECTION 5

INTERFERENCES IN PLATELET COUNTING

All platelet counting methods are potentially susceptible to interferences, but the degree of interference depends on the technology used in the analyzer. In general, one-dimensional methods like impedance are most prone to interference, since particles having similar size as platelets are counted as platelets. The two-dimensional optical methods are less sensitive to interference and interference in the immunological methods is virtually nonexistent.

Interfering factors can lead to both spuriously low and spuriously high platelet counts, where overestimation is the more common phenomenon. Overestimation is of major clinical significance, as it may give a false sense of security in patients with severe thrombocytopenia and may result in withholding platelet transfusions. On the other hand, underestimation of platelet count may lead to unnecessary additional diagnostic investigations and unnecessary therapeutic interventions. Therefore, it is imperative that clinical laboratory professionals are fully aware of the limitations of their platelet counting method(s) and have alternatives available that can provide the accuracy and precision required for the patient's clinical condition. **Table 10** gives an overview of possible interferences in platelet counts.

FALSE INCREASES	Overestimation may give a false sense of security in patients with severe thrombocytopenia and may result in withholding platelet transfusions.
RBC fragments	• Extremely microcytic erythrocytes as in iron deficiency or thalassemia • Microangiopathic hemolysis with schistocytes • Acute burns with microspherocytes
WBC fragments	White cell cytoplasmic fragments (e.g. in acute leukemia or lymphoma)
Microcytes / Bacteria	Bacteria, fungi, malaria parasites
Protein aggregates	Cryoglobulins, Cryofibrinogen
Immune complexes chylomicrons	Hyperchylomicronemia, Lipid-rich parenteral nutrition
FALSE DECREASES	Underestimation may lead to unnecessary additional diagnostic investigations and unnecessary therapeutic interventions.
Platelet clumps	• EDTA-induced platelet aggregation • Platelet-neutrophil satellitism • Poor sample quality due to clotting
Activated platelets	Degranulated platelets (when optical methods are used)
Abnormal platelet size	• Giant platelets e. g. in Bernard-Soulier syndrome, May-Hegglin anomaly, myelodysplastic syndromes, essential thrombocythemia • Micro platelets as in Wiskott-Aldrich syndrome

Table 10. Reasons for spurious platelet counts[1]

RBC AND WBC FRAGMENTS

In hospitalized patients, circulating cellular fragments of non-platelet origin are not uncommon. Fragmented erythrocytes, or schistocytes, are the hallmark of micro-angiopathic conditions such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura. Microspherocytes and other RBC fragments can be seen in patients with severe burns as well. In some types of leukemia or lymphoma, cytoplasmic fragments can split off from the malignant leukocytes, and may circulate in blood. [54-56] If these cellular fragments have similar size as platelets, it is very difficult or even impossible to distinguish them from platelets, thus resulting in falsely increased platelet counts. **(Figure 11.1, Figure 11.2, Figure 11.3, Figure 11.4)**

The above mentioned clinical conditions are all associated with thrombocytopenia and therefore the influence of platelet count overestimation due to interference by RBC or WBC fragments unfortunately becomes increasingly apparent and clinically more relevant.

Size-based platelet counting methods like impedance are predisposed to showing this type of interference. The optical method of platelet counting is less sensitive to interfering non-platelet fragments, but sometimes there may be overlap between platelets and non-platelet fragments even in a two-dimensional scatterplot. The immunological method is the only one that will give reliable platelet counts in this condition.

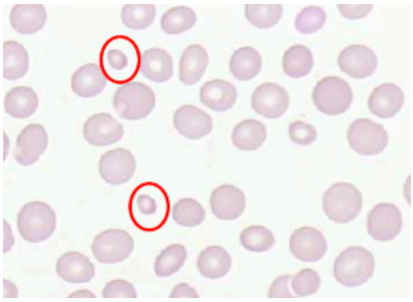

Figure 11.1. RBC Microcytes

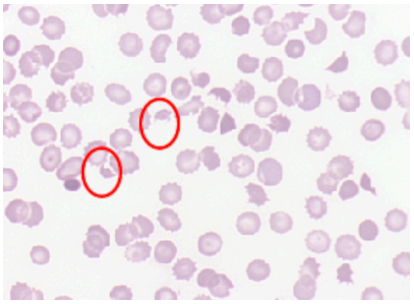

Figure 11.2. RBC Schistocytes

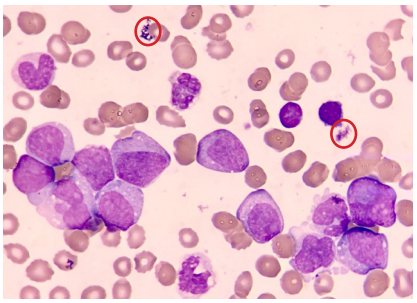

Figure 11.3. WBC Fragments: Acute Monocyte Leukemia

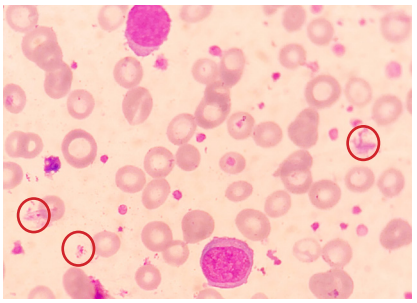

Figure 11.4. WBC Fragments: Acute Megakaryoblastic Leukemia

MICROCYTES / BACTERIA

Micro-organisms in blood are an exceptionally rare finding.[1] Yet, these cells may be responsible for unreliable platelet counts. There are case reports of spurious increased platelet counts caused by bacteria,[72,73] fungi[74,75] and malaria parasites **(Figure 12).**[76]

PROTEIN AGGREGATES

A less common type of interference resulting in falsely elevated platelet counts is caused by abnormal proteins that aggregate and precipitate in vitro at room temperature. Cryoglobulins,[1,57-60] immune complexes[61] and M-proteins[62] have been described as interfering substances in this category.

If the protein aggregates have similar size as platelets **(Figure 13)**, impedance methods tend to be affected most, and some optical methods are susceptible, too.[63] In many cases of cryoglobulinemia, optical methods are able to separate the proteins from platelets, but occasionally there are cases where the separation is incomplete. Like in other conditions of interference, immunological methods are not affected and provide the correct platelet count.

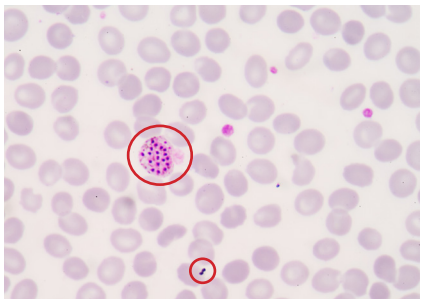

Figure 12. Intracellular parasites: Malaria

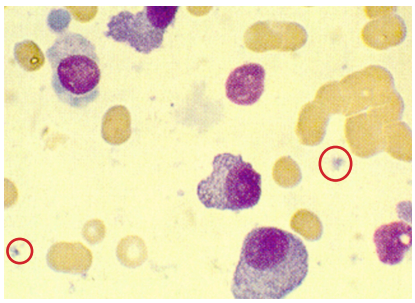

Figure 13. Myeloma showing background blue-staining small aggregates of protein

IMMUNE COMPLEXES CHYLOMICRONS

Few reports have been published on interference of lipid particles in platelet counts.[65,66] The lipids were either from endogenous hypertriglyceridemia or from fat emulsions administered as parenteral nutrition. The interference is caused by the high refractive index of lipid droplets,[1] which makes it plausible that the degree of interference depends on specific analyzer characteristics such as light source wavelength and angle of light scatter detection. Perfluorocarbon, that contains lipids as an emulsifying agent, caused significant overestimation of platelet counts in two impedance analyzers and to a lesser degree in an optical counting method.[67]

PLATELET CLUMPS

Probably the most frequent cause of false thrombocytopenia is the occurrence of EDTA-induced platelet agglutination **(Figure 14.2, Figure 14.3)**, as already mentioned in Section 3. Platelet aggregates are best visible in the 90° against 0° scatterplot as a large diagonal black cluster **(Figure 14.1)**. However, platelet counts and other platelet results are invalidated by the platelet clump flag. Since the multi-dimensional approach allows platelet aggregates to be well separated from the WBC, reliable WBC counts and differentials can still be reported.

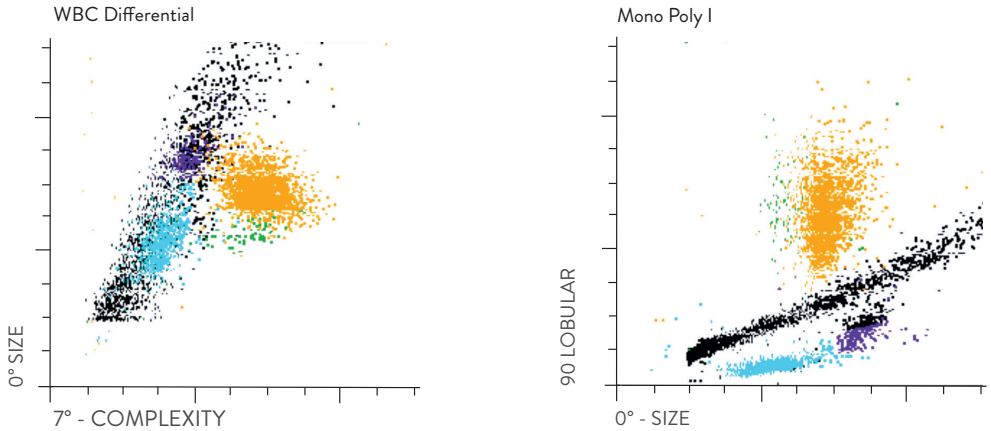

Figure 14.1. WBC scatterplots of a blood sample containing large platelet clumps (right panel, the black cell cluster). Thanks to the multi-angle approach it is possible to completely exclude the platelet clumps from the WBC analysis and report the true WBC count and differential (left panel).

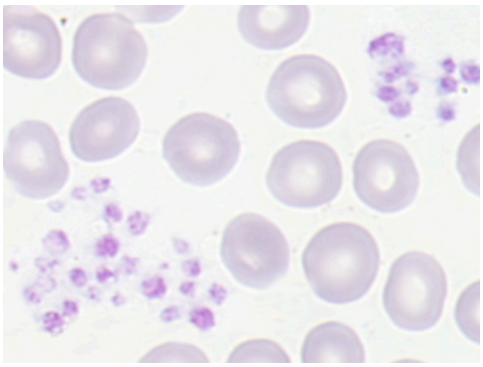

Figure 14.2. Platelet Clumps

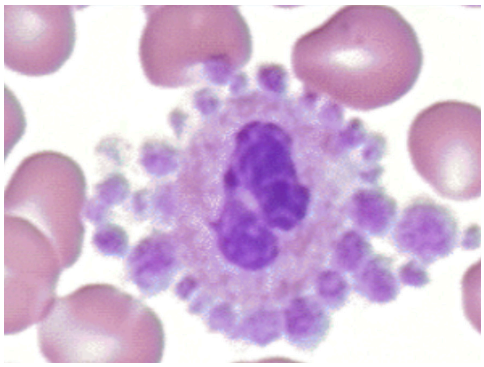

Figure 14.3. Platelet Satellitism

ACTIVATED PLATELETS

Activation of platelets is a potential interference typical for optical analyzers. These instruments depend on light scattering by platelet granules and upon activation, platelets can release (part of) their granules. As a consequence, degranulated platelets may fall outside the usual position in scatterplots, thus causing spuriously low values.[68] This phenomenon has been reported to occur in blood banks during the production of platelet concentrates for transfusion.[68-71] Impedance methods are less sensitive and immunologic methods are not at all subject to this type of interference.

ABNORMAL PLATELETS SIZE

GIANT PLATELETS

There exist some rare hereditary platelet disorders that are associated with unusually large platelets, or giant platelets, such as Bernard-Soulier syndrome and May-Hegglin anomaly.[52] In addition, giant platelets may occur in acquired diseases, too, for example in essential thrombocythemia and myelodysplastic syndromes.

It is not uncommon that platelets in these conditions are as large as or even larger than normal RBC. In impedance analyzers this inevitably leads to misclassification of giant platelets as RBC and thus to spuriously low platelet counts. Optical methods may also exclude the largest platelets and report falsely low platelet counts. The immunological methods measure platelets independent of their size and provide the correct count.[53] **(Figure 15.1, 15.2)**

MICRO PLATELETS

On the other side of the size spectrum one may sometimes find exceptionally small platelets, for example in patients with the rare Wiskott-Aldrich syndrome. These platelets may be so small, that they fall below the lower analysis threshold, which is approximately 3 fL. Again, impedance methods are most sensitive and will report falsely low platelet counts. Optical methods can be affected as well and only immunological platelet counts are reliable, as they do not take platelet size into account. **(Figure 16)**

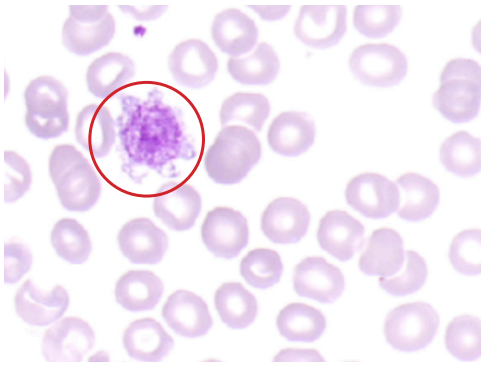

Figure 15.1. Bernard-Soulier Syndrome

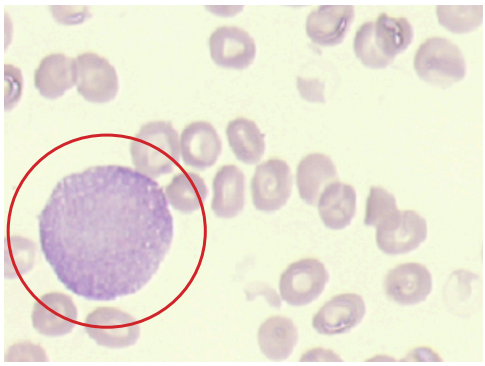

Figure 15.2. Myeloproliferative Disease

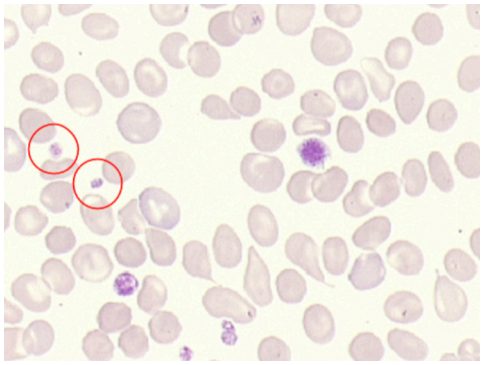

Figure 16. Sample containing small platelets

MISCELLANEOUS INTERFERENCES

The only known pitfall of the CD61 immunoplatelet method is in patients with Glanzmann thrombasthenia. This is a rare hereditary disorder that is characterized by deficiency of platelet glycoprotein IIb/IIIa, which constitutes the binding site for the CD61 antibodies. Thus, Glanzmann's platelets will not be stained by the antibodies, resulting in spuriously low immunoplatelet counts. However, as the platelets are otherwise normal, optical and impedance counts will be correct in this disease.

QUIZ QUESTIONS

1. Which of the following interfering factors is/are associated with a spurious increase in the platelet count?

   A. RBC fragments
   B. Leukocyte fragments
   C. Protein aggregates
   D. Platelet clumps

2. Which of the following interfering factors is/are associated with a spurious decrease in the platelet count?

   A. WBC fragments
   B. Platelet clumps
   C. Activated platelets
   D. Abnormal platelet size

SECTION 6

THROMBOCYTOPENIA AND PLATELET TRANSFUSION

The inverse relationship between platelet count and bleeding is well-known: the lower the platelet count, the higher the bleeding risk. This is crucial for patients who have prolonged, severe thrombocytopenia due to their disease (bone marrow failure) or the treatment of their disease (chemotherapy for cancer). This is best illustrated in **Figure 17,** which comprises patients with acute leukemia, in whom the bleeding risk strongly increased when platelet concentrations decreases to values below 50 x 10^9/L: patients with platelet counts < 5 x 10^9/L have approximately 50 times higher risk of severe bleeding than when the platelets are > 50 x 10^9/L.[80] Fortunately, bleeding risk can be mitigated by transfusing platelet concentrates. However, there is almost permanent global shortage of platelet donors and platelet concentrates are expensive. Moreover, platelet transfusions can infer risk to the recipients, so they should be given only when strictly indicated.

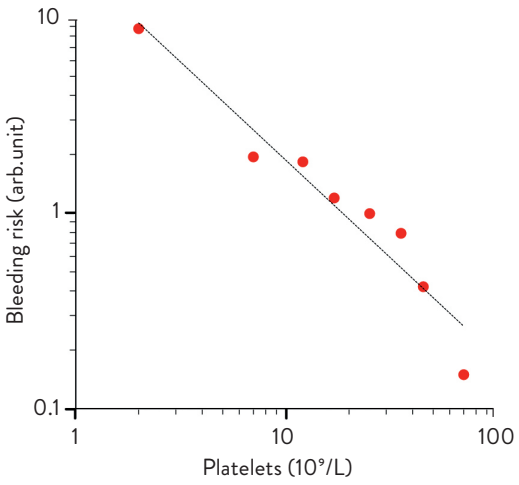

Figure 17. Exponential relationship between bleeding risk and platelet count (adapted from Heddle et at.)[80]

There are two major indications for administering platelet transfusions to thrombocytopenic patients. Patients with active bleeding due to thrombocytopenia usually receive platelet concentrates irrespective of their platelet count. Non-bleeding patients with thrombocytopenia due to chemotherapy are candidates for platelet concentrates as well, but the decision to transfuse is made on the basis of the platelet count. Traditionally, prophylactic platelet transfusion was given if the platelet count dropped below 20 x 10^9/L and there was no additional bleeding risk. Current European and US guidelines have adopted 10 x 10^9/L as a transfusion trigger for prophylactic use of platelet transfusion in patients treated with chemotherapy.[81-86]

A recent overview of external quality assessment data in thrombocytopenic samples demonstrated that the majority of analyzers currently in routine use overestimated platelet counts in comparison with the international reference method.[87] These authors also found that in all instrument groups the coefficient of variation increased as the platelet count decreased. This combination of imprecision and inaccuracy at clinical decision levels make it hazardous to introduce a lower transfusion trigger on a large scale. Several authors have shown that the risk of undertransfusion not only depended on the value of the transfusion trigger, but also on the specific hematology analyzer used for platelet counting.[41,89-91] Whereas undertransfusion obviously needs to be avoided for reasons of patient safety, overtransfusion due to the platelet counting method seems to be less frequent.[41,89] Inappropriate prophylactic platelet transfusion, which was observed in 34 % of hematology patients[85] is medically less risky, but is actually a waste of precious resources.

In conclusion, each laboratory should be aware of the limitations of its platelet counting method(s) and have procedures in place for an alternative method that provides the accuracy and precision that are required for clinical decision making. Currently, only laboratories that are able to use an immunoplatelet method in daily routine can comply with the requirements that would warrant application of 5 x 10^9/L as a trigger for prophylactic platelet transfusions.[90]

QUIZ QUESTIONS

1. True or False? The relationship between platelet count and bleeding is an inverse relationship.

   A. True

   B. False

SECTION 7

RETICULATED PLATELETS

DEFINITION AND PHYSIOLOGY

Reticulated platelets (retPLT) are platelets that are newly released from bone marrow megakaryocytes and do still contain RNA.[92] The initial description of retPLT dates back to 1969, when Ingram and Coopersmith observed coarsely punctuated reticulum in canine platelets after supravital staining with new methylene blue.[93] retPLT are the youngest platelets in the circulation, they have a short lifespan (less than 1 day) and therefore they reflect current megakaryopoietic activity, similar to what reticulocytes do in erythropoiesis.[94,95]

retPLT are considered to have clinical utility because they can help identifying the cause of thrombocytopenia and can serve as an early predictor of bone marrow recovery after chemotherapy or transplantation.

RETICULATED PLATELET COUNTING METHODS

FLOW CYTOMETRY

Kienast and Schmitz were the first to describe a flow cytometric technique for analyzing retPLTs, which was based on staining with thiazole orange.[96] Various modifications of this method have been described, resulting in reference values in presumed healthy adults ranging from 1 % to 15 %.[97,98] This wide range can be explained by lack of standardization of the technique (variation in incubation time, gate settings) as well as non-specific blinding of thiazole orange.

CELL-DYN Sapphire measures retPLT as an integral part of the reticulocyte assay. The method is based on the fluorescent dye CD4K530[101] and an optimized flow cytometric method.[97] **Figure 18** provides an example of the scatterplot used for the retPLT analysis.

Reference ranges for retPLT are between 0.35 and 2.2 %[102] or 0.4–6.0 %,[8] when expressed relative to platelets. When expressed in absolute concentration units, the retPLT reference range is 1.1 x10^9/L and 18.7 x 10^9/L.[8]

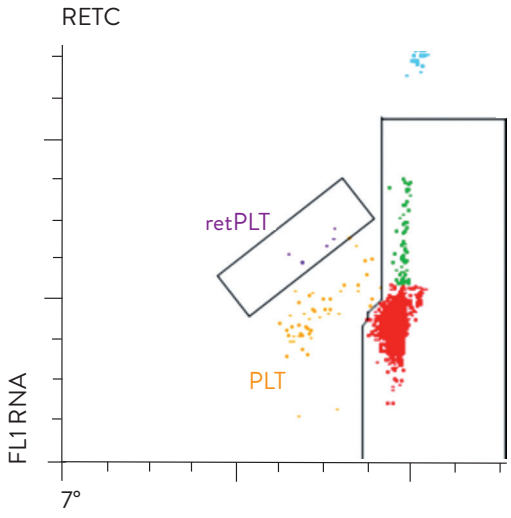

Figure 18. CELL-DYN Sapphire scatterplot for analyzing retPLT. Mature platelets are colored orange, retPLT purple, mature RBC are red, reticulocytes green and WBC blue. The retPLT gate is normally invisible; it is only displayed here for the purpose of illustration. Further, the actual number of events is approximately 10 times higher than the number of dots in the graph count (adapted from Heddle et at.80)

LARGE PLATELETS

Many studies have shown that accelerated megakaryopoiesis is associated with both increased IPF (immature platelet fractions) and elevated MPV (mean platelet volume). Apparently this has led to the widespread belief that immature platelets are synonymous with large platelets. [107,108] However, the biology of platelet production suggests that reticulated platelets can also be small, particularly when they are produced by high-ploidy megakaryocytes.[5,6] Therefore, although some hematology analyzers use IPF and MPV as markers of accelerated platelet production, these two parameters do not correlate well and should not be used interchangeably.[106]

CLINICAL UTILITY OF RETICULATED/IMMATURE PLATELETS

One of the main clinical usefullness of reticulated platelets is as a possible differential diagnostic aid in patients with thrombocytopenia. Since megakaryopoetic activity is low in patients with bone marrow failure, the assumption was that consequently reticulated platelets would be low, too. In contrast, conditions with peripheral platelet destruction like immune thrombocytopenia are characterized by highly active megakaryopoiesis and hence the reticulated platelet count would be increased. Indeed, many studies have now confirmed that reticulated platelets are low in thrombocytopenia due to bone marrow failure, while they are increased in most patients with thrombocytopenia due to peripheral destruction.[96,103,106,113-115]

Another useful application of reticulated platelets is monitoring the thrombocytopenic phase after chemotherapy and transplantation for hematological malignancies. It is well accepted that the increase in reticulated platelets precedes the increase in platelet count by 2–3 days on average.[116-119] A rise in reticulated platelets, therefore, heralds bone marrow recovery and creates the opportunity to defer platelet transfusions as opposed to when transfusion decisions were only based on platelet counts. While there have been reports on reticulated platelets in a variety of other diseases like myelodysplastic syndromes,[120,121] disseminated intravascular coagulation[122] and acute coronary syndromes[123,124], it is still too early to define the clinical utility in these conditions.

QUIZ QUESTIONS

1. The youngest platelets released from the bone marrow that reflect megakaryopoietic activity are known as _____.

2. True or False? The increase in reticulated platelets precedes the increase in platelet count by 2-3 days on average.

   A. True

   B. False

SECTION 8

CONCLUSIONS AND FUTURE PERSPECTIVE

This monograph has highlighted the analytical characteristics of platelet counting methods that are determinant factors for their clinical use. Platelet counts are particularly important for clinical decisions on administering platelet concentrates to patients with thrombocytopenia. Unfortunately, most methods for platelet counting are not very precise in the low range and their accuracy can be compromised by propensity to interferences. From a perspective of precision and accuracy, laboratories should refrain from using microscopic platelet count as their routine method. As for selecting automated analyzers for platelet counting, one should consider the type of techonolgy use to enumerate platelets between optical method, impedance technology or immunological methods.

There are guidelines that suggests withholding platelet transfusions is safe in non-bleeding patients with certain types of thrombocytopenia, but such policies can only be introduced in clinical practice when precise and accurate platelet counting methods are available to clinicians. The analytical limitations of platelet counting pose restrictions on widespread introduction of lower platelet threshold values. Thus, the diagnostic industry faces the challenge of developing analyzers in which the performance of platelet counting is in better agreement with clinical needs. Technological progress in engineering and optics is expected to bring improved platelet methods within each laboratory's reach before the end of the current decade.

In the domain of platelet transfusions, analytical performance of platelet counting is not the only factor of relevance. There is also a promising role for reticulated platelets to help clinicians in deciding whether to give platelet concentrates to patients who still have thrombocytopenia, but whose megakaryopoiesis is already recovering.

APPENDIX AND REFERENCES

APPENDIX: QUIZ ANSWERS

SECTION 1 THE DISCOVERY OF PLATELETS. PLATELETS FUNCTION

1. Max Schultze; Guilius Bizazarro
2. D
3. A

SECTION 2 MEGAKARYOPOIESIS AND THROMBOPOIESIS

1. A
2. Reticulated platelet
3. B

SECTION 3 PRE-ANALYTICAL FACTORS OF PLATELET COUNTING

1. B
2. B

SECTION 4 PLATELET COUNTING METHODS

1. Impedance
2. A
3. C

SECTION 5 INTERFERENCES IN PLATELET COUNTING

1. A, B, C
2. B, C, D

SECTION 6 THROMBOCYTOPENIA AND PLATELET TRANSFUSION

1. A

SECTION 7 RETICULATED PLATELETS

1. Reticulated platelets
2. A

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