Platelet detection and the importance of a reliable count

Platelet formation

As described by Wright for the first time [1], platelets are small non-nucleated 'extensions' of megakaryocytes – the platelet precursor cells present in bone marrow that develop from a stem cell in the course of thrombopoiesis (platelet generation). In the process of platelet generation, membranes form around the cytoplasmic granules of megakaryocytes, which are later differentiated as platelet subunits. After a certain degree of maturation, platelets are released into peripheral blood.

Platelets are the smallest blood corpuscles with a diameter of $1-4\,\mu m$ and a cell volume of 2 to 20 fL, with younger platelets being larger than the older ones (Fig. 1). They have no cell nucleus but residual mRNA originating from the megakaryocytes.

The normal concentration of platelets in human blood is $166-308 \times 10^9/L$ for men and $173-390 \times 10^9/L$ for women [2]. Values outside this range do not necessarily indicate disease. It is recommended to always examine reference ranges for suitability in a given patient population according to the method recommended by the International Federation of Clinical Chemistry and Laboratory Medicine [3].
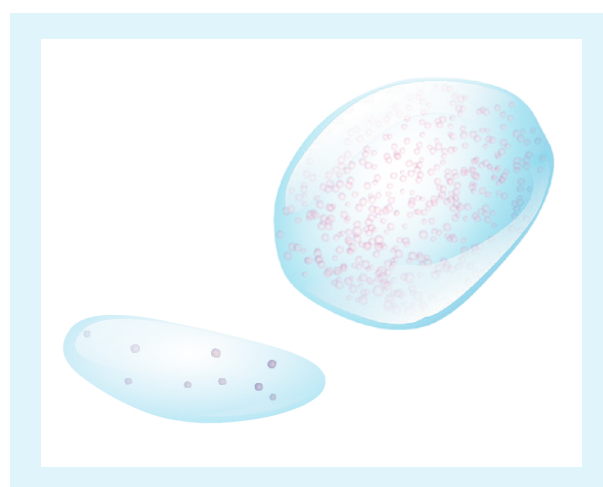

Fig. 1 *Illustration of a mature (left) and an immature platelet (right)*

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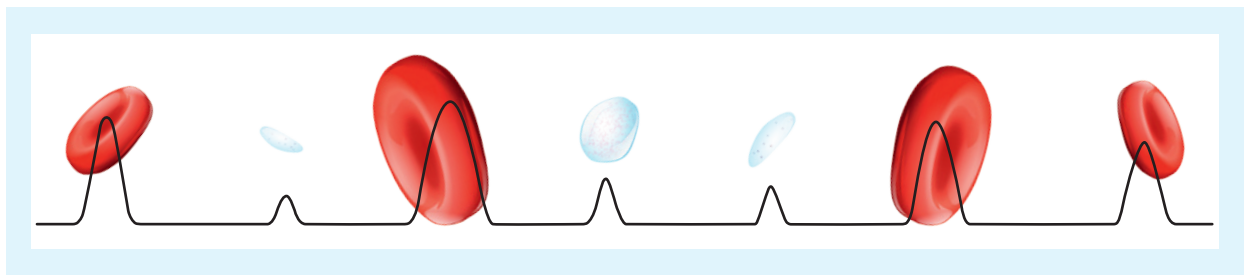*Sysmex Educational Enhancement and Development | August 2016*

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Fig. 2 Representation of the electrical resistance signal produced proportionately to cell volume

Assessment of platelet counts

There are several ways to assess the concentration of platelets circulating in peripheral blood. Initially this was done in a counting chamber, but nowadays platelets are almost exclusively measured by automated haematology analysers within the scope of the complete blood count.

Automated determination of platelet counts has replaced chamber counts in laboratories already for years and has greatly simplified and clearly improved cell count determination. One of the most important reasons for automated counting is – aside from time savings – the clearly lower statistical imprecision due to the greater number of cells examined. Yet, the technical validation of the platelet count is not all that simple and sometimes requires verification by smear review.

The impedance measurement principle

This is the method used by all the analysers that are currently in the market and that has been used for many years. It works very well when no interferences are present.

With an impedance measurement, cells are passing one after the other through a capillary opening – the aperture. There are electrodes on each side of the aperture – and direct current passes through these electrodes. The passing cells produce a change in the direct current resistance and thus an electronic signal which is proportionate to its volume (Fig. 2). Hence, the cells are identified based on their size and get represented in a volume distribution curve, the so-called 'histogram' (Fig. 3), which is defined by the sum of impulses within a certain size distribution.

This measuring principle was further improved in newer analysers through hydrodynamic focusing. This centred stream principle will 'jacket' the stream of particles by a sheath flow so that the particles are passing centrally and one after the other through the measuring capillary (Fig. 4). This almost excludes interference factors such as double passages by coincidence, recirculation, etc. and cells will therefore be counted with greater precision.
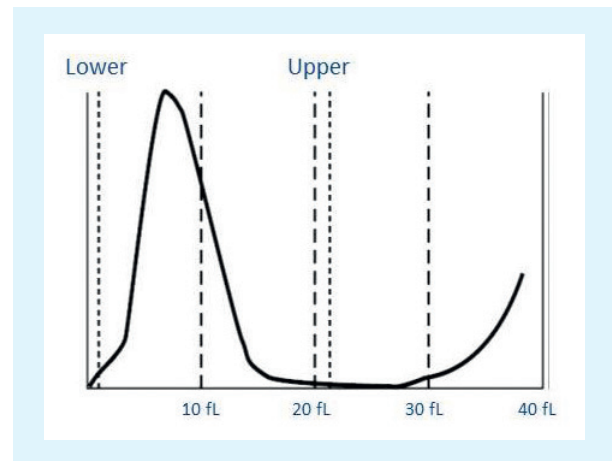

Fig. 3 Example of a platelet histogram

Under physiological conditions, red blood cells (RBC) and platelets differ clearly in their cell size and can be distinguished easily by the impedance method. Due to flexible discriminators (lower and upper discriminator; Fig. 3), the populations will be optimally separated from each other. The upper discriminator is the one that separates platelets (physiological size of 8 – 12 fL and measured between 2 – 30 fL) from RBC (physiological size of 80 – 100 fL and measured between 25 – 250 fL). But under pathological conditions, where platelets are larger than 30 fL (e.g. giant platelets) or when red blood cells are smaller than 25 fL (e.g. fragmentocytes) a clear separation cannot be guaranteed. In such a case the haematology analyser usually provides a corresponding warning message, and the user must perform a plausibility check of the sample with a different method.
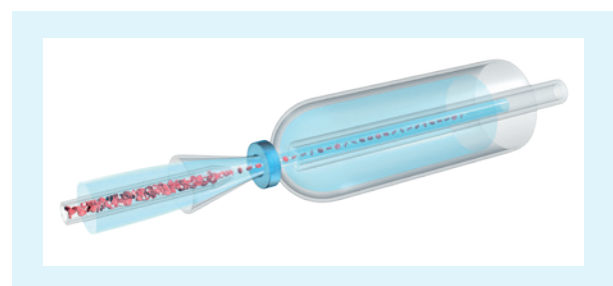

Fig. 4 Sheath flow with a hydrodynamic focusing method

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Possible interferences when using the impedance method

Analytical interferences can result in a falsely low or falsely high platelet count. The following constituents can cause interferences when the impedance measurement is used.
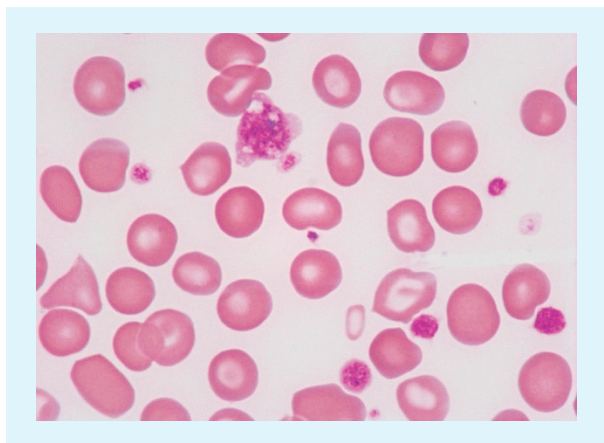

Fig. 5 *Giant platelet rom a patient with essential thrombocythaemia (ET)*

Giant platelets

Very large platelets – bigger than 'average' RBC – are called giant platelets (example shown in Fig. 5). They can be found in congenital diseases, like the Bernard-Soulier syndrome and myeloproliferative diseases such as MDS, AML and essential thrombocythaemia.

Due to their size – similar to the size of red blood cells – giant platelets can exceed the normal platelet size threshold value. This explains why a counting problem may arise with the automated impedance measuring method, which discriminates particles only according to their size. Nevertheless, this is accordingly indicated by a warning message. The large platelets are erroneously counted as RBC and this – if the warning message is ignored – might result in pseudothrombocytopenia. For the determination of a correct platelet count, the value must be reassessed with an alternative method.

Platelet aggregation and platelet agglutination

A falsely low platelet count is often due to the presence of platelet clumps, more rarely, to satellite formation in the blood sample. We can differentiate between platelet aggregation and agglutination. Platelet aggregation is the clumping together of platelets in the blood. It is part of the sequence of events leading to the formation of a thrombus (clot), and it is irreversible. It is mostly occurring due to platelet activation after a faulty venepuncture.

On the other hand, platelet agglutination (Fig. 6) is a reversible process, caused usually by the presence of antibodies in the blood. An example is cold-antibodies, where the proteins attached to platelets at temperatures below 34°C producing spurious thrombocytopenia. This is an *ex vivo* artefact that doesn't appear in the physiological temperature range.

Other causes that may lead to a false thrombocytopenia is the incompatibility with the EDTA anticoagulants or, in rarer cases, heparin or citrate. Platelet satellitism development is also an antibody-mediated, EDTA-dependent phenomenon. All these phenomena are entirely *in vitro* technical issues and do not occur *in vivo*.

In many cases, the haematology system generates a corresponding warning message and flags an abnormal distribution curve. But it has to be taken into consideration that the platelet aggregate might not have been aspirated by the system due to the inhomogeneous distribution within the sample, and that it might still be present in the blood tube. In that case, clumps can't be detected and a falsely negative result is obtained, as well as a falsely low count. Generally, a platelet value which is considered implausible despite additional examination of the blood film should be reassessed in a freshly taken blood sample.
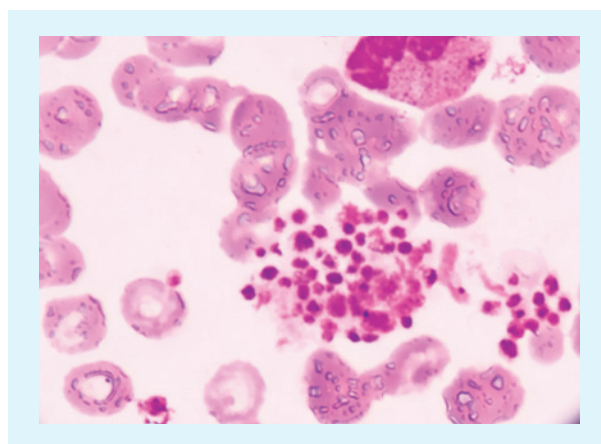

Fig. 6 *Platelet agglutination*

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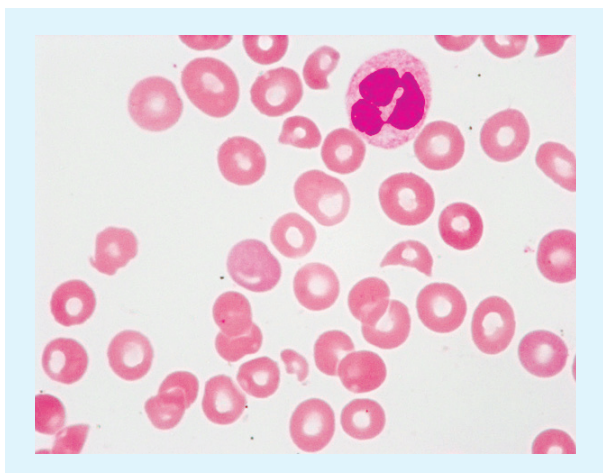*Sysmex Educational Enhancement and Development | August 2016*

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Fig. 7 *Fragmented RBC in the case of a thrombotic thrombocytopenic purpura (TTP)*

Fragmentocytes

Falsely elevated platelet values can be caused by red blood cell fragments or dysplastic erythrocytes (e. g. with MDS patients; Fig. 7). Numerous fragments are smaller than 25 fL and therefore fall below the upper discriminator value for platelets. Even with variable discriminator values, it is not always possible to separate very small red blood cells or red cell fragments from the platelets. In these cases, a precise platelet count can be determined using RNA-labelling dyes or immune labelling, or even by chamber counting when taking the statistical imprecision into account.

This phenomenon can also occur if fragments of white blood cells are present. Such particles can also interfere and be counted as platelets when using the impedance method.

Microcytic RBC

Microcytic RBC are classified as unusually small by their measured mean corpuscular volume [4] and can also interfere with the platelet count. There are many causes of microcytosis, a term which is essentially only a size descriptor. Cells can be small because of mutations affecting the formation of red blood cells (hereditary microcytosis) or because they do not contain enough haemoglobin, as in microcytosis associated with iron deficiency. Microcytic RBC are often counted as platelets by the impedance method of haematology analysers, since their size can be the same as that of platelets.
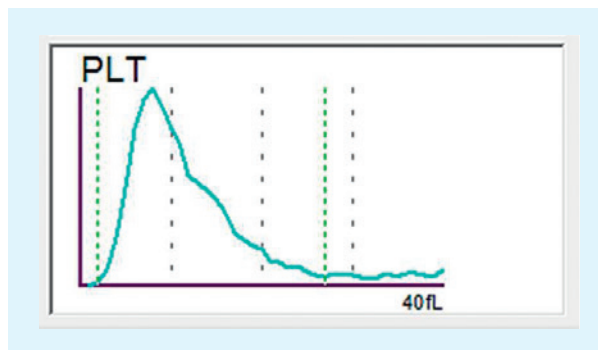

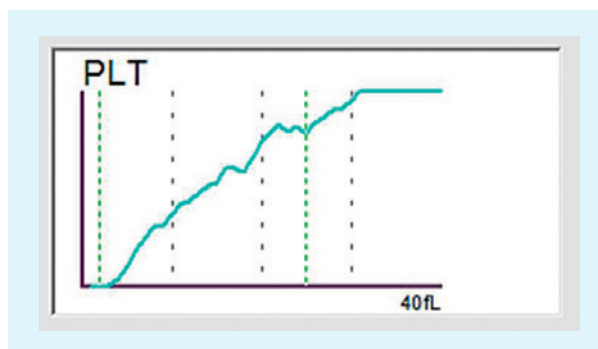**Fig. 8** *Interferences caused by platelet clumps*

Fig. 9 *Interferences caused by fragments*

Bacteria

Very small particles, such as WBC fragments or 'non-human cell particles' like bacteria, can be also erroneously counted as platelets. However, since the particle size is usually less than 2 fL, the system generates a warning message, and the user is requested to review the background value and to solve the problem if the warning message is repeated.

A couple of examples of platelet's histograms affected by interferences can be seen in Fig. 8 and 9. As a summary, the potential interferences are listed in the following table (Table 1).

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Table 1 *Constituents potentially causing interference with platelets when performing impedance measurements*

Constituent	Effect on PLT count	Actions to be taken
Giant platelets	Lower than true value	Evaluate histogram Repeat measurement with fluorescence method, if available
Fragmentocytes	Higher than true value	Evaluate histogram (fragmentocytes and platelets have no clear volume separation) Repeat measurement with fluorescence method, if available
Microerythrocytes	Higher than true value	Evaluate histogram (microcytes and platelets have no clear volume separation) Repeat measurement with fluorescence method, if available
Non-human cell particles	Higher than true value	Evaluate histogram Histogram needs to be evaluated Repeat measurement with fluorescence method, if available
Platelet aggregates	Lower than true value	Evaluate histogram Check for EDTA-induced pseudothrombocytopenia in cases of implausible thrombocytopenia Repeat the measurement with a fresh sample

Alternatives to the impedance measurement

There are various possibilities for re-examining the blood platelet concentration if a false analysis of platelets is suspected: for instance a counting chamber, immunological cell labelling or the use of fluorescence measurement by means of the haematology analyser.

Chamber count

A manual platelet count is usually performed in a counting chamber. Because of the high abundance of red blood cells in a blood sample it is essential to perform a preliminary lysis step to remove the red cells. As already mentioned, however, the determination of platelets in a counting chamber has been largely abandoned in laboratory routine. One of the most important reasons is the high statistical imprecision – apart from the time saved with automated counting. Particularly with low platelet numbers, where higher precision is necessary, the manually determined platelet count is very imprecise.

Platelet estimate according to Fonio

This method is useful for a quick guess of accuracy check of a given (automated) platelet count. It is performed via microscopy on a normal blood film at 1.000 times magnification (ocular 10, lens 100). There, the number of platelets in one visual field is counted and then projected to the total platelet count: 1 platelet per microscopic field equals to 20.000/µL platelets in circulating blood. Obviously, the values estimated by this method always just represent an approximation and cannot be exact.

Immunological labelling CD61/CD41

Immunological labelling of platelet surface receptors is currently the ICSH/ISLH reference procedure for platelet enumeration. The method's principle is to label the platelet surface receptors CD61 and CD41 with fluorescence-labelled monoclonal antibodies and to detect them in a flow cytometer. This method has replaced chamber counting as reference method. It has the advantage of an extremely reliable detection of all platelet sizes, although that can be laboratory dependent. It is not standardised and not automated and results can vary widely from lab to lab. Other disadvantages are that it is rather complicated and not always suitable for laboratory routine and, unfortunately, it is also very expensive.

Fluorescence labelling and flow cytometry

A fast and inexpensive alternative is to measure blood platelet concentrations via fluorescence labelling of the platelets' RNA. This nucleic acid labelling will enable the system to determine the exact platelet value in an automated manner on the basis of the cells' fluorescence activity and volume. It will also properly classify giant platelets (same volume as red blood cells, but differing in RNA content) and fragmentocytes (having no RNA). In a two-stage reaction, the cell membranes of the platelets are first perforated, whereby the cells remain largely native. Subsequently, a fluorescence marker specifically labels the platelets, and in doing so almost completely masks the interfering particles (e.g. other cells or fragments of a similar size) to minimise the interference. The measured fluorescence signal is directly proportional to the degree of maturity of the platelets, so additional information on immature platelets can become available.
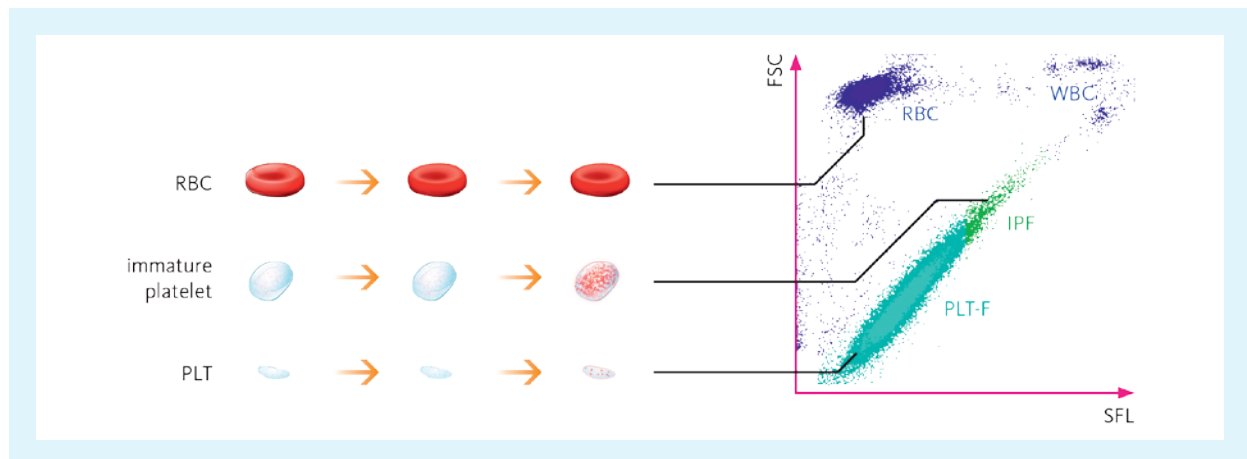
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Fig. 10 *PLT-F channel scattergram*

A good example of fluorescence labelling is the PLT-F channel (Fig. 10) in the XN-Series haematology analyser (Sysmex), which has been reported to produce reliable platelet counts that highly correlate with the reference method of immuno-flow cytometry [5 – 8], even with thrombocytopenic samples. The specific reagents used as well as the 5-fold counting volume compared to the system's impedance measurement provides a highly reliable count even for thrombocytopenic samples. One of the latest published papers from Wada *et al.* revealed that the labelling property of the PLT-F reagents, by which platelets and fragmented red blood cells are clearly distinguished, contributes to the platelet-counting accuracy of the PLT-F system [9].

In the following image (Fig. 11) it can be seen how the PLT-F channel provides a more accurate count in the case of inter-ferences due to the presence of fragments. PLT-I value (impedance) is 180 x 10^3/µL whereas the PLT-F channel points to 31 x 10^3/µL, exposing a thrombocytopenia that would have been missed only using the impedance count.

The PLT-F is the expert channel for platelet management. Not only provides an accurate and precise platelet count, it also determines the immature platelet fraction in the blood by means of the IPF parameter. IPF allows the evaluation of the current thrombopoiesis status, giving information about the platelet production in the bone marrow [10 – 14].*

With severely thrombocytopenic samples and those with an action message regarding an unreliable impedance platelet result, the XN rule set automatically triggers the measure-ment of PLT-F as a reflex test. Recently, the newly developed TWO algorithm has been incorporated in the *Extended* IPU, which optimises PLT-F triggers and supports the differential diagnosis of thrombocytopenic patients and their monitoring.

The PLT-F channel represents a fast and accurate method to ensure a reliable platelet count. With the IPF parameter, it also supports clinical questions by providing information that allows distinguishing between increased platelet destruc-tion and bone marrow dysfunction.
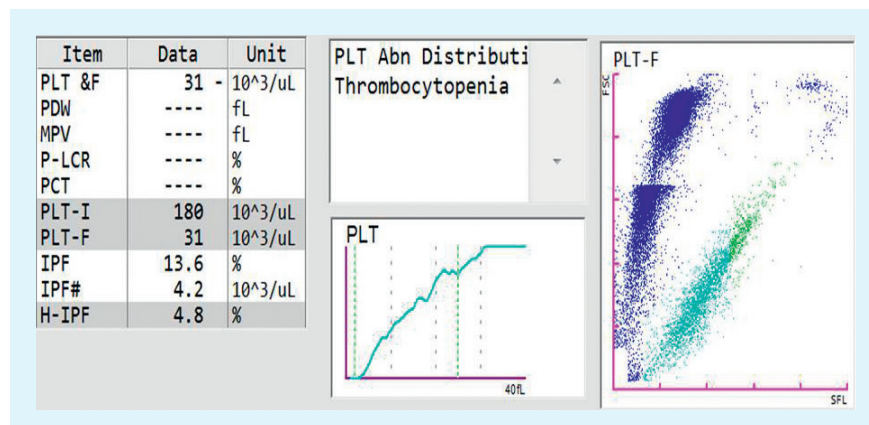

Fig. 11 *PLT-F channel scattergram*

* For more information please read the SEED paper 'The importance of thrombocytopenia and its causes' or the white paper entitled 'Differential diagnosis of thrombocytopenia'

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