Review

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Advances and challenges in platelet counting: evolving from traditional microscopy to modern flow cytometry

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Abstract: Platelet counting is a fundamental clinical test for diagnosing haemorrhagic diseases, coagulation abnormalities, and certain autoimmune disorders, and it also serves as a critical basis for decisions regarding platelet transfusion. Common automated methods for platelet counting include the international harmonization protocol (IHP) based on flow cytometry, CD61 immunoplatelet count (CD61-imm), impedance platelet count (PLT-I), hybrid platelet count (PLT-H), optical platelet count (PLT-O), and fluorescence platelet count (PLT-F). The IHP, based on flow cytometry, is recommended as the reference measurement procedure (RMP) by the Ministry of Health of the People's Republic of China, the International Council for Standardization in Hematology (ICSH), and the International Society of Laboratory Hematology (ISLH) due to its superior precision and accuracy. Despite the significant improvements in efficiency and standardization brought about by automation, traditional blood smear microscopic examination (PLT-M) remains indispensable in specific scenarios, such as low platelet counts or abnormal platelet morphology, to ensure the accuracy and reliability of platelet counting results from automated methods.

Keywords: platelet count; the international harmonization protocol; CD61 immunoplatelet count; impedance platelet count; automated methods; microscopic methods

Introduction

The platelet count (PLT) is essential in diagnosing hemorrhagic diseases, coagulation disorders, and certain autoimmune diseases. It is also a key indicator for assessing infection and inflammatory responses and serves as a critical basis for decisions regarding platelet transfusion [1]. Despite the widespread use of automated hematology analyzers in modern laboratories, which significantly improve work efficiency due to their multifunctionality and convenience, these devices still face challenges regarding accuracy in interference and low-concentration platelet counts.

Current platelet counting methods encompass the international harmonization protocol (IHP) based on flow cytometry, CD61 immunoplatelet count (CD61-imm), impedance platelet count (PLT-I), hybrid platelet count (PLT-H), optical platelet count (PLT-O), fluorescence platelet count (PLT-F), and microscopic examination (PLT-M) [2]. However, despite these methods striving to enhance measurement accuracy, accurate platelet counting remains constrained by various biological and technical factors [3].

To provide more accurate platelet counts in daily clinical practice, it is essential to understand the principles and limitations of these methods. This understanding not only helps laboratory staff and clinicians maintain confidence in platelet count results but also promotes the development and application of new technological approaches to meet the growing clinical demands for precision in platelet counts.

Platelets

Platelets are produced by megakaryocytes in the bone marrow [4], originating from the cytoplasm of mature megakaryocytes. Typically, they are biconvex discs with a diameter of about 2–5 μm. Although lacking a nucleus, they contain organelles such as the endoplasmic reticulum, Golgi apparatus, and mitochondria. Their primary functions are to promote hemostasis and accelerate coagulation while maintaining the integrity of the vascular endothelium.

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Normally, healthy individuals have a circulating platelet count of (150–400) $\times 10^9$/L [5].

Newly produced platelets first pass through the spleen [6], where about one-third are stored, freely exchanging with platelets entering the circulation to maintain a normal level in the blood. Platelets have a lifespan of 7–10 days [7], with newly formed, immature (reticulated) platelets being larger and mature platelets being smaller. Most aging platelets are cleared in the spleen, and interestingly, platelets are found only in mammals [8].

Recent studies have shown that platelets not only play a vital role in hemostasis and coagulation but also sense invading pathogens and the internal inflammatory environment, participating in the body's innate and adaptive immune responses [9, 10]. Consequently, platelets are crucial in the development of various diseases, including cardiovascular and cerebrovascular diseases, autoimmune diseases, infections, and tumor evasion.

Platelet counting

Platelet counting is a fundamental clinical test for diagnosing hemorrhagic diseases, coagulation abnormalities, and certain autoimmune disorders, and it also serves as a critical basis for decisions regarding platelet transfusion. Automated methods for platelet counting include the IHP, CD61-imm, PLT-I, PLT-H, PLT-O, and PLT-F, as well as the manual method of PLT-M. The IHP, based on flow cytometry, is recommended as the reference measurement procedure (RMP) by the Ministry of Health of the People's Republic of China, the International Council for Standardization in Hematology (ICSH), and the International Society of Laboratory Hematology (ISLH) due to its superior precision and accuracy [11–14].

Before implementing automated methods for platelet counting, it must be demonstrated that the method is precise, meaning it exhibits minimal imprecision and provides linear results within the reportable range stated by the manufacturer. Most manufacturers indicate a linear range for platelet (PLT) counts from 0 to 1,000 $\times 10^9$/L [15], while some extend the reportable range up to 7,000 $\times 10^9$/L [16]. Such an extended range is essential for managing extreme cases; for instance, platelet counts in patients with essential thrombocythemia (ET) have been reported to reach as high as 3,602 $\times 10^9$/L [17], though such high counts are seldom encountered in clinical practice. At the lower end, automated platelet counting methods should accurately measure counts down to 5–10 $\times 10^9$/L, as the transfusion monitoring threshold for patients with chronic thrombocytopenia or aplastic anemia falls within this range [18, 19]. Furthermore, it is important to note that the World Health Organization (WHO) provides general guidelines, recommending platelet transfusions in patients with counts ≤10 $\times 10^9$/L (or ≤20 $\times 10^9$/L when accompanied by infections or fever), ≤50 $\times 10^9$/L for those undergoing surgery or lumbar puncture, and ≤100 $\times 10^9$/L for those requiring neurosurgery [18].

For blood samples with factors that interfere with platelet counting, it is crucial that the analyzer can detect such interferences and, ideally, directly address them, enabling laboratory personnel to promptly correct erroneous counts and report accurate results to the clinic. Furthermore, the results obtained using different methods for the same sample should be comparable, with minimal methodological differences between different analyzers.

The International Harmonization Protocol (IHP)

The International Harmonization Protocol (IHP), as defined in the latest ISO 17511:2020 and recommended as the reference measurement procedure for platelet counting [20], includes the platelet-specific monoclonal antibody labeling technique and multi-parameter flow cytometry [14]. The principle involves pre-diluting EDTA-anticoagulated blood samples with sterile buffer (phosphate-buffered saline (PBS), pH 7.4) to prepare a suspension. Specific fluorescent antibodies are then used to stain platelets. The stained sample is delivered to the flow chamber via the sampling system, forming a flow focus with the sheath fluid, which ensures that cells pass through the detection area one by one. Platelets illuminated by a laser form scattered light (forward and side scatter, same wavelength as the laser) and fluorescence (wavelength longer than the laser, generated by excited antibody dyes). Forward scatter (FSC), side scatter (SSC), and fluorescence are set to logarithmic amplification, and platelet and red blood cell gates are set as shown in Figure 1 to detect the RBC/PLT ratio. An automatic blood cell analyzer using single-channel impedance accurately counts RBCs, and the platelet count is calculated by dividing the RBC count by the RBC/PLT ratio.

In clinical settings [21–23], this protocol is particularly valuable for accurate platelet quantification in conditions such as severe thrombocytopenia, which may occur following treatments for malignancies, such as radiochemotherapy and immunotherapy, as well as post-bone marrow transplantation and in cases of bone marrow dysfunction. The dual-platform absolute counting technique, which integrates flow cytometry with automated hematology analysis, has reached a level of maturity [24]. Although

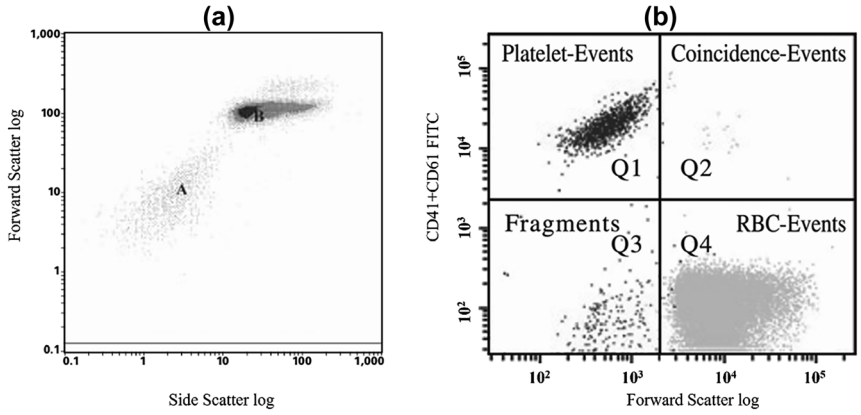

Figure 1: Flow cytometry scattergram. (a) The platelet cloud is shown on the lower left side (A) and is clearly resolved from the RBC cloud on the upper right side (B); (b) FITC, fluorescein isothiocyanate.

highly automated and requiring minimal technical expertise, this dual-platform approach involves multiple operational steps and can be time-consuming. It has not yet resolved all standardization challenges [15]. Moreover, specific conditions, such as elevated platelet counts (platelet activation), the presence of autoantibodies, congenital platelet disorders, platelet aggregation, platelet-white blood cell adherence, cold agglutinins, fragmented red blood cells, fragmented white blood cells (including apoptotic fragments), and abnormal platelet size, can affect the accuracy of absolute platelet counts [14].

Note: The specific fluorescent antibodies used for platelet counting in this method are fluorescein isothiocyanate-labeled CD41 and CD61 antibodies. These antibodies can bind to the platelet membrane glycoprotein IIb/IIIa complex [25]. Laboratories should verify that each batch of antibodies provides sufficient staining of platelets with fluorescence. The fluorescent platelets should be clearly resolved from noise and debris, RBCs, and RBC/platelet coincidence events.

CD61-immuno platelet count

The CELL-DYN Sapphire, developed by Abbott Diagnostics, offers a variant of the ICSH reference method. It employs a fully automated monochrome flow cytometry technique using fluorescein isothiocyanate (FITC)-conjugated CD61 monoclonal antibodies (MoAb) to stain and specifically identify platelets by targeting glycoprotein (gpIIIa) on their surface. During the analysis, the analyzer dispenses a small blood sample into a tube containing lyophilized FITC-conjugated CD61 monoclonal antibodies. As the reaction mixture incubates, the CD61 monoclonal antibodies bind to specific epitopes on the platelet surface. Subsequently, the mixture is further diluted and passed through the analyzer's optical flow cell, which measures two light scattering angles (7° and 90°) and the FL1 fluorescence signal from the FITC-bound cells, representing CD61. By accounting for the dilution ratio, flow rate, and analysis duration, the instrument directly calculates the platelet concentration, i.e., CD61-positive events. CD61-negative non-platelet events are automatically excluded from the analysis (Figure 2). The fully automated instrument configuration and optical settings enable easy operation in routine settings without the need for experienced flow cytometry personnel. This method is essentially the same as that developed for the CELL-DYN 4000 by Abbott Diagnostics in the 1990s [26, 27].

Numerous studies have shown a very close correlation between the CD61 immunoplatelet method and the ICSH reference method [28, 29]. Additionally, the precision of the

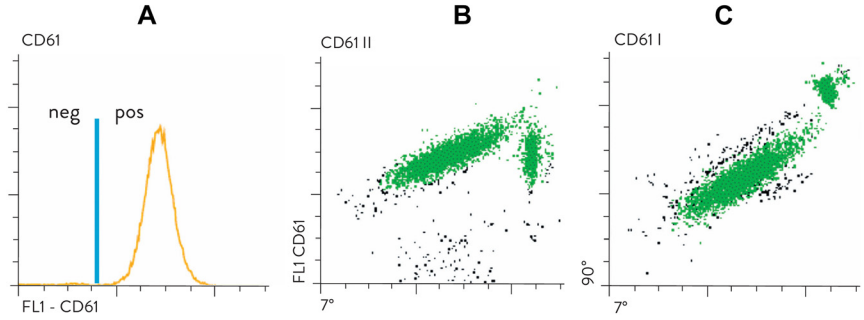

Figure 2: CELL-DYN Sapphire CD61 immunoplatelet method. (A) Histogram of FL1 fluorescence defining CD61-positive events as platelets; (B) 90° vs. 7° scatter plot, where all CD61-positive events are shown in green, and non-platelet events in black; (C) FL1 vs. 7° scatter plot showing platelets (large green cluster) and platelet-red cell coincidence events (small cluster on the right).

Sapphire CD61 method has been demonstrated to be very high, possibly even superior to the CD41/CD61 ICSH reference method. In severe thrombocytopenia, the coefficient of variation for CD61 testing ranges only from 1.6 to 2.3 % when platelet counts are between 5×10^9/L and 10×10^9/L, compared to 3.8–5.6 % for the corresponding ICSH reference method [30]. Some authors recommend using the CD61 immunoplatelet detection as the preferred method for low platelet counts and neonatal samples [31]. CD61 immunoplatelet detection technology offers significant advantages and may be particularly useful in clinical situations where accurate platelet counting is essential, although the overall cost per test will inevitably increase.

Microscopic methods (PLT-M)

Manual direct platelet counting

Despite the widespread use of automation in most laboratories, manual direct platelet counting (using phase contrast microscopy) is still conducted in laboratories with limited resources and without specialized equipment [32]. This method, which involves direct platelet counting, was recommended as the International Reference Method for platelet counting by the International Council for Standardization in Hematology (ICSH) in 1988 [33]. It is typically employed in special circumstances where automated hematology analyzers are inadequate, such as when the instrument reports abnormally low platelet counts or the presence of atypical platelets. Manual counting involves diluting the EDTA-anticoagulated blood sample in an ammonium oxalate solution to lyse red cells and differentiate platelets from leukocytes, followed by visual counting using a precisely designed Neubauer counting chamber, with the aid of phase contrast microscopy to determine the number of platelets.

Although manual counting can provide reliable clinical data for immediate comparison, it is labor-intensive and highly subjective. The coefficient of variation (CV) of the counting results can be as high as 25 %, and for samples with low platelet counts, it may reach up to 40 % [5, 13]. This variability is primarily attributed to the operator's skill, the uniformity of platelet distribution, and the efficiency of chamber filling. These limitations significantly affect the accuracy of manual counting and limit its feasibility for large-scale application. Therefore, while the manual method is an important and indispensable technique, its use is generally limited to specific clinical scenarios or as a supplement to automated methods (such as validating low PLT concentrations reported by a hematology analyzer). It is not recommended as a clinical counting method for patients with thrombocytopenia.

Manual indirect platelet counting

Manual indirect platelet counting provides a fundamental yet significant method for assessing platelet count, size, and morphology in peripheral blood smears [34]. Although more rudimentary, this method generally correlates well with results from automated hematology analyzers [35]. The preparation and staining of blood smears according to the methods recommended by the International Council for Standardization in Hematology (ICSH) are crucial for accurate counting. Before counting platelets under a microscope, a thorough examination of the entire blood smear should be conducted [36], particularly at the edges, to identify factors that might lead to an underestimation of counts, such as platelet clumps, fibrin strands or leukocyte satellitism. However, it is undeniable that any specific manual indirect platelet counting method shares the same, if not greater, level of inaccuracy as manual direct platelet counting. Therefore, its use is typically limited to specific clinical scenarios or as a supplement to automated methods [37].

In an EDTA-anticoagulated blood film, the ideal platelets should be evenly distributed, about one-fifth the volume of red blood cells, and appear round, oval, or slightly irregular in shape [38]. The manual indirect platelet counting method is straightforward and convenient, but the estimation of platelet counts is influenced by multiple factors and steps, including smear technique, cell distribution, selection of microscope examination areas, and the accuracy of WBC and RBC counts from automated hematology analyzers. Although mastering the ratio method can reduce the coefficient of variation (CV) in estimation results, the blood smear estimation method cannot replace automated hematology analyzer methods, nor can it replace reference methods. Its primary purpose is to confirm instrument count results, promptly identify and eliminate factors that may affect counting results, such as instrument malfunctions, sample clots, abnormal aggregation, pseudodecreases, and hematological diseases. This reduces significant testing errors, avoids misleading clinical decisions, and provides a basis for deciding whether to recheck and whether to use a reference method for rechecking.

Factors leading to an underestimation of counts

When observing platelets under a microscope, it is crucial not only to estimate their quantity but also to examine their morphology and identify any aggregation, fibrin strands, or

leukocyte satellitism. In cases of mild platelet aggregation, automated blood analyzers typically produce accurate platelet counts [39]. However, in cases of moderate or severe platelet aggregation, fibrin strands, or leukocyte satellitism, corrective measures must be taken to address the abnormal results (see Supplementary Materials). These measures may include switching to alternative testing methods, such as changing the anticoagulant or opting for direct blood collection at the machine. Manual direct platelet counting is also an option [34].

There are various methods for assessing platelet numbers on blood smears, and the main ones are introduced below.

Ratio method

The principle of the ratio method for estimating platelet counts is that a certain number of uniformly distributed red or white cells on a blood smear represents a specific volume of the original sample. The number of platelets observed in the same field of view as the red or white cells represents the number of platelets in that volume [40, 41]. The platelet/white cell ratio method can yield more accurate estimation results. When there are fewer white cells, the platelet/red cell ratio method might be more accurate. However, it should be noted that the ratio method, as an estimation technique, is relatively unreliable in terms of repeatability and accuracy compared to automated methods, and its application should be significantly limited.

Platelet/red cell ratio method

Count a certain number (over 1,000) of uniformly distributed red cells in an area while simultaneously recording the number of platelets, and calculate the ratio of platelets to red cells. This ratio, when multiplied by the red cell count determined by an automated hematology analyzer for the same sample, gives the estimated platelet count.

Platelet/white cell ratio method

At the tail end of the smear, count 100 white cells while recording the number of platelets observed. The estimated platelet count ($\times 10^9$/L) is calculated by dividing the number of platelets counted per 100 white cells by 100, and then multiplying by the white cell count ($\times 10^9$/L). The white cell count is obtained from the automated hematology analyzer.

R value method

Under oil immersion, observe at least 10 fields, counting the total number of platelets seen across these fields. Calculate the average number of platelets per oil immersion field, and then determine the estimated platelet count ($\times 10^9$/L) using the formula: average number of platelets per oil immersion field $\times$ R $\times 10^9$/L. The R value, also known as the field factor, is traditionally set at 20 [42]. Generally, obtaining the R value involves selecting a certain number of samples and conducting a count both by smear examination and an automated hematology analyzer, then analyzing the results using least-squares regression to simplify the linear equation into the form Y=bX, and rounding off the coefficient for practical use.

The R value method is the simplest for estimation but is also notably prone to error. Firstly, the R value is calculated under relatively ideal conditions and assumes a uniform distribution of cells and consistent platelet counts in the same field of view, without considering influences such as hematocrit (HCT). However, in actual practice, due to variations in smear thickness, red cell coverage, and differences in microscope eyepiece diameter, results can vary significantly from those obtained by instruments or other methods. Moreover, the more severe the anemia, the greater the potential estimation error. Therefore, unless laboratory conditions are limited, the priority for using this method should be the lowest.

Hemoglobin factor method

Under oil immersion, observe at least 10 fields and calculate the average number of platelets per oil immersion field. The estimated platelet count ($\times 10^9$/L) is calculated using the formula: average number of platelets per oil immersion field $\times$ hemoglobin concentration (g/dL) $\times 10^9$/L. The hemoglobin factor method is less accurate in estimating the number of platelets compared to the R value method [43]. If experimental conditions allow, this method should not be considered for issuing clinical reports.

Linear equation method

Select a certain number of samples, prepare blood smears as described above, and count the number of platelets in 10 fields at the tail end of the smear under high magnification. Calculate the average number of platelets per high-power field. Use this value as the X-axis and the platelet count from the automated hematology analyzer as the Y-axis to perform linear regression and obtain a linear equation similar to Y=a+bX. When used, the average number of platelets obtained under high magnification can be substituted into the equation for calculation. However, this method is not reliable for very low platelet counts [44]. Similarly, this method should not be prioritized.

Impedance platelet count (PLT-I)

The PLT-I counting mode is the most widely used method for platelet counting in clinical practice due to its low cost and high efficiency [45]. The impedance method treats platelets as completely non-conductive particles. As platelets pass through a detection aperture, they generate pulses; the volume of the platelets is determined by the height of these pulses, while the number of pulses determines the platelet count. Impedance counting is essentially a one-dimensional method, focusing solely on particle volume. All particles with volumes within a range defined by the specific analyzer are considered platelets, regardless of whether they are actually platelets. Significant variation may occur in the results of testing the same sample on different impedance analyzers due to differences in analytical methods, linearity across the entire measuring range, and the actual number of cells counted.

Different brands of impedance-based hematology analyzers determine platelet counts by calculating the number of blood components within a specific size range. These analyzers generate a platelet volume histogram, from which a log-normal curve is fitted to calculate the final data. For example, the Sysmex XN-1000 platelet counting system uses three thresholds to generate a platelet distribution histogram (Figure 3). One threshold is fixed at 12 fL, while lower and upper limits are set; the counting range is 2–30 fL, with the lower limit fluctuating between 2 and 6 fL and the upper limit covering a wide range from 12 to 30 fL [46]. The upper limit is determined by the lowest intersection point between the descending PLT curve and the ascending RBC curve. These thresholds are set to accurately distinguish platelets from larger red blood cells, red cell fragments, and smaller particles. Impedance-based hematology analyzers generally provide accurate counts down to 20×10^9/L, but below this level, accuracy may be affected due to reduced statistical confidence, fewer analyzed events, and increased background noise and interference from non-platelet particles [46].

The advantages of the impedance method for counting platelets include its ability to count a large number of particles with good precision, repeatability, and low cost. However, there are also drawbacks; one is that the impedance method detects both red blood cells and platelets in the same channel [47], and it can only detect platelets within a specific size range. Even with hydrodynamic focusing, it can be difficult to distinguish large platelets from small red blood cells or red cell fragments. When red cell fragments, leukocyte cytoplasmic fragments, small red cells, cryoglobulins, immune complexes, lipids, bacteria, or fungi are present, this can lead to falsely elevated platelet counts [48, 49].

On the other hand, the presence of large platelets, microplatelets, and platelet clumps (such as Ethylene diamine tetra-acetic acid pseudo-thrombocytopenia, EDTA-PTCP) can lead to falsely decreased platelet counts (See Supplementary Materials) [50]. It is important to note that large platelets have higher biochemical and metabolic activity compared to regular platelets, a higher density, and can produce and release more platelet-active substances. They also bind to fibrinogen more rapidly and have a stronger thrombotic effect [51]. Therefore, accurate counting of large platelets is crucial for reliable platelet count results.

Similarly, the platelet satellitism phenomenon (platelets adhering around neutrophils), or even platelets being engulfed by neutrophils, can cause falsely lowered platelet counts, although these phenomena may lack clinical significance [52]. In such cases, re-collection and correction with sodium citrate anticoagulation or peripheral blood is necessary. Additionally, immediate testing of whole blood

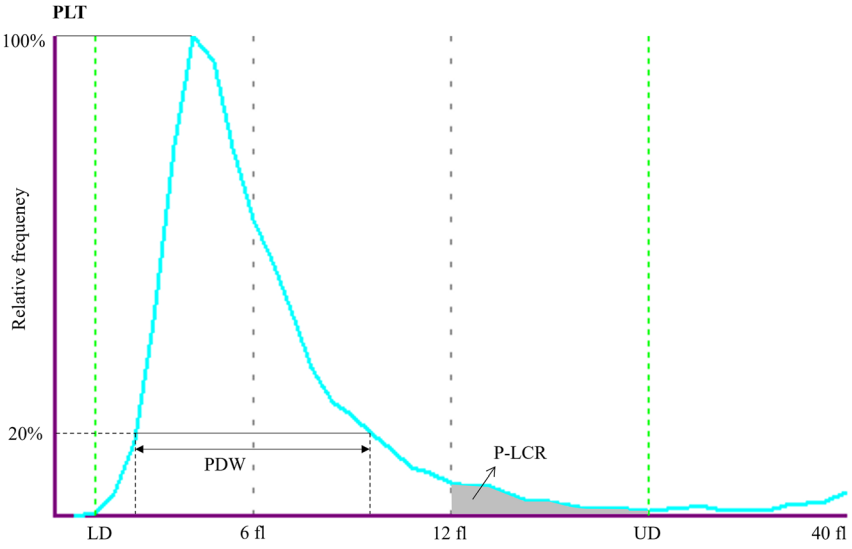

Figure 3: Typical platelet size distribution by impedance method in the Sysmex XN series automated hematology analyzers. LD, lower discrimination for platelet size distribution; PDW, platelet distribution width; P-LCR, platelets-large cell ratio; UD, upper discrimination for platelet size distribution.

samples after collection can also falsely lower platelet counts, possibly related to reversible platelet aggregation; in such cases, it is advisable to retest after letting the sample rest for 30 min [53]. Improper blood collection (such as difficult venipuncture, inadequate mixing, inappropriate draw volume, etc.) can also occasionally lead to falsely reduced platelet counts [54].

Optical platelet count (PLT-O)

Two-dimensional laser light scatter

To enhance the accurate discrimination of platelets from nonplatelet particles, a two-dimensional laser light scatter method was developed. This measurement is based on Mie theory [5], which describes the light scattering behavior of homogeneous spheres. Before analysis, platelets are typically treated with sodium dodecyl sulfate (SDS) and glutaraldehyde to make them spherical without altering their volume [55]. Laser scattering and hydrodynamic focusing enhance platelet counting. The scattered light is directly related to the size (area), surface irregularities, and refractive index of the illuminated particles or cells. When two detectors are used for low-angle and high-angle light scattering measurements, platelet size is represented by low-angle scatter, while platelet density is indicated by high-angle scatter.

The ADVIA 120 detects platelets by measuring laser diffraction (Figure 4A) at two angles: 2–3° for converting to volume (platelet size) and 5–15° for converting to refractive index (platelet density). This technology can distinguish between large platelets and cell debris and measures platelets ranging in size from 1 to 60 fL. The Cell-Dyn Sapphire instrument also uses optical platelet counting but with different angles (Figure 4B) from the Siemens system (7° and 90°, respectively) [22]. It is more accurate because it can monitor cell counts as platelet events accumulate. If the analyzer detects a reduction in platelet count, it automatically extends the platelet data acquisition time, thereby increasing the number of platelet events counted. This ultimately improves the accuracy of the platelet count. Abbott's latest MAPSS technology enables measurements at multiple angles for better discrimination (Figure 4C).

The advantage of the two-dimensional method is that the resolution between platelets and nonplatelet particles is not solely based on size, making it more specific. Although scatter plots from different instruments may appear different, this method allows for an accurate and specific division of platelet populations. Although less susceptible to interference from nonplatelet particles than impedance technology, optical methods can still be sensitive to interference in rare cases, particularly as the proportion of small and hypogranular platelets increases [56].

Optical fluorescence platelet count

Optical platelet counting is a parameter within the reticulated red channel of automated hematology analyzers (Figure 5). This method employs a combination of semiconductor lasers and fluorescent dyes (polymethine) for staining RNA and DNA within cells [47]. In the laser flow channel, cells are illuminated with lasers of specific wavelengths, producing forward scatter, side scatter, and side fluorescence for differentiated counting. Forward scatter reflects the size of the cell, side scatter indicates the size and quantity of intracellular particles, and side fluorescence

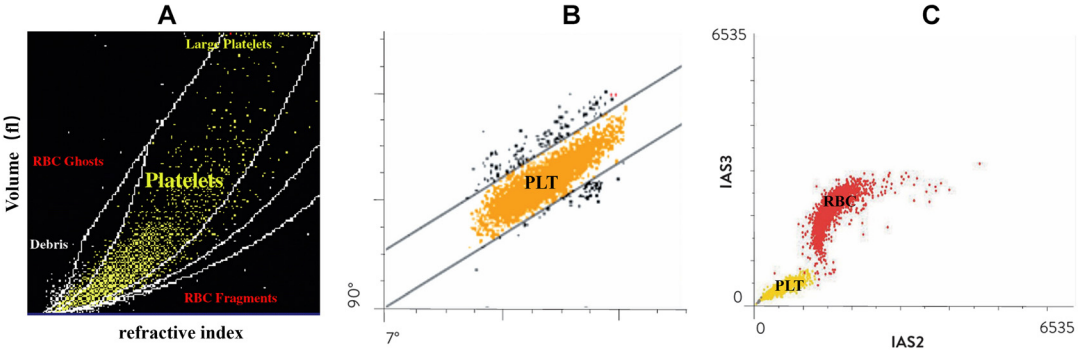

Figure 4: Optical platelet scattergrams. (A) ADVIA 120 (Siemens) platelet scattergram. This scattergram displays low and high angle scatter converted to cell size and cell refractive index. Debris, shadow cells; RBC fragments, red blood cell fragments. (B) CELL-DYN Sapphire optical platelet scattergram. This scattergram shows 90° vs. 7° light scatter. Two dynamic thresholds are used to separate platelets (yellow dots) from non-platelet particles (black dots), which may be located above or below the platelet population. (C) Intermediate angle light scatter plot. This scattergram shows the separation of PLT and RBC populations based on two intermediate angle light scatters (IAS2 vs. IAS3). Smaller RBCs (red) are located near the platelet population (yellow); PLT, platelets; RBC, red blood cells.

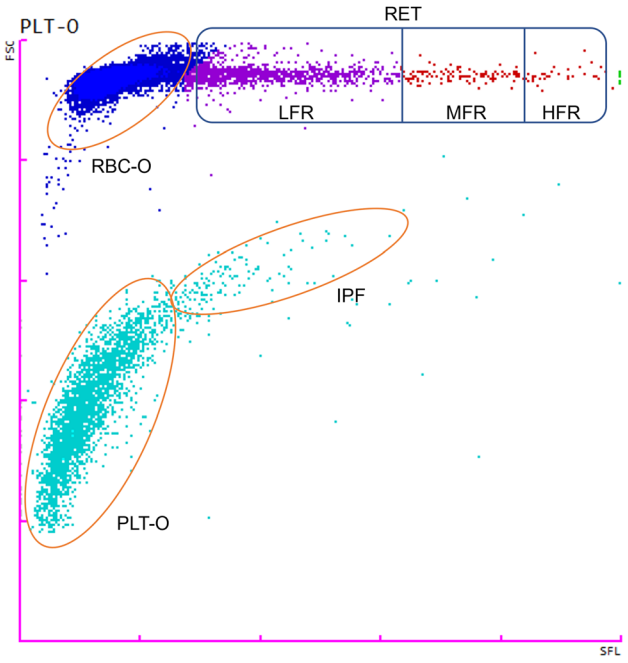

Figure 5: Typical scattergram of normal platelets by optical method. SFL, side fluorescence; FSC, forward scatter; RBC-O, optical red blood cells; PLT-O, optical platelets; IPF, immature platelets; RET, reticulated red cells; LFR, low fluorescence reticulated red cells; MFR, medium fluorescence reticulated red rells; HFR, high fluorescence reticulated red cells.

reflects the amount of nucleic acids in the cells. Platelets contain small amounts of nucleic acids, giving them a higher side fluorescence intensity compared to mature red cells; they are smaller in volume than red cells and are distributed below them in scatter plots. Theoretically, the optical method can detect platelets of all sizes.

With further technological advancements, the Mindray BC-6800 hematology analyser's optical channel has also utilized PLT disaggregation technology [39, 57]. This technique involves adding a disaggregating agent (Butylamine Canamycin) under controlled temperature and pH conditions, followed by high-speed physical stirring to prevent the re-aggregation of platelet particles and promote their separation [58]. However, when the number of aggregated platelet particles is high (PLT>20) or the aggregation is particularly dense, the PLT-O channel may not fully disperse the platelet clumps.

In summary, the optical method can effectively eliminate interference from small red cells, red cell fragments, and large platelets. Some automated hematology analyzers even possess PLT disaggregation technology, enhancing the accuracy of platelet counts. However, since the optical method counts fewer absolute numbers of PLTs per test compared to the impedance method, its accuracy is relatively lower for atypical samples with low platelet concentrations or those containing abnormal cells, particularly blood specimens from patients with severe burns [59]. Additionally, leukocyte fragments may also be stained, potentially leading to falsely elevated platelet counts [60], which warrants attention.

Hybrid platelet count (PLT-H)

Recently, a new hybrid channel platelet counting method (PLT-H) has been introduced on the BC-780 fully automated hematology analyzer. In this method, small platelets (≤10 fL) are counted in the impedance channel to avoid interference from small red blood cells and fragments. Large platelets (>10 fL) are counted in the white blood cell differential (DIFF) channel to

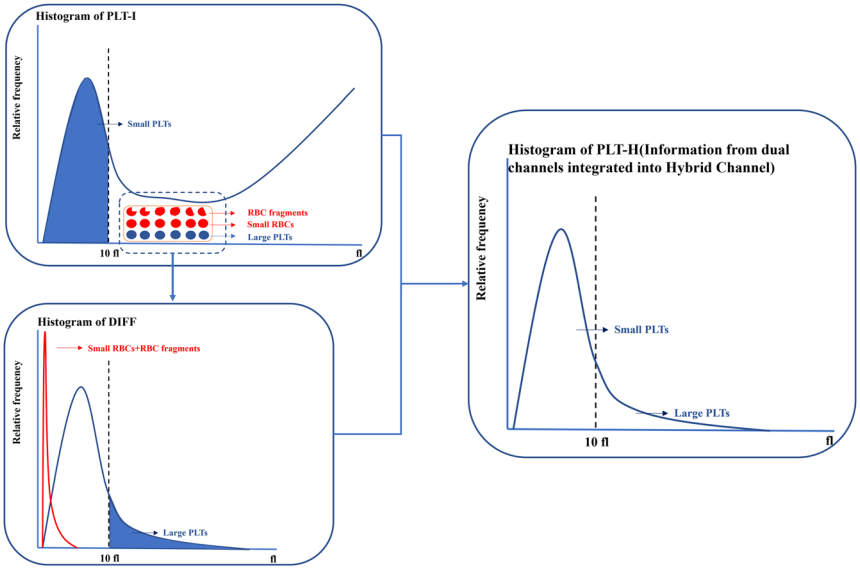

Figure 6: Schematic diagram of the PLT-H principle. In the impedance channel, large platelets are interfered with by small red blood cells and fragments, while small platelets remain unaffected. In the DIFF channel, red blood cells are lysed by specific reagents, and the platelet structure remains intact, with large platelets being detected through a precise optical method. By combining the small platelets from the traditional impedance method and the large platelets from the optical method, an accurate platelet count is obtained.

maintain their intact structure and avoid the effects of hemolysis. The PLT-H is a new platelet counting method [23], or more accurately, a new data manipulation technique (Figure 6), as it combines the detection of small platelets via the impedance channel with the detection of large platelets via the DIFF channel to achieve accurate platelet counting. This approach is more akin to data manipulation obtained from signals in two separate measuring channels, and it does not increase additional reagent costs or require extra testing. Eakachai Prompetchara suggests that PLT-H offers better reproducibility and interference resistance than PLT-I and correlates well with PLT-O, although it is still susceptible to interference from severely hemolyzed samples [61].

Fluorescence platelet count (PLT-F)

The fluorescence platelet count (PLT-F, Figure 7) operates on the principle of flow cytometry [62], utilizing a unique fluorescence dye (primarily oxazine) that specifically binds to mitochondrial DNA (MtDNA) and ribosomal RNA in the endoplasmic reticulum of platelets [59], which differs from the optical method. This increases the fluorescence intensity difference between platelets and fragmented red cells, enhancing specificity. Inside the laser flow channel, similar to the PLT-O principle, a semiconductor laser of the corresponding wavelength illuminates the platelets, plotting the forward scatter light and side fluorescence intensity on a two-dimensional scatter diagram, enabling more accurate identification and counting of platelets based on the differences in fluorescence intensity. The fluorescent method can achieve very good precision. However, this method appears to suffer from systematic bias when compared to the international harmonization protocol [60, 63].

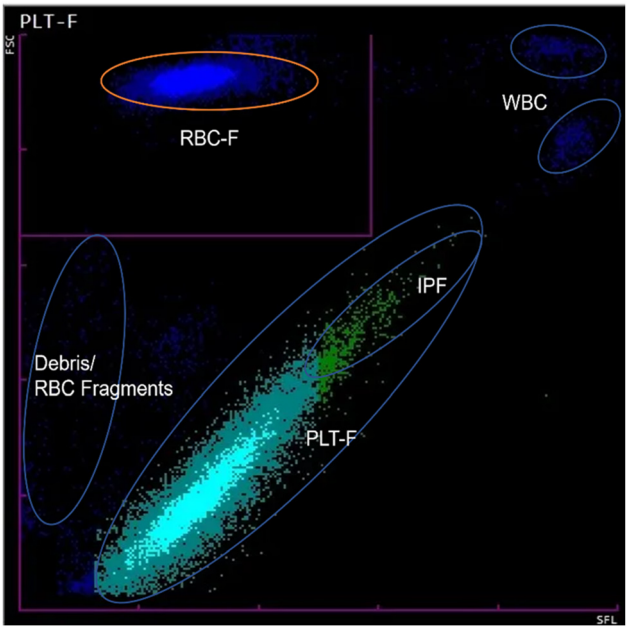

Figure 7: Typical fluorescence method mormal platelet scattergram. SFL, side fluorescence; FSC, forward scatter; WBC, white blood cells; RBC-F, fluorescent red blood cells; IPF, immature platelets; PLT-F, fluorescent platelets; debris/RBC fragments, shadow cells/red blood cell fragments.

Comparison of platelet measurement methods

Compared to the impedance method and PLT-O, the advantage of PLT-F is that special fluorescent dyes specifically stain platelets [59], making it difficult for reticulated red cells, regular red cells, and other cells to be stained, thus avoiding interference from red cells and providing high specificity. Moreover, PLT-F can exclude large platelets, microparticles, cell debris, small red cells, cryoglobulins, lipids, and other impurities that may affect platelet counts [64, 65]. It also analyzes five times more particles, enhancing the precision of the detection results, especially in low-value platelet count testing and guidance on platelet transfusion, where the PLT-F channel has shown excellent performance [60].

However, compared to the conventional impedance method, the PLT-F channel requires a larger sample volume, longer counting times, and higher costs for fluorescent dyes. Studies have shown that for samples with elevated platelet counts, the PLT-F channel does not demonstrate significant advantages [47]. Therefore, it is more suitable as a confirmatory test for samples where the accuracy of the impedance method is in question. Additionally, since RNA degrades at room temperature, the PLT-F channel is better suited for use with fresh whole blood samples.

Indeed, as the clinical demand for precise diagnostics and treatment increases, the provision of accurate platelet counts by laboratories becomes especially critical. Technologies like PLT-F, PLT-O, and IHP not only provide platelet counts but also measure the fraction of immature platelets (IPF) [66], which is an important parameter for assessing bone marrow hematopoietic function. Since immature platelets are larger, PLT-I often falls short in identifying these cells. Measuring IPF has significant implications for distinguishing the causes of thrombocytopenia, assessing recovery after bone marrow or stem cell transplantation, evaluating the risk of cardiovascular diseases, and assessing responses to antiplatelet medications [67–69]. Each detection method has its strengths and

Table 1: Comparison of seven platelet counting methods.

Methodology	Accuracy	Precision	Cost	Advantages	Limitations	Description basis
IHP	High	High	High	Strong specificity; robust interference resistance; accurate counting; the international harmonization protocol for platelet counts.	Multiple steps in testing, time-consuming; high cost.	[11–14]
CD61-imm	High	High	High	Strong specificity; robust interference resistance; accurate counting;	Time-consuming; high cost.	[28–31]
PLT-M	Medium	Low	Low	Direct counting of platelets; low cost; can detect abnormalities in platelets and other blood cells.	Many influencing factors; low absolute count of platelets, slow detection speed; highly subjective; poor repeatability.	[5, 13, 32–37]
PLT-I	Medium	High	Low	Good precision; low cost; counts many particles;	Many influencing factors; poor stability in low-value platelet counts.	[45–54]
PLT-H	High	High	Low	Low cost; strong interference resistance.	Severe hemolysis can affect results; a new counting method, limited application.	[23, 61]
PLT-O	High	Medium	Medium	Strong interference resistance; can reduce interference from platelet aggregation; can count IPF.	Precision not as good as PLT-I; leukocyte fragments may also be stained; leading to falsely elevated counts; poor stability at low values.	[21, 47, 56, 58–60, 66]
PLT-F	High	Medium	High	Strong specificity; strong interference resistance; stable counting of low-value platelets; minimizes interference from platelet a ggregation; can count IPF.	Requires larger sample volume; suitable for fresh whole blood; time-consuming; high cost.	[47, 59, 60, 63–66]

Low, medium, and high only indicate relative performance levels.

weaknesses, and the presence of various interfering factors presents challenges to the accuracy of platelet count testing methods. Table 1 details a comparison of seven platelet counting methods.

Summary and outlook

In this review, we comprehensively examine current platelet counting technologies, including IHP, CD61-imm, PLT-M, PLT-I, PLT-H, PLT-O, and PLT-F. We focus on evaluating the advantages and limitations of these technologies in both clinical and laboratory settings. PLT-I offers simple and rapid operation but may be influenced by red blood cell fragments, leading to falsely elevated platelet counts. PLT-M is usually limited to specific clinical situations or used as a supplement to automated methods, despite being complex and time-consuming to operate. CD61-imm has been proven to be highly accurate and is the preferred method for low platelet counts and neonatal samples. PLT-H does not incur additional reagent costs and offers better reproducibility and resistance to interference compared to PLT-I, though it remains susceptible to interference from severely hemolyzed samples. PLT-O and PLT-F, as newer technologies, provide more accurate platelet counts, particularly excelling in cases involving low counts or samples with interfering substances. The International Harmonization Protocol, utilizing specific fluorescent labeling and a complex optical system, provides precise platelet counting and cell characteristic analysis, making it especially suitable for the accurate measurement of complex samples.

Looking forward, the development of platelet counting techniques should focus on improving accuracy and simplifying operational processes. As platelet counting technologies continue to evolve, future research should explore the applicability and accuracy of these technologies in specific clinical scenarios to better meet the needs of clinical practice and research, ensuring the reliability and practicality of the results.

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