



Review Article

Flow cytometry for evaluating platelet immunophenotyping and function in patients with thrombocytopenia

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ABSTRACT

Platelets play an essential role in primary hemostasis through bleeding and thromboembolism. Thus, the diagnosis or evaluation of impaired hereditary, acquired, and drug-related platelet dysfunction has become imperative. The assessment of the platelet function is too complex for routine platelet function study. The major methods involved in platelet function study include platelet function analyzer testing, thromboelastography, thromboelastometry, light transmission aggregometry, and flow cytometry. The current review article focuses on the methods with flow cytometry for immunophenotyping of platelet and evaluating platelet function for platelet disorders, especially in patients with thrombocytopenia. According to the consensus published by the International Society on Thrombosis and Haemostasis, for inherited and acquired platelet disorders, the two major measures by which flow cytometry determines platelet function are glycoprotein IIb/IIIa/P-selectin (CD62p) expression and percentage of leukocyte-platelet aggregates. Using flow cytometry to determine platelet function has several advantages, including good sensitivity to low platelet counts, small blood volume required, and the nonnecessity of centrifugation. However, flow cytometry has still many limitations and challenges, with standardization for routine laboratory testing also proving difficult. Although flow cytometry is available for multipurpose and sensitive study of platelet functions at the same time, the challenging analysis gradually increases and needs to be addressed before reality.

KEYWORDS: *Flow cytometry, Glycoprotein, Leukocyte-platelet aggregates, Platelet function, Thrombocytopenia*

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INTRODUCTION

Platelets play an essential role in primary hemostasis through bleeding and thromboembolism. Well-balanced activation and inhibitory pathways are responsible for adhesion, activation (including granule release), aggregation, and then thrombus formation. The known causes of bleeding have been associated with platelet number and function [1]. Previous reports have shown that patients with defective platelet function are at considerably higher risk for perioperative bleeding compared to those with thrombocytopenia [1]. This highlights the importance of diagnosing or evaluating impaired hereditary and acquired platelet dysfunction. Platelet function studies have become necessary in clinical practice, and for research of platelet pathophysiology and dysfunction, all kinds of platelet function tests were developed. However, there exists no universal test which is sensitive to all platelet dysfunction [2]. Skilled personnel and customized laboratories are requiring by most platelet function tests. Moreover, the assessment of platelet function is exceedingly complex for

routine coagulation laboratory testing due to time constraints, poor standardization, and the need for extensive experience. Limitations in platelet function study have also been noted [2].

Thrombocytopenia, the most common bleeding disorder among medical and surgical patients, especially in the hemato-oncologic departments and intensive care units, is characterized by abnormally low levels of platelets [3]. Several platelet function studies evaluating platelet aggregation defects are insensitive to low platelet counts (i.e., $<150 \times 10^9/L$), jeopardizing the validity of the results [4,5]. The principle of flow cytometry is based by single-cell analysis, which allows for platelet study independent of platelet count [6]. As such, flow cytometry has been recommended as the first-line

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screening assay for the evaluation of platelet pathophysiology and dysfunction [7,8].

The current review focuses on flow cytometry for platelet immunophenotyping and platelet function evaluation for inherited and acquired platelet disorders, especially in patients with thrombocytopenia.

EVALUATION OF PLATELET FUNCTION

The major methods for determining platelet function in clinical use include platelet function analyzer (PFA) testing, thromboelastography (TEG®), light transmission aggregometry (LTA), and flow cytometry.

The PFA (including PFA-100/200®) had originally been developed as a rapid method to evaluate bleeding time *in vitro*, under the agonists, including collagen/adenosine diphosphate (ADP) or collagen/epinephrine; the occlusion time is measured in whole blood. Thus, von Willebrand factor (vWF)-dependent primary hemostasis and drug-related platelet dysfunction with aspirin have been screened, although previous data have shown that such tests lack the specificity and sensitivity for mild platelet disorders. The important limitations include low hematocrit levels (<30%) and thrombocytopenia when platelet count <100 × 10⁹/L. The PFA-100/200 is no longer suggested by the International Society on Thrombosis and Haemostasis (ISTH) for the assessing platelet dysfunction [9,10].

The viscoelastic method, which includes TEG and thromboelastometry (ROTEM®), measures the parameters of coagulation in whole blood, including clot formation, strength, stiffness, clot resolution, and lysis. The main applications were for the evaluation of coagulation and decision for transfusion management during peri- and postsurgery. The methods have also been updated to the Platelet Mapping™ system for the study of platelet function. Therefore, the sensitivity of the TEG system to platelet dysfunction is still limited for mild defects. Moreover, evidence has shown that clot strength is affected by thrombocytopenic samples, too [11,12].

LTA is still the gold standard for the evaluation of platelet function. It is based on the light transmission, which increases with platelet aggregation by the addition with multiple agonists [13]. Conventional LTA has several disadvantages. It needs strict preanalytical measures for reliable results [14]. Large blood volumes are required for the study, and experienced research technicians for interpreting results are also necessary [13]. LTA measurements also require a normal platelet count, which ranges between 150 and 600 × 10⁹/L, in platelet-rich plasma. Low platelet counts affect the sensitivity of the test and consequently the validity of the evaluation of platelet aggregation defects [4,5].

Flow cytometry has been used to evaluate the expressions of platelet surface glycoproteins (GP), including GPIIb/IIIa, GPIb/IX/V, GPIa/IIa, P-selectin (CD62p), CD63, lysosomal-associated membrane protein 1, and mepacrine [15-18]. Flow cytometry allows for the quantification of platelet surface receptors and activation markers *in vivo* in citrated whole blood [2,19]. The major

advantage of flow cytometry for the evaluation of platelet function is that it only needs a minimal amount of blood and platelets, especially in children [20]. Second, because of flow cytometry-based single-cell analysis and independent of platelet count, it could be used for severe thrombocytopenic patients [6]. Third, whole blood without centrifugation is used for flow cytometry, which could prevent platelet activation. In addition, under advanced protocols for fixation, shipped blood samples become feasible [21].

One study by Sharma *et al.* compared the ability of flow cytometry and LTA to detect platelet dysfunction. Flow cytometry can be useful for the evaluation of platelet functional defects, with potential use with thrombocytopenia [14]. Navred *et al.*, who also compared the performance of flow cytometry under platelet activation to LTA as the diagnostic test for inherited platelet disorders, showed that flow cytometry had a higher negative predictive value [22]. As such, flow cytometry is recommended as a first-line screening assay for specific defects in phenotype and function of platelets [7,8]. The advantages and disadvantages of the platelet function studies are summarized in Table 1.

FLOW CYTOMETRY FOR ASSESSING MARKERS OF PLATELET FUNCTION

Principle of flow cytometry

The principle of flow cytometry is based on evaluating optical and fluorescence expressions from single cells. The powerful tool can simultaneously detect and measure physical and chemical characteristics of a population of cells. Physical characteristics, including size by forward scatter and internal complexity by side scatter, can analyze different cell populations. Chemical properties, such as fluorescent dyes, can interact with different cellular components, and fluorochrome-labeled antibodies make it possible to simultaneously detect multiple proteins on cell membranes or inside cells [23-25].

By Dave *et al.*, under comparing the flow cytometry and LTA, flow cytometry had excellent agreement with LTA (sensitivity: 94.1% and specificity: 98.5%). Flow cytometry could be used in thrombocytopenic patients [26]. Another study was published by Saraymen *et al.* Flow cytometric analysis with GPs was used in the diagnosis of Glanzmann's thrombasthenia. Flow cytometry is the most sensitive method [27].

Glycoprotein IIb/IIIa and P-selectin

The human platelet membrane GPIIb/IIIa (integrin αIIbβ3) complex is a significant protein on platelet membrane. The widely expressed membrane protein has been described to have around 80,000 copies existing on the surface of unstimulated platelets in circulation [28]. The GPIIb/IIIa binding motifs is able to bind adhesive proteins by recognizing the peptide sequence arginine-glycine-aspartic acid known to be present in fibronectin, thrombospondin, vWF, vitronectin, and fibrinogen [29]. When platelets are stimulated by different agonists, the activated form of GPIIb/IIIa undergoes a conformational change that exhibits high affinity for binding plasma fibrinogen [30]. This explains platelet GPIIb/IIIa

Table 1: Compared different platelet function tests

	Advantages	Disadvantages	Platelet function detection
PFA-100/200	Semiautomated Simple and rapid No sample preparation (whole blood) Assess platelet function in presence of erythrocytes and high shear	Only sensitive to aspirin and von Willebrand disease Dependent on hematocrit and platelet counts	Platelet adhesion and aggregation
Thromboelastography	No sample preparation (whole blood) Platelet clot formation and lysis data	Properties only Insensitive to aspirin Limited study Expensive	Platelet aggregation
Light transmission aggregometry	Historical gold standard Widely available Correlated with clinical events Measure platelet function over time	Large blood volume Sample needs centrifuge Time-consuming Operator- and interpreter-dependent Assess platelet function in the absence of erythrocytes and blood flow Not possible in samples with low platelet counts	Platelet aggregation
Flow cytometry	No sample preparation (whole blood) Small blood volume No contraindication due to thrombocytopenia Allow platelet reactivity testing	Specialized operator Difficult to standardize Only endpoint measurements Not aspects and signaling induced by platelet-platelet-contacts Not include any shear stress component Uncertain correlation with clinical events	Circulating resting and activated platelets Leukocyte-platelet aggregates Procoagulant platelet-derived microparticles

PFA: Platelet function analyzer

function in normal hemostasis, pathological thrombosis, and its important tribute [31].

P-selectin, also named as CD62p, is a surface protein only expressed on the membrane of activated platelets [32]. When platelet was activated, P-selectin is mobilized from intracellular granules to the external plasma membrane, after which fibrinogen aggregates adjacent platelets by bridging GPIIb/IIIa. P-selectin on circulating activated platelets is necessary to recruiting monocytes, neutrophils, and natural killer cells, as well as driving immune cells to thrombi [33] and inducing fibrin production during hemostasis [34].

The agonists usually used to activate platelets in flow cytometry include thrombin or synthetic thrombin receptor agonist peptide (TRAP) and ADP. ADP and its analogs bind to purinergic P2 receptors on platelets. Platelets express three types of receptors, P2X1, P2Y1, and P2Y12. Platelets that possess P2Y1 and P2Y12 are required for full activation and aggregation in response to ADP [35]. After complex signal cascade processes, platelets tend to activate and aggregate [36,37].

Thrombin has been the first agonist used, given its ability to induce total secretion. The activation signaling of platelets GPIIb/IIIa is initiated by thrombin, which binds to G protein-coupled seven-transmembrane receptors [38]. TRAP is a synthetic peptide that has been shown to mimic several effects of thrombin. After activation with TRAP, GPIIb/IIIa expression on the platelets' surface also increases significantly [39].

Flow cytometric methods for determining platelet activation status have been well described, with evidence showing GPIIb/IIIa and P-selectin to be suitable candidates. The PAC-1 antibody (mouse monoclonal antibody) can recognize the activated form of GPIIb/IIIa complex [40,41], and the test evaluating activated GPIIb/IIIa on platelets is well described [42]. P-selectin is also included when measuring activated GPIIb/IIIa flow systems [43]. Recently, most P-selectin binding ligand scaffolds have been used in thrombolysis and imaging thrombosis.

Leukocyte-platelet aggregates

Platelets can bridge to circulating leukocytes, particularly neutrophils and monocytes, through its selectin ligand to form leukocyte-platelet aggregates (LPAs) [44,45]. The critical interactions between CD40 ligand (CD40L) and P-selectin on platelets, as well as P-selectin glycoprotein ligand-1 (CD162) and Mac-1 (integrin α M β 2) on leukocytes are the major receptors recruited in LPA phenomenon [46-48]. Importantly, LPAs can trigger granular content release and activation on platelets and leukocytes, finally lead to modulate immune responses by leukocyte function [49].

Circulating monocyte-platelet aggregation has become a better marker for platelet activation study *in vivo* than platelet surface P-selectin [50]. Flow cytometry offers a highly sensitive and rapid method that can simultaneously analyze circulating LPAs and platelet activation in one sample [51]. The difference in sensitivity between these methods can be attributed to the fact that even low-expression platelet surface P-selectin can still trigger leukocyte binding, and

flow cytometry can easily detect platelets associated with leukocytes through diverse platelet markers.

Flow cytometry assay of LPAs is based on fluorescence-labeling antibody, which can recognize variant surface markers expressed on platelets and leukocytes in blood sample. The gating strategy has been shown to identify platelet aggregates with different leukocyte subtypes of interest. For instance, the target cell populations gated in the side scatter and CD45 (a ubiquitous marker of white blood cells) were transferred into the CD14 (a monocyte marker) and CD45 plots. The expression of CD42b (a platelet marker) on monocytes was regarded as platelet–monocyte complexes [52].

FLOW CYTOMETRY FOR CLINICAL BLEEDING DISORDERS

As mentioned earlier, platelet function has become more important for inherited and acquired bleeding disorders, and flow cytometry is a suitable method to determine platelet function under thrombocytopenic conditions [53]. The methods and molecules used for different platelet disorders in flow cytometry are summarized in Table 2.

For inherited bleeding disorders

Inherited platelet disorders are important etiologies of abnormal hemorrhage [54]. Accordingly, the guideline of the diagnosis with inherited bleeding disorders by the Scientific and Standardization Committee (SSC), Subcommittee of ISTH, recommended for the diagnostic approach by flow cytometry [7]. Flow cytometry for platelet function study is

indicated for the inherited bleeding disorders, especially under thrombocytopenic conditions. Some of these diseases may be included Glanzmann’s thrombasthenia with decreasing of GPIIb/IIIa, Bernard–Soulier syndrome with the deficiency of the GPIb-IX-V, von Willebrand disease, platelet type, with large platelets caused by increasing in GPIBA, and collagen receptor deficiency with abnormal expression with $\alpha 2 \beta 1$ and Gp VI [1,55,56].

While LTA remains the gold standard, investigations via flow cytometry have been increasingly used due to several advantages [57,58]. First, flow cytometry is used to evaluate GP deficiencies of platelet surface, abnormal adhesion, activation or aggregation, and dysfunctions in platelet procoagulant [16,22,59,60]. Whole blood, low platelet counts, or minimal blood volume are still helpful for investigations in children [20,61]. According to the guideline by ISTH, flow cytometry has been used successfully for the diagnosis of GP deficiencies among patients with Glanzmann’s thrombasthenia, Bernard–Soulier syndrome, and collagen receptor deficiencies [59,60].

For acquired bleeding disorders

Acquired bleeding disorders from platelets are a heterogeneous group. Accordingly, thrombocytopenia is more frequently found under systemic or chronic diseases. Previous studies have provided minimal evidence suggesting the association between the risk of hemorrhage and platelet counts in acquired thrombocytopenic patients due to hematological malignancies, infection, and hepatic and nephrotic disease [6-8], with other studies showing impaired platelet function in these disease entities [9-12]. Consensus recommendations on flow cytometry for the evaluation of acquired platelet function disorders were reported by the ISTH SSC Subcommittee [62].

Notably, among patients with hematologic disease, those with acute myeloid leukemia and myelodysplastic syndrome had thrombocytopenia, decreasing immature platelet fraction, and substantially decreasing the expression of platelet surface markers [63-65]. Immune thrombocytopenia has also been reported. Higher expressions of P-selectin and GPIIb/IIIa-positive under TRAP stimulated activated platelets were significantly decreasing bleeding events [64,66].

Among patients with nonhematologic disorders, those with uremia revealed inhibition of GPIIb/IIIa by uremic plasma [67,68]. Patients with alcoholic liver cirrhosis had reduced platelet activation and platelet aggregation based on flow cytometry data [69]. Thrombocytopenia with platelet dysfunction is also frequently found in infection and sepsis. Acquired platelet GP VI receptor dysfunction has also been observed in critically ill patients with infection [70]. In another report, higher platelet activation was found in the patients with septic shock significantly. Increasing platelet surface-bound fibrinogen, CD63, and decreasing surface-bound P-selectin were also noted [71].

For antiplatelet drugs

Dysfunction of platelets and acquired thrombocytopenia frequently occurs at the same time in hospitalized patients due

Table 2: Methods and molecules used for different platelet disorders in flow cytometry

Platelet disorders	Methods	Molecules
Inherited bleeding disorders		
Glanzmann’s thrombasthenia	Decrease in fluorescence level with GPIIb/IIIa complex	GpIIb/IIIa
Bernard-Soulier syndrome	Decrease in fluorescence level with GPIb receptor	GPIb-IX-V
von Willebrand disease, platelet type	Enhanced of fluorescence level with GPIb α receptor	GPIb α
Collagen receptor deficiency	Decrease in fluorescence level with GpIa/IIa and GPVI	GpIa/IIa and GpVI
Acquired bleeding disorders		
Acute myeloid leukemia and myelodysplastic syndrome	Decrease in fluorescence level with GPIIb/IIIa and CD62p	GPIIb/IIIa and CD62p
Immune thrombocytopenia	Decrease in fluorescence level with GPIIb/IIIa and CD62p	GPIIb/IIIa and CD62p
Uremia	Decrease of fluorescence level with GPIIb/IIIa receptor	GpIIb/IIIa
Infection and sepsis	Increased of fluorescence level with GPVI, CD63, and CD62p	GPVI, CD63, and CD62p
Drugs		
Antiplatelet drugs	Decrease in fluorescence level with GPIIb/IIIa and CD62p	GPIIb/IIIa and CD62p
Tyrosine kinase inhibitors	Decrease in fluorescence level with GPIIb/IIIa and CD62p	GPIIb/IIIa and CD62p

CD62p: P-selectin, GP: Glycoproteins

to iatrogenic causes [53]. During the last two decades, flow cytometry started to be used for monitoring of antiplatelet therapy. Initially, under antiplatelet drugs in cardiovascular diseases, flow cytometry had been used for the variable platelet function study. High platelet reactivity has been associated with higher risk for thromboembolism, and platelet inhibition detected by flow cytometry and antiplatelet therapy were more consistent by multiple reported [72-76]. Lee *et al.* also detected the effect of dual-antiplatelet therapy by flow cytometry, including P-selectin and activated GPIIb/IIIa [77]. Overall, evidence suggests that flow cytometry has become more important for determining drug-related platelet dysfunction.

Except antiplatelet drugs in cardiovascular diseases, several tyrosine kinase inhibitors for cancer treatment have been found to inhibit platelet function with bleeding symptoms [78]. Tyrosine kinase inhibitors, including ibrutinib [79], imatinib [80], dasatinib [81], nilotinib [82], sunitinib, sorafenib [83], and gefitinib [84], had been reported previously. Flow cytometry of platelet function has recently been used, given the limitations with LTA and the side effect of drugs in patients with anemia or thrombocytopenia. Flow cytometry could allow platelet reactivity testing and measurement of surface receptor expression [85].

CONCLUSION

Flow cytometry for platelet function exhibited better sensitivity under low platelet counts compared to LTA or other methods. Other advantages of flow cytometry include the small blood volume requiring and nonnecessity of centrifugation for sample preparation [53]. However, flow cytometry still faces several limitations and challenges, including its inability to study aspects and signaling induced by contacts between platelets and platelets. Sample preparation remains labor intensive and requires skilled personnel, and several of the proposed protocols have yet to be validated by studies [86]. For procedure standardization, two consensus studies have been published by the ISTH SSC Subcommittee for inherited [7] and acquired [62] platelet disorders. Although flow cytometry is available for multipurpose and sensitive study of platelet functions at the same time, the challenging analysis gradually increases and needs to be addressed before reality.

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Conflicts of interest

There are no conflicts of interest.

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