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Consensus recommendations on flow cytometry for the assessment of inherited and acquired disorders of platelet number and function: Communication from the ISTH SSC Subcommittee on Platelet Physiology

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Running title: Flow cytometry for platelets

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Abstract
Flow cytometry is increasingly used in the study of platelets in inherited and acquired disorders of platelet number and function. However, wide variation exists in specific reagents, methods, and equipment used, making interpretation and comparison of results difficult. The goal of the present study was to provide expert consensus guidance on the use of flow cytometry for the evaluation of platelet disorders.
A modified RAND survey method was used to obtain a consensus among 11 experts from 10 countries across 4 continents, on the appropriateness of statements relating to clinical utility, pre-analytical variables, instrument and reagent standardization, methods, reporting, and quality control for platelet flow cytometry. Feedback from the initial survey revealed that uncertainty was sometimes due to lack of expertise with a particular test condition rather than unavailable or ambiguous data. To address this, the RAND method was modified to allow experts to self-identify statements for which they could not provide expert input.

There was uniform agreement among experts in the areas of instrument and reagent standardization, methods, reporting and quality control and this agreement is used to suggest best practices in these areas. However, 25.9% and 50% of statements related to pre-analytical variables and clinical utility, respectively, were rated as uncertain. Thus, while citrate is the preferred anticoagulant for many flow cytometric platelet tests, expert opinions differed on the acceptability of other anticoagulants, particularly heparin. Lack of expert consensus on the clinical utility of many flow cytometric platelet tests indicates the need for rigorous multi-center clinical outcome studies.

Key words: platelet, flow cytometry, RAND survey, SSC Platelet Physiology

Introduction
Flow cytometry is increasingly used in platelet research, particularly in the characterization of acquired and inherited platelet disorders [1-4], and the guidance document on the diagnosis of inherited platelet disorders by the Platelet Physiology SSC advises the use of flow cytometry as first and second steps in the diagnostic approach [5]. Flow cytometry has been used for a long time to assess deficiencies in platelet surface glycoproteins (GP) [e.g., GPIIb/IIIa (integrin αIIbβ3, CD41/CD61), GPIb/IX/V, GPIa/IIa (integrin α2β1), GPVI] [1, 6, 7], agonist-induced secretion of α-granules and dense granules (e.g. P-selectin [CD62P], CD63, LAMP-1, and mepacrine) [8-12]. A major advantage of flow cytometry over other methods used to evaluate platelet function is that it requires a minimal numbers of platelets, allowing investigation of platelet function in young children and in patients with thrombocytopenia [2, 13]. In addition, over the last two decades, a major new use of flow cytometry has been monitoring of antiplatelet therapy [14, 15]. High platelet reactivity, as measured by a number of methods, in patients treated with antiplatelet agents has been associated with increased risk for ischemic events [15], and flow cytometric tests show greater and more consistent platelet inhibition in patients treated with second [16, 17] and third generation $P2Y_{12}$ antagonists [18].

A worldwide survey on the diagnosis of inherited platelet disorders showed that flow cytometry is among the most widely used methods in platelet diagnostic laboratories [19]. However, despite years of widespread use, great variation exists in equipment, reagents and methods used for flow cytometric analysis of platelet function, making interpretation and comparison of results difficult. A previous consensus protocol for the flow cytometric characterization of platelet function was published in 1998 [20] and in 2008 a multicenter effort was undertaken to standardize the measurement of platelet surface P-selectin [21]. Beyond this, only a small number of studies have provided information on pre-analytical factors, reproducibility and clinical utility of platelet flow cytometric tests [4, 5, 22-31]. We therefore undertook an international survey of experts to identify current practices and recommendations.

Methods

A literature search was performed to identify published methods related to standardization of flow cytometry for the assessment of inherited and acquired platelet disorders. Due to the limited number of prospective studies, the RAND method [32] was used to obtain a consensus among experts in the field. An expert panel was assembled based on recommendations from members of the Platelet Physiology SSC and of individuals publishing in the area and/or known to lead laboratories performing flow cytometric analysis of platelets. The resulting panel comprised 11 experts representing 10 countries on 4 continents (see Acknowledgements). The RAND approach required members of the expert panel (blinded to the responses of other panel members), to score statements regarding the topic from 1 – 9, where 1 is completely inappropriate and 9 is fully appropriate. The highest and lowest scores were discarded and the median of the remaining scores calculated. Statements were then classified as inappropriate (scores of 1 – 3), uncertain (scores of 4 – 6), or appropriate (scores of 7 – 9).

Statements on flow cytometry of platelets were drafted by the Principal Investigators (ALF and JR) and circulated among the members of the Platelet Physiology SSC for input. The refined survey containing a total of 75 survey statements covered the following areas: 1) clinical utility – 14 statements, 2) pre-analytical variables – 27 statements, 3) instrument and reagent standardization – 10 statements, 4) methods – 12 statements, 5) reporting – 7 statements, and 6) quality control – 5 statements. **Supplemental Table S1A** shows an example of the statements scoring system sent and used by the experts in the first round survey. The full list of statements used in this first round survey, and their final scores, is provided in **Supplemental Tables S2–S7**.

A second survey round was deemed necessary based on experts feedback regarding expertise in specific tests and ambiguity of some statements. Feedback revealed that among the variety of platelet parameters evaluated by flow cytometry, uncertainty was sometimes due to lack of expertise with a particular test condition rather than unavailable or ambiguous data. To address this, the RAND method was modified allowing experts to self-identify statements for which they could not provide expert input. Moreover, ambiguity of statements regarding pre-analytical conditions were clarified by specifying a particular assay endpoint and asking the experts to rank common pre-analytical variables

(anticoagulant, whole blood *vs.* platelet-rich plasma, time interval between sample collection and processing, fresh vs. fixed, *etc.*) as: 1) recommended, 2) acceptable, 3) not recommended 4) uncertain (lack of data) or 5) no experience. Subsequent results were summarized only for those experts with experience. **Supplemental Table S1B** shows an example of the statements scoring system sent and used by the experts in the second round survey. The full list of statements used in the second survey is provided in **Supplemental Tables S8–S11**.

Results of the initial survey, and the revised questions from the second survey, are presented as the median and range of the scores of the eleven experts (**Supplemental Tables S2–S7 and S11)**. The level of agreement between expert panel members with respect to each statement was modeled after Fitch *et al.*, [32] and judged according to the coefficient of variation (CV) of the numerical responses as follows: CV <32% indicated agreement (+); CV >42% indicated disagreement (-); CV between 32% and 42% indicated inconclusive agreement. These arbitrary cut-offs provide an objective means to assess the level of agreement with a high CV (>42%) generally being the result of panelist ratings in each extreme (1-3 and 7-9). Results of the second survey regarding pre-analytical conditions divided by assay endpoint are presented as percent of experts, among those stating to have knowledge or experience, who selected 1) recommended, 2) acceptable, 3) not recommended, or 4) uncertain (lack of data) for specific pre-analytical variables for specific platelet tests (**Supplemental Tables S8–S10)**.

Results

First, we found good agreement among panel experts in the areas of instrument and reagent standardization, methods, reporting, and quality control **(Supplemental Tables S4, S5, S6 and S7)**. In general, the practices identified are common good laboratory procedures applicable to all areas of testing.

Expert recommendations with regard to "Instrument and reagent standardization"

In this section of the survey, all statements were rated as appropriate (panel median ratings of 7–9) by the experts (**Supplemental Table S4).** A good agreement (CV < 32%) was seen for all but one statement, where there was inconclusive agreement (CV between 32% and 42%) :

- Each day that samples are analyzed, a quality control check of the flow cytometer should be run using standardized fluorescent calibration beads.

Recommendations on "Platelet flow cytometry methods"

Also in this part, experts rated all statements as appropriate (**Supplemental Table S5),** and good agreement (CV < 32%) was seen for all but one statement, where there was inconclusive agreement (CV between 32% and 42%):

- Methods should include the methods used to train individuals performing these tests and procedures for documenting their training.

Recommendations on "Reporting and quality control"

Here, all statements included in the survey were rated as appropriate by the experts and there was good agreement (CV<32%) in this rating (**Supplemental Tables S6 and S7)**

Expert recommendations with regard to "Clinical utility and pre-analytical variables"
There was good agreement between panel members that flow cytometry is useful for 1) the diagnosis of inherited deficiencies of platelet surface glycoproteins, 2) the diagnosis of disorders that result in abnormal platelet procoagulant activity such as Scott syndrome and Stormorken syndrome, 3) the diagnosis of platelet α granule secretion defects, 4) the diagnosis of defects in specific platelet activation (signaling) pathways, and 5) the determination of the fraction of immature platelets.
However, 7 out of 14 statements were rated as uncertain **(Supplemental Table S2)** including the utility of flow cytometry to diagnose dense granule and cytoskeletal defects, to recognize heparin-induced thrombocytopenia, and to monitor platelet inhibition by aspirin.

Twenty of 27 statements related to pre-analytical variables of flow cytometry for assessment of inherited and acquired platelet disorders were judged to be appropriate with good agreement between experts while 7 statements were judged to be uncertain **(Supplemental Table S3)**. Pre-analytical variable statements rated as appropriate and with good agreement between experts included careful documentation of potentially interfering medications, sample collection times, and steps to avoid platelet activation and/or hemolysis during sample collection. Statements rated as uncertain included use of heparin, ACD-A or EDTA as anticoagulants and the minimum and maximum acceptable time interval between sample collection and analysis. Expert panel feedback indicated that uncertainty was in part due to differences in the requirements for different flow cytometric platelet tests.

Second survey
Feedback from expert panel members indicated that statements that were rated as "uncertain" in the initial survey, particularly with respect to clinical utility, were inconclusive in part due to lack of specificity regarding pre-analytical variables, as the requirements may differ between analysis of abnormal levels of platelet surface molecules and agonist-stimulated changes in platelet activation markers. In addition, expert panel feedback identified areas which were not adequately covered in the initial survey and revealed that not all experts had experience in some of the less frequently used platelet flow cytometric tests. Based on this feedback, a second survey was developed which allowed collection of expert opinion on the interaction of pre-analytical variables with clinical utility and also allowed panel members to distinguish between areas in which data was lacking or inconclusive vs. areas where they were unable to provide an informed opinion. **Supplemental Table S1B** shows an example of such a revised statements and their scoring by the experts and **Supplemental Table S11** shows the results for the other revised and added statements.

Table 1 shows the expert consensus of pre-analytical variables for the assessment of inherited and acquired abnormal levels of platelet surface molecules. Results shown are the percentage of experts, among those who self-identified as having knowledge or expertise, at each level of recommendation (Recommended, Acceptable, Not Recommended, Uncertain). Not surprisingly, citrate, the most commonly used anticoagulant for platelet studies, was recommended for flow cytometry studies of GPIIb/IIIa, GPIb/IX/V, GPIa/IIa, and GPVI. Whole blood was generally preferred for these studies, but platelet-rich plasma (PRP) was rated acceptable or recommended by most experts. Comments from the expert panel revealed that acceptability of formalin fixation depended on the use of antibodies that react with the receptor following fixation.

Early testing (within 30 min of sample collection) was recommended by the majority of experts in the context of in vivo platelet-leukocyte aggregate characterization, although testing within this time frame may not be achievable in a clinical setting (**Table 1**). In contrast, a substantial portion of experts indicated that it may be acceptable to test for platelet glycoproteins up to 24 hours after sample collection.

Similarly, citrate anticoagulation and early testing was recommended by the majority of experts for agonist-stimulated changes in platelet surface glycoproteins (**Supplemental Table S8**), but approximately 30% of experts find testing within 24 hours to be acceptable. For these types of analyses, a resting period before analysis was considered appropriate with good agreement between experts, and also that a parallel sample without activation should be included and reported to take into account the potential presence of primed/pre-activated platelets (**Supplemental Table S11**).

Most experts (>50%) did not have experience with flow cytometry for measurement of von Willebrand factor (VWF) binding to platelets, which can be useful for Bernard Soulier syndrome (BSS) and platelet-type von Willebrand disease (PT-VWD) characterization (**Supplemental Table S9**). However, several publications provide good technical detail on this procedure [33-35]. Similarly, many experts did not have experience with different permeabilization methods for measurement of platelet intracellular markers such as Wiskott-Aldrich syndrome protein (WASp) and phosphorylated vasodilator-stimulated phosphoprotein (VASP-P) (**Supplemental Table S10**). Of note, a standardized flow cytometry assay for the measurement of VASP-P in patients treated with inhibitors of the platelet $P2Y_{12}$ ADP receptor was developed [14, 36] and is commercially available (PLT VASP P2Y12, BioCytex-Stago Group, Asnières-sur-Seine, France).

Major recommendations

A summary of the major recommendations, as rated by expert consensus, for flow cytometric analysis of inherited and acquired platelet disorders is shown in **Table 2**.

Discussion

This report provides recommendations based on input from an international panel of experts on pre-analytical variables, instrument and reagent standardization, methods,

reporting, and quality control, for the assessment of inherited and acquired disorders of platelet number and function by flow cytometry. Minimum data standards were consistent with MIFlowCyt recommendations [37]. Remarkably, there was broad agreement across a wide range of statements, which could be viewed as good laboratory practice (**Table 2**). The effect of sample volume and needle size on platelet flow cytometry was recently analyzed [38] and was not included in the survey. While a consensus emerged regarding pre-analytical variables, the survey revealed a surprisingly broad range of pre-analytical conditions to be acceptable. For example, while anticoagulation with citrate was preferred, experts were divided on the acceptability of heparin, with 4 of the 11 experts reporting no pertinent experience with the use of heparin for the specific tests. Similarly, while the preferred time interval between blood collection and testing was 30 min or less for in vivo platelet activity / platelet-leukocyte aggregate formation assays, 30% of experts indicated that testing of samples at up to 24 hours after collection for their glycoprotein levels or functional responses to activation in vitro was acceptable. Indeed, large distances between patients and testing centers contribute to the need to test samples at up to 24 hours after collection. However, data to support stability of untreated whole blood overtime are currently scarce and time frames for platelet analysis can be different for GP expression and for platelet activation testing [27, 28, 39].

Several laboratories have sought to address this issue by stabilizing samples through addition of a fixative solution, such as FACS lysing solution [BD Biosciences, San Jose, CA] [16, 40], formalin or formaldehyde containing buffer [17, 41-43], or PAMFix [Platelet Solutions Ltd, Nottingham, UK] [24, 25], at the point of collection prior to sending the sample to the testing center. Addition of fixative to freshly collected platelet samples has the advantage that it can preserve samples for later analysis, but sensitivity of certain epitopes to fixation (notably the binding site on activated GPIIb-IIIa for fibrinogen which is recognized by the monoclonal IgM antibody, PAC-1) can limit this approach. Hence, the use of fresh samples rather than fixed samples was generally favored by the panel members (**Table 1**). An approach that was not specifically addressed in the survey statements, but which has been used in some studies [4, 17, 22, 24, 25, 42, 43], is to provide prepared aliquots of fluorescent antibodies, with or without agonist, to sites where samples are collected. Thus, with minimal sample manipulation, freshly collected blood can be reacted with antibodies then stabilized by fixation for later analysis at a core facility. This approach has been commercialized by at least two groups [24-26, 38, 44].

The inconclusive agreement regarding the use of a daily quality control check of the flow cytometer using standardized fluorescent calibration beads may be related to the fact that this statement referred not only to the need to run a quality control, but also that this should be done daily and using calibration beads. Certain instruments or FC applications (e.g. in haematology) require stabilized blood and not calibrated beads as QC [45]. This may have contributed to the uncertain rating of this statement, and should not be taken as an uncertainty around the need for calibration. Guidance on this topic as well as panel design may be drawn from recent reviews [46, 47].

Survey results showed that even among expert panel members with extensive background in the analysis of platelets by flow cytometry, few had direct experience with flow cytometry for measurement of VWF, which can be useful for the diagnosis of PT-VWD [34] and the characterization of BSS, particularly those variant forms of BSS which have normal or minimally affected GPIb/IX/V expression. Recommendations for this specific assay are needed. Similarly, most experts reported no experience/knowledge regarding different fixation and permeabilization methods for staining of intracellular platelet proteins. This is only partly addressed by a recent publication on technical considerations for platelet phosphoflow cytometry and barcoding [48]. These findings highlight the need for future standardization studies comparing flow cytometric methods and testing conditions in order to establish best practice, also regarding issues such as determination of saturation for antibodies and gating strategies. Indeed, lack of expert consensus on the clinical utility of many flow cytometric platelet tests indicates the need for rigorous multi-center clinical outcomes studies. In addition, the creation of shared data repositories and more advanced multi-color protocols may be other areas of future development in this rapidly expanding field.

Author Contributions

ALF and JR designed the study, identified the expert panelists, conceived the first and secondary survey statements, analyzed data and wrote the manuscript. DEC, KF, AG, PG, PH, SK, ML, ADM and SR contributed expertise, provided feedback following the first survey and critically revised the manuscript. ALF, ML, SR and PG revised the manuscript following peer review. PG chaired the Platelet Physiology SSC and oversaw the project. All authors reviewed and approved the final manuscript for publication.

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Disclosures

The authors declare no conflict of interest.

References

1. Jennings LK, Ashmun RA, Wang WC, Dockter ME. Analysis of human platelet glycoproteins IIb-IIIa and Glanzmann's thrombasthenia in whole blood by flow cytometry. *Blood*. 1986; **68**: 173-9.
2. Michelson AD. Flow cytometry: a clinical test of platelet function. *Blood*. 1996; **87**: 4925-36.
3. Blair TA, Frelinger III AL, Michelson AD. Flow cytometry. In: Michelson AD, Cattaneo M, Frelinger III AL, Newman PJ, eds. *Platelets*, 4th edn. San Diego: Elsevier, 2019, 627-52.
4. Ramstrom S, Sodergren AL, Tynngard N, Lindahl TL. Platelet Function Determined by Flow Cytometry: New Perspectives? *Semin Thromb Hemost*. 2016; **42**: 268-81. 10.1055/s-0035-1570082.
5. Gresele P, the Subcommittee on Platelet P. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. *J Thromb Haemost*. 2015; **13**: 314-22. https://doi.org/10.1111/jth.12792.
6. Marti GE, Magruder L, Schuette WE, Gralnick HR. Flow cytometric analysis of platelet surface antigens. *Cytometry*. 1988; **9**: 448-55. 10.1002/cyto.990090508.
7. Giannini S, Mezzasoma AM, Guglielmini G, Rossi R, Falcinelli E, Gresele P. A new case of acquired Glanzmann's thrombasthenia: diagnostic value of flow cytometry. *Cytometry Part B, Clinical cytometry*. 2008; **74**: 194-9. 10.1002/cyto.b.20396.
8. Febbraio M, Silverstein RL. Identification and characterization of LAMP-1 as an activation-dependent platelet surface glycoprotein. *J Biol Chem*. 1990; **265**: 18531-7.
9. Nieuwenhuis HK, van Oosterhout JJ, Rozemuller E, van Iwaarden F, Sixma JJ. Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood*. 1987; **70**: 838-45.
10. Stenberg PE, McEver RP, Shuman MA, Jacques YV, Bainton DF. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. *J Cell Biol*. 1985; **101**: 880-6.
11. Mumford AD, Frelinger AL, 3rd, Gachet C, Gresele P, Noris P, Harrison P, Mezzano D. A review of platelet secretion assays for the diagnosis of inherited platelet secretion disorders. *Thromb Haemost*. 2015; **114**: 14-25. 10.1160/TH14-11-0999.
12. Ramstrom AS, Fagerberg IH, Lindahl TL. A flow cytometric assay for the study of dense granule storage and release in human platelets. *Platelets*. 1999; **10**: 153-8. 10.1080/09537109976239.
13. Boknäs N, Macwan A, Södergren A, Ramström S. Platelet function testing at low platelet counts: When can you trust your analysis? *Res Pract Thromb Haemost*. 2019; **3**: 285-90. 10.1002/rth2.12193.
14. Aleil B, Ravanat C, Cazenave JP, Rochoux G, Heitz A, Gachet C. Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases. *J Thromb Haemost*. 2005; **3**: 85-92.
15. Frelinger AL, Gachet C, Mumford AD, Noris P, Mezzano D, Harrison P, Gresele P. Laboratory Monitoring of P2Y12 Inhibitors: communication from the SSC of the ISTH. *J Thromb Haemost*. 2018; **16**: 1-6. 10.1111/jth.14282.
16. Michelson AD, Frelinger AL, 3rd, Braunwald E, Downey WE, Angiolillo DJ, Xenopoulos NP, Jakubowski JA, Li Y, Murphy SA, Qin J, McCabe CH, Antman EM, Wiviott SD. Pharmacodynamic assessment of platelet inhibition by prasugrel vs. clopidogrel in the TRITON-TIMI 38 trial. *Eur Heart J*. 2009; **30**: 1753-63.

17. Wiviott SD, Trenk D, Frelinger AL, O'Donoghue M, Neumann FJ, Michelson AD, Angiolillo DJ, Hod H, Montalescot G, Miller DL, Jakubowski JA, Cairns R, Murphy SA, McCabe CH, Antman EM, Braunwald E, PRINCIPLE-TIMI Investigators. Prasugrel compared with high loading- and maintenance-dose clopidogrel in patients with planned percutaneous coronary intervention: the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction 44 trial. *Circulation*. 2007; **116**: 2923-32. 10.1161/CIRCULATIONAHA.107.740324.
18. Storey RF, Angiolillo DJ, Bonaca MP, Thomas MR, Judge HM, Rollini F, Franchi F, Ahsan AJ, Bhatt DL, Kuder JF, Steg PG, Cohen M, Muthusamy R, Braunwald E, Sabatine MS. Platelet Inhibition With Ticagrelor 60 mg Versus 90 mg Twice Daily in the PEGASUS-TIMI 54 Trial. *J Am Coll Cardiol*. 2016; **67**: 1145-54. 10.1016/j.jacc.2015.12.062.
19. Gresele P, Harrison P, Bury L, Falcinelli E, Gachet C, Hayward CP, Kenny D, Mezzano D, Mumford AD, Nugent D, Nurden AT, Orsini S, Cattaneo M. Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey. *J Thromb Haemost*. 2014; **12**: 1562-9. 10.1111/jth.12650.
20. Schmitz G, Rothe G, Ruf A, Barlage S, Tschope D, Clemetson KJ, Goodall AH, Michelson AD, Nurden AT, Shankey TV. European Working Group on Clinical Cell Analysis: Consensus protocol for the flow cytometric characterisation of platelet function. *Thromb Haemost*. 1998; **79**: 885-96.
21. Curvers J, de Wildt-Eggen J, Heeremans J, Scharenberg J, de Korte D, van der Meer PF. Flow cytometric measurement of CD62P (P-selectin) expression on platelets: a multicenter optimization and standardization effort. *Transfusion*. 2008; **48**: 1439-46. 10.1111/j.1537-2995.2008.01738.x.
22. Lindahl TL, Ramstrom S. Methods for evaluation of platelet function. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis*. 2009; **41**: 121-5. 10.1016/j.transci.2009.07.015.
23. Linden MD, Frelinger AL, 3rd, Barnard MR, Przyklenk K, Furman MI, Michelson AD. Application of flow cytometry to platelet disorders. *Semin Thromb Hemost*. 2004; **30**: 501-11.
24. Dovlatova N. Current status and future prospects for platelet function testing in the diagnosis of inherited bleeding disorders. *Br J Haematol*. 2015; **170**: 150-61. 10.1111/bjh.13405.
25. Dovlatova N, Lordkipanidze M, Lowe GC, Dawood B, May J, Heptinstall S, Watson SP, Fox SC, Group UGS. Evaluation of a whole blood remote platelet function test for the diagnosis of mild bleeding disorders. *J Thromb Haemost*. 2014; **12**: 660-5. 10.1111/jth.12555.
26. van Asten I, Schutgens REG, Baaij M, Zandstra J, Roest M, Pasterkamp G, Huisman A, Korporaal SJA, Urbanus RT. Validation of flow cytometric analysis of platelet function in patients with a suspected platelet function defect. *J Thromb Haemost*. 2018; **16**: 689-98. 10.1111/jth.13952.
27. Boknas N, Ramstrom S, Faxalv L, Lindahl TL. Flow cytometry-based platelet function testing is predictive of symptom burden in a cohort of bleeders. *Platelets*. 2018; **29**: 512-9. 10.1080/09537104.2017.1349305.
28. Rubak P, Nissen PH, Kristensen SD, Hvas AM. Investigation of platelet function and platelet disorders using flow cytometry. *Platelets*. 2016; **27**: 66-74. 10.3109/09537104.2015.1032919.

29. Andres O, Henning K, Strauss G, Pflug A, Manukjan G, Schulze H. Diagnosis of platelet function disorders: A standardized, rational, and modular flow cytometric approach. *Platelets*. 2018; **29**: 347-56. 10.1080/09537104.2017.1386297.
30. Pasalic L, Pennings GJ, Connor D, Campbell H, Kritharides L, Chen VM. Flow Cytometry Protocols for Assessment of Platelet Function in Whole Blood. *Methods in molecular biology*. 2017; **1646**: 369-89. 10.1007/978-1-4939-7196-1_28.
31. van Asten I, Schutgens REG, Urbanus RT. Toward Flow Cytometry Based Platelet Function Diagnostics. *Semin Thromb Hemost*. 2018; **44**: 197-205. 10.1055/s-0038-1636901.
32. Fitch K, Bernstein S, Aguilar M, Burnand B, LaCalle J, Lazaro P, van het Loo M, McDonnell J, Vader J, Kahan J. The RAND/UCLA Appropriateness Method User's Manual. Santa Monica, CA: RAND Corporation, 2001.
33. Giannini S, Mezzasoma AM, Leone M, Gresele P. Laboratory diagnosis and monitoring of desmopressin treatment of von Willebrand's disease by flow cytometry. *Haematologica*. 2007; **92**: 1647-54. 10.3324/haematol.11313.
34. Giannini S, Cecchetti L, Mezzasoma AM, Gresele P. Diagnosis of platelet-type von Willebrand disease by flow cytometry. *Haematologica*. 2010; **95**: 1021-4. 10.3324/haematol.2009.015990.
35. Mina A, Favaloro EJ, Koutts J. A novel flow cytometry single tube bead assay for quantitation of von Willebrand factor antigen and collagen-binding. *Thromb Haemost*. 2012; **108**: 999-1005. 10.1160/TH12-05-0294.
36. Siller-Matula JM, Panzer S, Jilma B. Reproducibility and standardized reporting of the vasodilator-stimulated phosphoprotein phosphorylation assay. *Platelets*. 2008; **19**: 551-4.
37. Lee JA, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M, Furlong J, Gasparetto M, Goldberg M, Goralczyk EM, Hyun B, Jansen K, Kollmann T, Kong M, Leif R, McWeeney S, Moloshok TD, Moore W, Nolan G, Nolan J, Nikolich-Zugich J, Parrish D, Purcell B, Qian Y, Selvaraj B, Smith C, Tchuvatkina O, Wertheimer A, Wilkinson P, Wilson C, Wood J, Zigon R, International Society for Advancement of Cytometry Data Standards Task F, Scheuermann RH, Brinkman RR. MIFlowCyt: the minimum information about a Flow Cytometry Experiment. *Cytometry A*. 2008; **73**: 926-30. 10.1002/cyto.a.20623.
38. Pedersen OH, Nissen PH, Hvas AM. Platelet function investigation by flow cytometry: Sample volume, needle size, and reference intervals. *Platelets*. 2018; **29**: 199-202. 10.1080/09537104.2017.1353684.
39. Huskens D, Sang Y, Konings J, van der Vorm L, de Laat B, Kelchtermans H, Roest M. Standardization and reference ranges for whole blood platelet function measurements using a flow cytometric platelet activation test. *PLoS One*. 2018; **13**: e0192079. 10.1371/journal.pone.0192079.
40. Barnard MR, Linden MD, Frelinger AL, 3rd, Li Y, Fox ML, Furman MI, Michelson AD. Effects of platelet binding on whole blood flow cytometry assays of monocyte and neutrophil procoagulant activity. *J Thromb Haemost*. 2005; **3**: 2563-70.
41. Furman MI, Krueger LA, Linden MD, Fox ML, Ball SP, Barnard MR, Frelinger AL, 3rd, Michelson AD. GPIIb-IIIa antagonists reduce thromboinflammatory processes in patients with acute coronary syndromes undergoing percutaneous coronary intervention. *J Thromb Haemost*. 2005; **3**: 312-20.
42. Frelinger AL, 3rd, Michelson AD, Wiviott SD, Trenk D, Neumann FJ, Miller DL, Jakubowski JA, Costigan TM, McCabe CH, Antman EM, Braunwald E. Intrinsic platelet reactivity before P2Y12 blockade contributes to residual platelet reactivity despite high-level P2Y12 blockade by prasugrel or high-dose clopidogrel. Results

from PRINCIPLE-TIMI 44. *Thromb Haemost*. 2011; **106**: 219-26. 10.1160/TH11-03-0185.
43. Psaila B, Bussel JB, Linden MD, Babula B, Li Y, Barnard MR, Tate C, Mathur K, Frelinger AL, Michelson AD. In vivo effects of eltrombopag on platelet function in immune thrombocytopenia: no evidence of platelet activation. *Blood*. 2012; **119**: 4066-72. 10.1182/blood-2011-11-393900.
44. Kicken CH, Roest M, Henskens YM, de Laat B, Huskens D. Application of an optimized flow cytometry-based quantification of Platelet Activation (PACT): Monitoring platelet activation in platelet concentrates. *PLoS One*. 2017; **12**: e0172265. 10.1371/journal.pone.0172265.
45. Barnett D, Reilly JT. Quality Control in Flow Cytometry. Totowa, NJ: Humana Press, 113-31.
46. Spurgeon BEJ, Naseem KM. Platelet Flow Cytometry: Instrument Setup, Controls, and Panel Performance. *Cytometry Part B, Clinical cytometry*. 2019. 10.1002/cyto.b.21774.
47. Busuttil-Crellin X, McCafferty C, Van Den Helm S, Yaw HP, Monagle P, Linden M, Ignjatovic V. Guidelines for panel design, optimization, and performance of whole blood multi-color flow cytometry of platelet surface markers. *Platelets*. 2020; **31**: 845-52. 10.1080/09537104.2019.1709630.
48. Spurgeon BEJ, Naseem KM. Phosphoflow cytometry and barcoding in blood platelets: Technical and analytical considerations. *Cytometry Part B, Clinical cytometry*. 2020; **98**: 123-30. 10.1002/cyto.b.21851.

Tables

Table 1. Expert consensus of pre-analytical variables for the assessment of inherited and acquired abnormal levels of platelet surface molecules. Results shown are the percent of experts supporting each level of recommendation, among the eleven experts of the panel which self-identified as having knowledge or expertise (N)

		GPIIb/IIIa	GPIb/IX/V	GPIa/IIa	GPVI
Citrate	Recommended	80%	80%	88%	80%
	Acceptable	20%	20%	13%	20%
	Not Recommended	0%	0%	0%	0%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	10	10	8	10
Heparin	Recommended	0%	0%	0%	0%
	Acceptable	43%	50%	33%	43%
	Not Recommended	57%	50%	67%	57%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	7	6	6	7
EDTA	Recommended	0%	17%	14%	17%
	Acceptable	43%	67%	29%	33%
	Not Recommended	57%	17%	57%	50%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	7	6	7	6
Whole Blood	Recommended	80%	70%	86%	80%
	Acceptable	10%	20%	0%	0%
	Not Recommended	10%	10%	14%	20%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	10	10	7	10
PRP	Recommended	30%	40%	38%	30%
	Acceptable	60%	40%	50%	50%
	Not Recommended	10%	20%	13%	20%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	10	10	8	X
Fresh/Not Fixed	Recommended	90%	90%	100%	90%
	Acceptable	10%	10%	0%	10%
	Not Recommended	0%	0%	0%	0%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	10	10	8	10
Formalin-fixed	Recommended	14%	29%	17%	17%
	Acceptable	86%	71%	83%	83%
	Not Recommended	0%	0%	0%	0%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	7	7	6	6
Test within 30 min	Recommended	67%	67%	63%	60%
	Acceptable	22%	22%	38%	30%
	Not Recommended	11%	11%	0%	10%
	Uncertain (lack of data)	0%	0%	0%	0%
	N	9	9	8	10
Test within 4 hr	Recommended	56%	56%	63%	50%
	Acceptable	33%	33%	13%	30%

	Not Recommended	11%	11%	13%	20%
	Uncertain (lack of data)	0%	0%	13%	0%
	N	9	9	8	10
Test within 24 hr	Recommended	11%	11%	0%	0%
	Acceptable	44%	44%	63%	63%
	Not Recommended	33%	33%	25%	38%
	Uncertain (lack of data)	11%	11%	13%	0%
	N	9	9	8	8
Test within 96 hr	Recommended	0%	0%	0%	0%
	Acceptable	14%	14%	17%	0%
	Not Recommended	71%	71%	67%	71%
	Uncertain (lack of data)	14%	14%	17%	29%
	N	7	7	6	7

Table 2. Catalog of the major recommendations as rated as by expert consensus, for flow cytometric analysis of inherited and acquired platelet disorders.

Topic	Recommendation
Clinical settings where flow cytometric analysis of platelets is useful	• Diagnosis of inherited or acquired deficiencies of platelet surface glycoproteins (BSS, GT, inherited or immune-mediated GPVI defects) • Diagnosis of platelet alpha granule secretion defects (such as Grey platelet syndrome) • Diagnosis of defects in specific platelet activation (signaling) pathways (such as RASGRP2, P2Y12 or TXA2R disorders) • Diagnosis of GFI1B macrothrombocytopenia associated to platelet expression of CD34 • Diagnosis of disorders of platelet procoagulant activity (such as Scott syndrome and Stormorken syndrome) • Assessment of increased platelet activation in prothrombotic syndromes (diabetes, anti-phospholipid syndrome or secondary to drug induced, non-immune platelet activation) • Monitoring, if applicable, pharmacodynamic effect $P2Y_{12}$ antagonists (ticlopidine, clopidogrel, prasugrel, ticagrelor, cangrelor) with specifically designed test such as VASP P2Y12 • Determination of the fraction of immature platelets
Pre-analytical variables	• Information should be collected at the time of the blood draw on the use of medications that interfere with platelet function (antihistamines, theophylline, antibiotics, tricyclic antidepressants, dipyridamole, P2Y12 antagonists and aspirin, among others) • Blood sampling should avoid procedures favoring platelet activation (prolonged or too tight tourniquet, small gauge needles, slow blood drawing, initial blood draw into non-anticoagulated syringes, difficult or discontinuous blood draw, etc.), and the first 1 – 2 mL of blood should be discarded • Citrate should generally be the first choice anticoagulant • Partial filling of anticoagulant tubes should be avoided • Date and times of blood sample collection and platelet analysis must be recorded • Blood samples for platelet testing ex vivo should be rested a minimum 30 min then stored at room temperature (20 – 25°C) prior to analysis • Analysis of platelet glycoproteins should be performed preferentially in fresh whole blood within 24h from collection • Analysis of agonist induced platelet activation (e.g. fibrinogen or PAC-1 binding, CD62P expression, or other functional tests) should be performed preferentially in fresh whole blood within 4h from collection • Analysis of in vivo circulating monocyte-platelet aggregates should be performed immediately after blood collection, or in fixed whole blood, to prevent ex vivo formation of these conjugates • A stabilizing solution (usually a fixative) may be used to allow later flow cytometric analysis (up to several days) of some platelet antigens

Instrument and reagents	• The minimum requirements of flow cytometry instruments are detection of forward and side light scatter, detection of at least two fluorescence signals with good sensitivity (i.e., detection of <1000 FITC molecules per cell) and allowing for fluorescence compensation (hardware or software) if two, or more, fluorophores are in use • Software for data acquisition should allow data storage preferentially in a standard, sharable format (*e.g.,* FCS-2.0 or FCS-3.0) • Flow cytometry instruments should undergo maintenance and qualification on a regular basis (*e.g.* by use of preventive maintenance and calibrations plan) • Pipettes should be calibrated on a regular schedule • Validated and commonly used antibodies or ligands should be used before the expiration dated indicated by the manufacturer • Novel or less commonly used antibodies should be validated (e.g., by Western blotting, immunoprecipitation, reactivity with known positive/negative cells, or other appropriate method)
Methods	• Flow cytometry protocols should contain at least: a) version number, dates of last update and approval; b) instrument description summary and any user-configurable settings; c) sufficient detail to allow the procedure to be reproduced by an independent party (source, type and processing of samples; full description of the antibodies, ligands or agonists to be used (source, catalog and lot number, clone, target protein, fluorophore, recommended concentration); d) positive and negative controls to be included in the assay;e) strategies for event gating and for event analysis; f) results reporting • The use of a fluorescent platelet-identifying antibody and setting the threshold accordingly is desirable to minimize interference by RBCs or other blood cells • Antibodies against target antigens should be used at saturating conditions. • For each assay, laboratories should generate their own reference ranges for healthy individuals.
Reporting and Quality Control	• A standardized laboratory report form should include: Date and times of sample collection and analysis; b) standard operating procedure used and reagents description; result description, (if applicable, international units, references range and test uncertainty); result interpretation • Stability and reproducibility of the flow cytometric methods in place should be documented • Documentation should allow external audit of: a) samples possession, processing, and analysis; b) the in date (not expired) use of all reagents; c) the regular maintenance and qualification of instruments; d) the training of the personnel performing the methods

Supplemental information

Consensus recommendations on flow cytometry for the assessment of inherited and acquired disorders of platelet number and function: Communication from the SSC of the ISTH

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FOR THE SUBCOMMITTEE ON PLATELET PHYSIOLOGY

Supplemental Table S1. Example of the statements scoring system used by the experts in the primary (A) and secondary (B) surveys. The secondary survey was specifically designed to capture recommended best pre-analytical variables for a specific clinical application of platelet flow cytometry.

A) Sample statements from primary survey.

Flow cytometry is useful in the determination of platelet count.	Inappropriate 1	2	3	4	5	6	7	8	Appropriate 9
	☐	☐	☐	☐	☐	☐	☐	☐	☒
ACD-A is an acceptable anticoagulant for blood collected for platelet testing by flow cytometry.	Inappropriate 1	2	3	4	5	Uncertain 6	7	8	Appropriate 9
	☐	☐	☐	☐	☐	☒	☐	☐	☐

B) Sample statements from secondary survey

For flow cytometry assessment of abnormal levels of platelet surface GPIIb/IIIa (integrin αIIbβ3, CD41/CD61) as in suspected Glanzmann thrombasthenia, sample recommendations are as follows (please check lowest number that applies in each row)

	recommended 1	acceptable 2	not recommended 3	uncertain (lack of data) 4	no experience 5
whole blood	☐	☒	☐	☐	☐
PRP	☒	☐	☐	☐	☐
citrate	☒	☐	☐	☐	☐
heparin	☐	☐	☐	☐	☒
EDTA	☐	☐	☐	☐	☒
fresh/not fixed	☒	☐	☐	☐	☐
formalin fixed	☐	☐	☐	☐	☒
test within 30 min	☒	☐	☐	☐	☐
test within 4 hr	☐	☒	☐	☐	☐
test within 24 hr	☐	☐	☐	☒	☐
test within 96 hr	☐	☐	☐	☐	☒
Other (please explain):					

Supplemental Table S2. Clinical Utility: Survey 1 statements and expert panel scores: 1 – 3 inappropriate, 4 – 6 uncertain, 7 – 9 appropriate. Agreement between expert panel members with respect to each statement was judged according to the coefficient of variation (CV) of the

numerical responses as follows: CV <32% indicated agreement (+); CV >42% indicated disagreement (-);.CV between 32-42% indicated inconclusive agreement (?). Based on the feedback from experts in Survey 1, some statements were removed (*) or revised (**) in Survey 2.

I.	**Clinical Utility**	Appropriateness Median, Range CV, Level of Agreement (+, ?, -)
I.1	Flow cytometry is useful in the determination of platelet count.	appropriate 7, 2 - 9 36.9%, ?
I.2	Flow cytometry is useful in the diagnosis of inherited deficiencies of platelet surface glycoproteins.	appropriate 9, 8 - 9 4.6%, +
I.3*	Flow cytometry is useful in the diagnosis of storage pool disease.	uncertain 6, 3 - 9 25.8%, +
I.4	Flow cytometry is useful in the diagnosis of disorders that result in abnormal platelet procoagulant activity such as Scott syndrome and Stormorken syndrome.	appropriate 8, 5 - 9 14.9%, +
I.5**	Flow cytometry is useful in the diagnosis of platelet cytoskeletal defects (*e.g.,* defects in filamin, Wiskott-Aldrich syndrome protein, ACTN1, MYH9-RD, TUBB1).	uncertain 5, 1 - 8 42.4%, -
I.6	Flow cytometry is useful in the diagnosis of defects in specific platelet activation (signaling) pathways.	appropriate 7, 4 - 8 16.9%, +
I.7*	Flow cytometry is useful in the diagnosis of heparin-induced thrombocytopenia.	uncertain 5, 3 - 8 24.2%, +
I.8	Flow cytometry is useful for the determination of the fraction of immature platelets.	appropriate 8, 2 - 9 27.7%, +
I.9*	Flow cytometry is useful in the monitoring of GPIIb/IIIa antagonist (abciximab, eptifibatide, tirofiban) therapy. (Please evaluate this statement independent of whether monitoring GPIIb/IIIa antagonist therapy is itself useful).	uncertain 6, 4 - 7 18.3%, +
I.10**	Flow cytometry is useful in the monitoring of $P2Y_{12}$ antagonist (ticlopidine, clopidogrel, prasugrel, ticagrelor, cangrelor) therapy. (Please evaluate this statement independent of whether monitoring $P2Y_{12}$ antagonist therapy is itself useful)	appropriate 7, 2 - 9 34.5%, ?

I.11**	Flow cytometry is useful in the monitoring of platelet inhibition by aspirin. (Please evaluate this statement independent of whether monitoring platelet inhibition by aspirin is itself useful)	uncertain 5, 2 - 7 43.0, -
I.12**	Flow cytometry is useful in the analysis of prothrombotic syndromes characterized by platelet activation (e. g. diabetes, anti-phospholipid syndrome or secondary to drug induced, non-immune platelet activation)	uncertain 6, 3 - 8 24.8, +
I.13	Flow cytometry is useful in the diagnosis of platelet alpha granule secretion defects.	appropriate 7, 6 - 9 15.0%, +
I.14	Flow cytometry is useful in the diagnosis of platelet dense granule secretion defects.	uncertain 6, 3 - 8 23.7%, +

Supplemental Table S3. Pre-analytical variables: Survey 1 statements and expert panel scores: 1 – 3 inappropriate, 4 – 6 uncertain, 7 – 9 appropriate.
Based on the feedback from experts in Survey 1, some statements were removed (*) or revised (**) in Survey 2.

II.	**Pre-analytical variables**	Appropriateness Median, Range CV, Level of Agreement (+, ?, -)
II.1	Where clinically feasible, patients should refrain from aspirin for at least 10 days and nonsteroidal anti-inflammatory drugs (NSAIDs) for at least 3 days prior to providing blood sampling for analysis of platelet function by flow cytometry.	appropriate 7, 3 - 9 27.6%, +
II.2	Information should be collected at the time of the blood draw on the use of medications that can interfere with platelet function, including antihistamines, theophylline and antibiotics (including penicillin, cephalosporin, nitrofurantoin, and similar antibiotics).	appropriate 9, 5 - 9 21.7%, +
II.3	Information should be collected at the time of the blood draw on the use of medications that can interfere with platelet function, including tricyclic antidepressants.	appropriate 9, 5 - 9 18.3%, +
II.4	Information should be collected at the time of the blood draw on the use of anti-platelet drugs (including dipyridamole, clopidogrel, prasugrel, ticagrelor and cangrelor).	appropriate 9, 5 - 9 15.3%, +
II.5	Information should be collected at the time of the blood draw with regard to recent food intake (fasting or fed), smoking history, alcohol consumption, recent exertion/exercise.	uncertain 6, 1 - 8 36.4%, ?
II.6	In order to document the time between specimen collection and analysis, date and time of sample collection must be recorded.	appropriate 9, 7 - 9 7.8%, +
II.7	Care should be taken when collecting blood for platelet testing by flow cytometry to avoid procedures (e.g. prolonged or too tight tourniquet, use of small gauge needles, collection into non-anticoagulated syringe prior to transfer to anticoagulant, difficult blood draw, etc.) that may cause platelet activation.	appropriate 9, 7 - 9 7.8%, +
II.8	Citrate is the best choice of anticoagulant for blood collected for platelet testing by flow cytometry.	appropriate 8, 5 - 9 19.1%, +

II.9	ACD-A is an acceptable anticoagulant for blood collected for platelet testing by flow cytometry.	uncertain 6, 2 - 9 38.0%, ?
II.10	EDTA is an acceptable anticoagulant for blood collected for platelet testing by flow cytometry.	uncertain 6, 1 - 7 42%, ?
II.11	Heparin is an acceptable anticoagulant for blood collected for platelet testing by flow cytometry.	uncertain 4.5, 2 - 7 44.4%, -
II.12	Partial filling of anticoagulant tubes may affect platelet testing by flow cytometry and therefore should be avoided.	appropriate 8, 5 - 9 15.8%, +
II.13	Difficulty in collecting the blood sample (difficulty drawing the blood or a blood draw that stops and starts) may affect platelet testing by flow cytometry and therefore should be avoided.	appropriate 9, 7 - 9 10.9%, +
II.14	EDTA should be avoided as anticoagulant for blood collected for platelet testing due to its effect respectively on glycoprotein structure.	appropriate 7, 3 - 8 29.2%, +
II.15	Heparin should be avoided as anticoagulant for blood collected for platelet testing due to its effect platelet microaggregate formation.	appropriate 7, 3 - 9 31.6%, +
II.16	Blood for platelet testing by flow cytometry should be collected only after a discard of the first 1 – 2 mL of blood.	appropriate 8, 1 - 9 39.3%, ?
II.17*	Blood for platelet testing by flow cytometry may be collected from an indwelling line after discarding a volume greater than the volume of the line.	appropriate 7, 5 - 9 21.1%, +
II.18	Platelet function testing by flow cytometry should not be performed on hemolyzed blood samples.	appropriate 9, 7 - 9 8.1%, +
II.19	Between sample collection and flow cytometric determination of platelet function, blood samples should be stored at room temperature (20 – 25°C)	appropriate 9, 7 - 9 9.7%, +
II.20**	Blood samples should be allowed to “rest” for 30 min at 37°C prior to flow cytometric determination of platelet function.	uncertain 5, 2 - 8 30.7%, +
II.21**	Flow cytometric analysis of platelet function must be performed within 4 hours of blood collection.	appropriate 7, 3 - 8 30.7%, +

II.22*	Flow cytometric analysis of platelet function may be performed up to 72 hours following blood collection, provided the blood has been maintained at room temperature.	uncertain 5, 1 - 7 55.4%, -
II.23**	Flow cytometric analysis of platelet function may be performed up to 72 hours following blood collection, provided blood from a healthy control subject is processed in parallel and the blood has been maintained at room temperature.	uncertain 6, 1 - 8 39.9%, ?
II.24	Flow cytometric analysis of circulating monocyte-platelet aggregates must be performed within 30 min of blood collection.	appropriate 7, 4 - 9 20.9%, +
II.25	For flow cytometric determination of some platelet antigens, whole blood may be stabilized for later analysis by addition of a stabilizing solution (usually a fixative).	appropriate 8, 7 - 9 10.3%, +
II.26	Stabilized samples may be analyzed several days after preparation, provided this is supported by stability studies.	appropriate 8, 1 - 9 30.8%, +
II.27	To minimize artifactual pre-analytical platelet activation it is preferable to use native anticoagulated whole blood for platelet analysis rather than PRP or washed platelets.	appropriate 7, 4 - 9 24.6%, +

Supplemental Table S4. Instrument and reagent standardization: Survey 1 statements and expert panel scores: 1 – 3 inappropriate, 4 – 6 uncertain, 7 – 9 appropriate.
Based on the feedback from experts in Survey 1, some statements were removed (*) or revised (**) in Survey 2.

III.	**Instrument and reagent standardization**	Appropriateness Median, Range CV, Level of Agreement (+, ?, -)
III.1	The most basic requirements for a flow cytometer to perform platelet analysis are detection of forward and side light scatter and at least two fluorescence signals with good sensitivity (equivalent to detection of ≤1000 FITC molecules per cell).	appropriate 8, 3 - 9 21.7%, +
III.2	Software for data acquisition should allow data storage in a standard, sharable format (*e.g.* FCS-2.0 or FCS-3.0).	appropriate 8, 5 - 9 18.8%, +
III.3**	Flow cytometry instruments used for analysis of platelet function should have a preventive maintenance check at least twice a year.	appropriate 8, 3 - 9 24.8%, +
III.4	Each day that samples are analyzed, a quality control check of the flow cytometer should be run using standardized fluorescent calibration beads.	appropriate 8, 2 - 9 33.0%, ?
III.5	Auditable records of instrument maintenance and daily calibration should be kept.	appropriate 9, 4 - 9 18.8%, +
III.6	Fluorescence compensation (hardware or software) is required whenever more than one fluorophore is used.	appropriate 9, 6 - 9 14.3%, +
III.7	Antibodies for platelet testing by flow cytometry must be validated.	appropriate 9, 7 - 9 8.1%, +
III.8	For well-established combinations of monoclonal antibodies and fluorophores (for example, FITC-PAC-1), validation provided by the manufacturer is sufficient.	appropriate 7, 1 - 8 30.1%, +
III.9**	For less commonly used antibodies, validation by methods such as Western blotting, immunoprecipitation, or reactivity with known positive and negative cell lines is required.	appropriate 7, 3 - 9 27.8%, +
III.10	Pipettes should be calibrated on a regular schedule.	appropriate 9, 3 - 9 25.6%, +

Supplemental Table S5. Methods: Survey 1 statements and expert panel scores: 1 – 3 inappropriate, 4 – 6 uncertain, 7 – 9 appropriate.

IV.	**Methods**	Appropriateness Median, Range CV, Level of Agreement (+, ?, -)
IV.1	Protocols which provide sufficient detail to allow the procedure to be reproduced by an independent party are required for platelet analysis by flow cytometry.	appropriate 8, 8 - 9 6.2%, +
IV.2	Protocols for platelet analysis by flow cytometry must capture a description of the flow cytometer (manufacturer and model number), its configuration and settings, and any user-configurable settings or modifications.	appropriate 9, 3 - 9 24.6%, +
IV.3	Protocols for platelet analysis by flow cytometry must capture the source and type of sample and any sample treatment descriptions.	appropriate 9, 3 - 9 22.4%, +
IV.4	Antibodies must be identified by source, catalog number, lot number, and expiration date, the name of the clone of the antibody used, fluorescent label, the antigen detected (usually by CD nomenclature) and the dilution or concentration of used.	appropriate 9, 3 - 9 21.6%, +
IV.5	Antibodies for platelet activation analysis by flow cytometry should be used at saturating conditions.	appropriate 8, 5 - 9 19.1%, +
IV.6	Studies should be performed to determine acceptable time windows between sample preparation and flow cytometric analysis.	appropriate 8, 7 - 9 9.7%, +
IV.7	Each laboratory should generate their own reference ranges for healthy donors for each assay.	appropriate 9, 6 - 9 11.0%, +
IV.8	Each assay must include a positive control (such as healthy donor blood, or in the case of activation-dependent antigens, healthy donor blood activated by *ex vivo* addition of a platelet agonist).	appropriate 8, 5 - 9 18.8%, +
IV.9	Each assay must include a negative control (e.g. blockade of specific binding by the immunizing peptide or a sample stained with an isotype-matched fluorescent normal Ig).	appropriate 8, 6 - 9 12.0%, +
IV.10	Methods for setting gates and positive analysis regions for the parameters to be reported should be standardized and detailed in the assay protocol.	appropriate 9, 7 - 9 9.7%, +

IV.11	Methods should include the methods used to train individuals performing these tests and procedures for documenting their training.	appropriate 8, 1 - 9 37.0%, ?
IV.12	Documented methods should contain version numbers, a revision history, date last updated, and date approved.	appropriate 9, 4 - 9 24.6%, +

Supplemental Table S6. Reporting: Survey 1 statements and expert panel scores: 1 – 3 inappropriate, 4 – 6 uncertain, 7 – 9 appropriate.

V.	**Reporting**	Appropriateness Median, Range CV, Level of Agreement (+, ?, -)
V.1	A standardized laboratory report form should document date and time of sample receipt, preparation and analysis as well as all technical steps of the analysis, e. g., instrument performance and reagent lot numbers.	appropriate 8, 6 - 9 12.9%, +
V.2	A standardized laboratory report form should provide the concentrations of any agonists used to stimulate platelet activation.	appropriate 9, 8 - 9 3.4%, +
V.3	A standardized laboratory report should provide results using internationally recognized standardized units where possible.	appropriate 9, 4 - 9 18.8%, +
V.4	Where results are provided as numerical values, the uncertainty of the test (analytical and biological variability) should also be reported. [Example: Vasodilator-stimulated phosphoprotein (VASP) platelet reactivity index 49%, (analytical and biological uncertainty +/- 10%).	appropriate 8, 5 - 9 15.4%, +
V.5	A standardized laboratory report form should contain an interpretation of test results.	appropriate 8, 7 - 9 9.7%, +
V.6	Interpretation of test results should include mention of pre-analytical and analytical factors that could influence the results.	appropriate 8, 7 - 9 11.2%, +
V.7	Interpretation of test results should consider the appropriateness of the normal reference range with respect to the individual case.	appropriate 8, 7 - 9 9.6%, +

Supplemental Table S7. Quality control: Survey 1 statements and expert panel scores: 1 – 3 inappropriate, 4 – 6 uncertain, 7 – 9 appropriate.

VI.	Quality control	
VI.1	Stability and reproducibility of the method should be analyzed based on a repetitive analysis of samples.	appropriate 9, 3 - 9 22.4%, +
VI.2	Documentation should be sufficient to provide an auditable trail of sample possession, processing, and data analysis.	appropriate 9, 3 - 9 25.5%, +
VI.3	Records should be kept to demonstrate that personnel performing the tests are qualified to do so.	appropriate 9, 7 - 9 11.1%, +
VI.4	Records should be kept to demonstrate that all instruments used to perform the studies are in good working order.	appropriate 9, 7 - 9 9.7%, +
VI.5	Records should be kept to demonstrate that all reagents used to perform the studies are in-date (not expired).	appropriate 9, 5 - 9 16.8%, +

Supplemental Table S8. Expert consensus of pre-analytical variables for the assessment of inherited and acquired abnormal agonist-stimulated levels of platelet surface GPIIb/IIIa, P-selectin and GPIb. Results shown are the percent of experts supporting each level of recommendation, among the eleven experts of the panel which self-identified as having knowledge or expertise (N).

		Agonist-stimulated platelet surface GPIIb/IIIa change	Agonist-stimulated platelet surface P-selectin change	Agonist-stimulated platelet surface GPIb change
Citrate	Recommended	100%	91%	88%
	Acceptable	0%	9%	13%
	Not Recommended	0%	0%	0%
	Uncertain (lack of data)	0%	0%	0%
	N	10	11	8
Heparin	Recommended	0%	0%	0%
	Acceptable	29%	33%	14%
	Not Recommended	71%	67%	86%
	Uncertain (lack of data)	0%	0%	0%
	N	7	9	7
EDTA	Recommended	0%	0%	0%
	Acceptable	0%	13%	0%
	Not Recommended	100%	88%	100%
	Uncertain (lack of data)	0%	0%	0%
	N	9	8	6
Whole Blood	Recommended	78%	80%	88%
	Acceptable	11%	10%	0%
	Not Recommended	11%	10%	13%
	Uncertain (lack of data)	0%	0%	0%
	N	9	10	8
PRP	Recommended	50%	45%	25%
	Acceptable	30%	36%	50%
	Not Recommended	20%	18%	25%
	Uncertain (lack of data)	0%	0%	0%
	N	10	11	8
Fresh/Not Fixed	Recommended	90%	91%	88%
	Acceptable	10%	9%	13%
	Not Recommended	0%	0%	0%
	Uncertain (lack of data)	0%	0%	0%
	N	10	11	8
Formalin-fixed	Recommended	0%	10%	0%
	Acceptable	0%	10%	0%
	Not Recommended	100%	80%	100%
	Uncertain (lack of data)	0%	0%	0%
	N	9	10	7
Test within 30 min	Recommended	90%	82%	88%
	Acceptable	10%	9%	0%
	Not Recommended	0%	9%	13%
	Uncertain (lack of data)	0%	0%	0%
	N	10	11	8
Test within 4 hr	Recommended	50%	45%	50%
	Acceptable	30%	36%	25%
	Not Recommended	20%	18%	25%

		Uncertain (lack of data)	0%	0%	0%
		N	10	11	8
Test within hr	24	Recommended	0%	10%	0%
		Acceptable	33%	30%	29%
		Not Recommended	56%	50%	43%
		Uncertain (lack of data)	11%	10%	29%
		N	9	10	7
Test within hr	96	Recommended	0%	0%	0%
		Acceptable	13%	20%	14%
		Not Recommended	75%	70%	71%
		Uncertain (lack of data)	13%	10%	14%
		N	8	10	7

Supplemental Table S9. Secondary survey statement on flow cytometry assessment of ristocetin-dependent VWF binding. Results shown are the percent of experts supporting each level of recommendation, among the eleven experts of the panel which self-identified as having knowledge or expertise (N).

For flow cytometry assessment of ristocetin-dependent VWF binding:					
	Recommended	**Acceptable**	**Not Recommended**	**Uncertain (lack of data)**	**N**
citrate	100%	0%	0%	0%	5
heparin	0%	0%	100%	0%	3
EDTA	0%	0%	100%	0%	4
whole blood	50%	25%	0%	25%	5
PRP	50%	25%	25%	0%	5
fresh/not fixed	100%	0%	0%	0%	5
formalin fixed	0%	50%	0%	50%	5
test within 30 min	75%	25%	0%	0%	5
test within 4 hr	50%	25%	25%	0%	5
test within 24 hr	0%	50%	25%	25%	5

Supplemental Table S10. Secondary survey statement on flow cytometry assessment of intracellular markers. Results shown are the percent of experts supporting each level of recommendation, among the eleven experts of the panel which self-identified as having knowledge or expertise (N).

For flow cytometry assessment of intracellular markers (such as cytoskeletal proteins such [e.g. WASp] or signaling pathways [*e.g.* VASP-P]):					
	Recommended	**Acceptable**	**Not Recommended**	**Uncertain (lack of data)**	**N**
citrate	83%	17%	0%	0%	6
heparin	0%	25%	75%	0%	4
EDTA	0%	33%	67%	0%	6
whole blood	83%	17%	0%	0%	6
PRP	33%	67%	0%	0%	6
formaldehyde followed by detergent	60%	40%	0%	0%	5
formaldehyde followed by methanol	0%	0%	0%	100%	1
methanol followed by detergent	50%	0%	0%	50%	2
acetone	50%	0%	0%	50%	2
anticoagulant and procedure depend on target	50%	50%	0%	0%	6

Supplemental Table S11. Revised and newly suggested statements. Results are presented as median rating (1 – 3 inappropriate, 4 – 6 uncertain, 7 – 9 appropriate), range, and the level of agreement between the eleven experts in the panel (+ = agreement, - = disagreement, ? = inconclusive).

Statement	Rating: median, range Level of Agreement
Flow cytometry may be used in the diagnosis of platelet cytoskeletal defects (e.g defects in filamin, Wiskott-Aldrich syndrome protein, ACTN1, MYH9-RD, TUBB1).	uncertain 6, 1 – 9 ?
Certain flow cytometry tests (for example, VASP $P2Y_{12}$) may be used to monitor the pharmacodynamic effects of $P2Y_{12}$ antagonist (ticlopidine, clopidogrel, prasugrel, ticagrelor, cangrelor). (Please evaluate this statement independent of whether monitoring $P2Y_{12}$ antagonist therapy is itself useful)	appropriate 9, 5 – 9 +
Flow cytometry can be used to detect the aspirin-induced defect in platelet function. (Please evaluate this statement independent of whether monitoring platelet inhibition by aspirin is itself useful)	uncertain 6, 1 – 9 ?
Flow cytometry can be used to detect increased levels of activated platelets in patients with prothrombotic syndromes (e. g. diabetes, anti-phospholipid syndrome or secondary to drug induced, non-immune platelet activation)	appropriate 8, 5 – 9 +
Blood samples should be allowed to “rest” prior to flow cytometric determination of platelet function.	appropriate 7, 3 – 9 +
Agonist (ADP or TRAP for example) stimulation of platelet samples for flow cytometric analysis of platelet activation markers (PAC-1 or platelet surface P-selectin for example) is preferably done within 4 hours of blood collection.	appropriate 9, 7 – 9 +
Agonist (ADP or TRAP for example) stimulation of platelet samples for flow cytometric analysis of platelet activation markers (PAC-1 or platelet surface P-selectin for example) may be performed up to 72 hours following blood collection, provided blood from a healthy control subject is processed in parallel.	uncertain 6, 2 – 7 ?
Flow cytometry instruments used for analysis of platelet function should be documented on a regular basis to be in good working order (for example by use of a preventive maintenance plan).	appropriate 9, 6 – 9 +

For less well-known antibodies, the specificity of the antibody for the intended target should be validated, for example by Western blotting, immunoprecipitation, or reactivity with known positive and negative cell lines (although reactivity of the antibody by these methods with the target does not guarantee the antibody will work in flow cytometry).	appropriate 8, 2 – 9 +
GFI1B macrothrombocytopenia may be screened by CD34 flow cytometry.	appropriate 7, 4 – 8 +
For flow cytometric tests of agonist-stimulated platelet activation markers, a parallel sample without activation (buffer activated) should always be run and the results reported to take into account primed/pre-activated platelets.	appropriate 9, 8 – 9 +
When whole blood samples are used, steps should be taken to minimize possible interference by RBCs during sample analysis.	appropriate 7, 6 – 9 +
When whole blood samples are used, the preferred approach to minimize possible interference by RBCs during sample analysis is RBC lysis.	uncertain 6, 1 – 8 -
When whole blood samples are used, the preferred approach to minimize possible interference by RBCs during sample analysis is use of a fluorescent platelet-identifying antibody and setting the threshold accordingly.	appropriate 9, 6 – 9 +