Application Note

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New Research Methods for Analyzing Platelet Activation with the Agilent NovoCyte Flow Cytometer

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Introduction

Platelets, also known as thrombocytes, are small discoid cells resembling small plates. Megakaryocytes release protoplatelets, which break apart and, once in circulation, form mature platelets with an average life span of 7 to 10 days. Platelets are important, both for their essential role in hemostasis and thrombosis, as well as their involvement in several biological processes, including immune regulation, vasculopathy, and fibrosis. Increased focus on platelet research has developed, with the potential of inhibiting platelets for diseases in which they are a contributing factor, such as atherothrombosis and inflammation. When activated by stimuli, platelets undergo rapid metamorphic changes to spread and adhere to endothelial surfaces, release granules, and aggregate with other platelets to propagate coagulation.

The gold standard used to measure platelet function is light transmission aggregometry (LTA) where platelet aggregation is measured by changes in light transmission on a spectrometer. However, LTA is cumbersome and results vary with platelet count, making it unsuitable for people with thrombocytopenia. In addition, LTA requires a large volume of blood while investigating only one aspect of platelet function. Therefore, improved research methods for measuring platelet function are needed. Flow cytometry has been widely used to identify several platelet disorders and, recently, techniques have been developed to measure platelet function. Measuring platelet activity by flow cytometry offers several advantages over LTA in that only small amount of blood volumes are needed, whole blood can be used, and results are independent of platelet count. Furthermore, the possibility of ready-made tubes with fixative would extend the time between blood collection and analysis, which is crucial for field testing. This application note measured platelet function in two ways: expression of CD62P and by frequency of platelet-leukocyte aggregates.

Platelet activation triggers CD62P expression on the cell surface

Measuring the upregulation of P-selection (CD62P) is an established research method for determining platelet function. Under normal conditions, CD62P is not expressed on the surface of platelets; however, upon activation, platelet granules are released and CD62P, a component of these granules, is expressed on the platelet surface. Aberrant expression of CD62P has been reported in people with platelet α-storage pool disease, which is characterized by reduced platelet function. Activation of platelets with adenosine diphosphate (ADP) was measured by CD62P expression on platelets (Figure 1). CD62P expression rapidly increased to ~40% of platelets within five minutes after addition of ADP, while <2% of activated platelets were observed in the untreated control sample. Nonspecific binding of antibodies was not observed; this is demonstrated by the absence of signal with an isotype control. Therefore, measuring CD62P is a specific and easy research method for measuring platelet function.

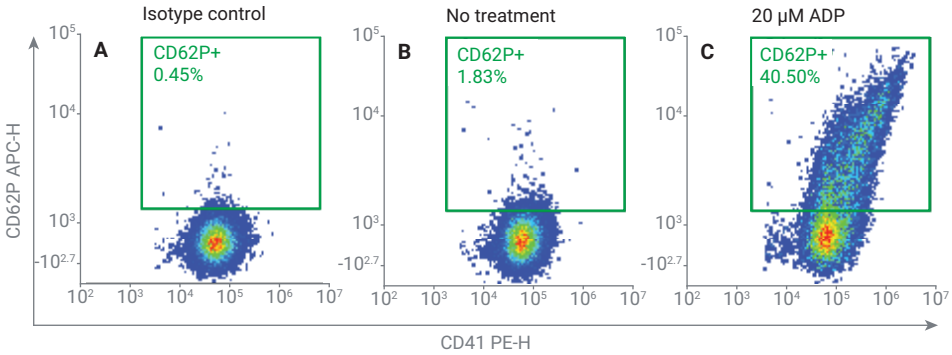

Figure 1. Measuring platelet activation by CD62P expression. Whole human blood was collected with a 21 g needle, and immediately placed in a sodium citrate anticoagulant tube. For platelet activation, 20 µM ADP was added to indicated sample and incubated for five minutes at room temperature. After activation, cells were stained with αCD41PE (to label platelets) and αCD62P antibody or isotype control (to measure platelet activation) for 20 minutes at room temperature, followed by fixation with 1% PFA. Cells were analyzed within 24 hours of blood collection. Representative plots are shown measuring CD62P+ cells within the platelet population (CD41+ cells) with and without treatment of 20 µM ADP.

Platelet activation results in aggregation with platelets

Platelets are a central cellular interface of thrombotic and inflammatory processes, and modulate this interface by binding to leukocytes and altering their function. CD62P also mediates this process, regulating adhesion of activated platelets to leukocytes through P-selectin glycoprotein ligand 1 (PSGL1). Analysis of platelet-leukocyte aggregates (PLA) has been suggested as a more sensitive, earlier marker of platelet activation, especially as a research method to investigate the dose response of antiplatelet agents. Platelet activation was measured by changes in PLA frequencies within white blood cell (WBC) populations after treatment with ADP (Figure 2). First, WBC populations were identified by forward scatter (FSC), side scatter (SSC), and CD45 surface expression. Then, each population was monitored for the presence of CD41, a marker for platelet cells, to demonstrate aggregation of platelets on the cell surface. Minimal spontaneous PLA formation was seen in the untreated sample (upper row). Upon addition of ADP, the frequency of CD41+ cells rapidly increases, specifically within the granulocyte and monocyte populations; 45% of granulocytes and 70% of monocytes were aggregated with platelets. This data indicates that PLA formation is a fast and easy indicator of platelet activation.

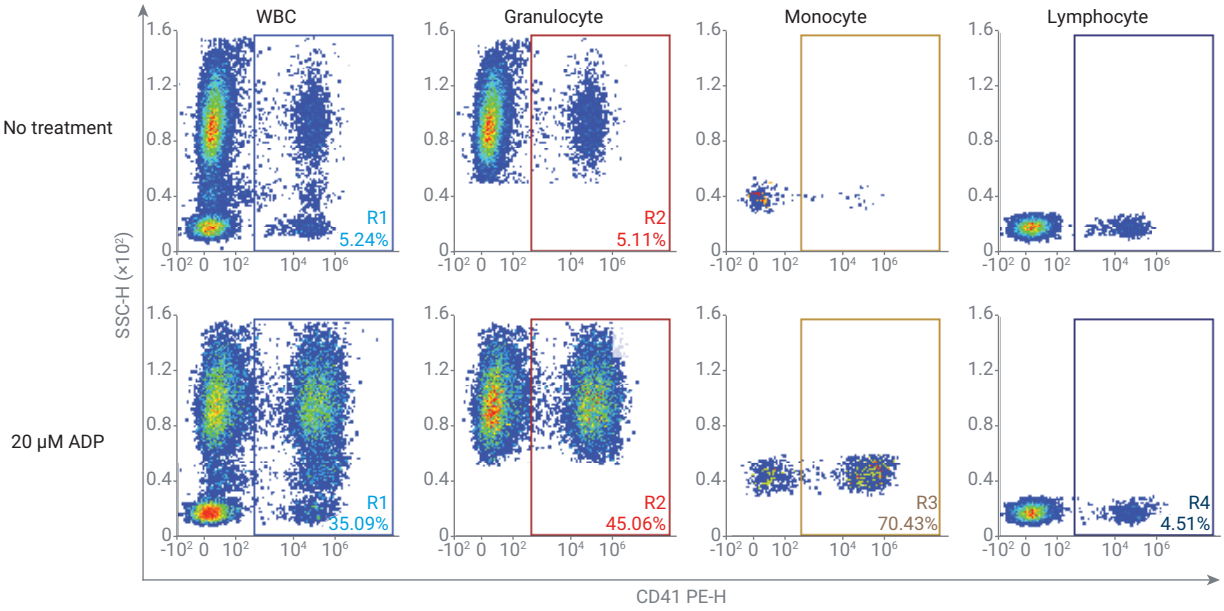

Figure 2. Measuring platelet activation by leukocyte-platelet aggregation. Whole human blood was collected with a 21 g needle and immediately placed in a sodium citrate anticoagulant tube. For platelet activation, 20 µM ADP was added to indicated sample and incubated for 30 minutes at 37 °C followed by fixation in 2% PFA for 10 minutes on ice. Cells were diluted in water to dissolve RBCs followed by antibody staining with αCD45FITC (to distinguish WBC) and αCD41PE (to label platelets) for 20 minutes at room temperature. Cells were analyzed within 24 hours of blood collection. Representative plots measuring CD41+ cells (platelet-leukocyte aggregates) within total WBCs as well as individual granulocyte, monocyte, and lymphocyte populations.

Conclusion

Improved research methods are needed to assess platelet activity *in vivo*, specifically a method independent of platelet number. Flow cytometric analysis of surface receptors such as CD62P, as well as quantification of platelet-leukocyte aggregates, enhances current research methods for assessing platelet activity at the single cell level. In conclusion, flow cytometry is a valuable tool to include in the assessment of platelet numbers and activity.

References

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