Flow Cytometry and Platelet Analysis: Technical Tips

Stephen F. Garner

Platelets have multiple roles in both health and disease and are easily accessible in peripheral blood, two factors that have lead to extensive characterisation by flow cytometry. Assays that have complemented or replaced more conventional techniques are used to study aspects of platelet biology such as functional responses, antigen quantitation and antibody detection.

Prior to the development of whole blood flow cytometric assays, platelets were often purified as platelet-rich plasma or even washed platelets. The separation techniques involved in preparing purified platelets may however lead to loss of platelet sub-populations and to process-induced activation, and consequently compromise the value of any assay results. The ability of flow cytometers to identify and discriminate platelets in whole blood, using simply light scatter properties or light scatter combined with fluorescence derived from platelet specific monoclonal antibodies has eliminated the need to separate platelets from red and white cells. Indeed, one needs as little as five microlitres of whole blood to perform a flow cytometric platelet function assay.

Excellent review articles can help those new to the field of study (Michelson 1996) and there are even consensus protocols, which cover aspects ranging from sample type through to result evaluation (Schmitz *et al.* 1998). Furthermore, literature searches will often find a multitude of studies relevant to specific clinical or research applications.

The relative simplicity of the assays however may obscure potential technical pitfalls which can markedly influence results, and these problems can arise at all stages from initial sample collection to final data analysis.

With regard to the flow cytometric aspects of the assays, initial identification of platelets may prove challenging to workers accustomed to analysing leucocytes or cell lines. Due to their small size and variation in shape, platelets are identified and analysed using logarithmic settings for light scatter properties rather than the linear settings more familiar to leucocyte-based studies.

Although whole blood can be used, a dilution step prior to analysis is usually required due to the excess number of red cells compared with platelets. Buffers used for incubation steps with agonists and monoclonal antibodies prior to dilution are relatively simple, but can be a major source of problems. Phosphate or HEPES buffered saline (HBS) are often used as buffers, with additives such as calcium being included for some activation-based assays. Bovine serum albumin (BSA) may also be added for some assays as a source of protein to reduce non-specific binding and loss of antibody; EDTA can also be added to inhibit platelet activation and subsequent aggregation. Additives however, like buffers can be a major source of problems. BSA for example can support bacterial contamination, and bacteria may have light scatter properties similar to platelets. As a general rule, problems can be avoided by filtering (0.2 µm) all buffer components and the final buffer. The components should be stored at +4°C, and BSA is best stored as small single use aliquots. In addition, working aliquots of buffers should be made up on the day required, and should not be stored.

The flow cytometer itself can also be a source of problems; generating events with light scatter characteristics similar to platelets. Reagents such as sodium hypochlorite, distilled water and commercially available detergents are often processed on a flow cytometer as cleaning agents, with the idea that after cleaning there should be very few detectable events when sheath fluid alone is run. The instrument settings used to run cleaning agents can however generate a false sense of security for subsequent platelet analysis. Running distilled water using settings appropriate for leucocyte work may show a clean system, but adjusting the settings to those required for platelet work can reveal a large number of events that were too small to be visible on the leucocyte settings. Hence, for platelet studies all cleaning protocols and assessment of cleanliness should be performed using instrument settings suitable for platelets, and where appropriate, filtered reagents.

Sheath fluid, filters, and tubing can be sources of unwanted debris that by forward and side light scatter analysis look like platelets. Figure 1A shows the expected forward and side scatter profile of platelets. Figure 1B shows an unexpected population with greater side scatter, while Figure 1C has an unexpected population with lower side scatter. In both instances the unexpected populations were not visible when running the manufacturers' standard cleaning reagents using the instrument settings supplied, but they were present when running filtered deionised water using platelet settings. The unexpected events in Figure 1B were no longer seen after the flow cytometer's sheath filter was replaced, while re-tubing the entire flow cytometer was required to eliminate the unexpected events in Figure 1C.

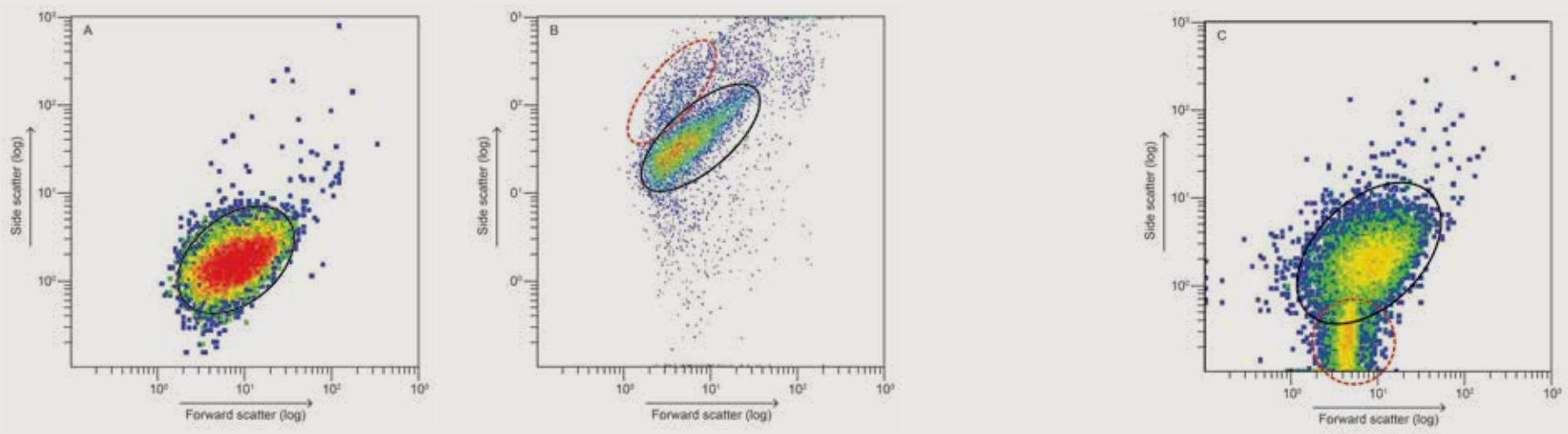


Figure 1. Aberrant forward and side scatter profiles due to debris within the flow cytometer. (A) Normal forward and side scatter profile, (B) Additional population with increased side scatter, (C) Additional population with decreased side scatter. (Normal populations are shown within black ovals, while unexpected populations are within red ovals).

Even with clean buffers problems with unexpected cell populations can occur. Figure 2A shows the expected forward and side scatter profile of platelets, while Figure 2B shows an additional unexpected population of cells. The cause of this population was obscured by the system of data acquisition. With older flow cytometers having relatively limited data storage space there was a tendency to use an acquisition gating strategy that excluded events felt to be irrelevant. In platelet function assays these irrelevant events were red cells and as they significantly outnumber platelets they were excluded to reduce the size of data files. If data from red cells in this example had been acquired it would have been clear that they had abnormal forward and

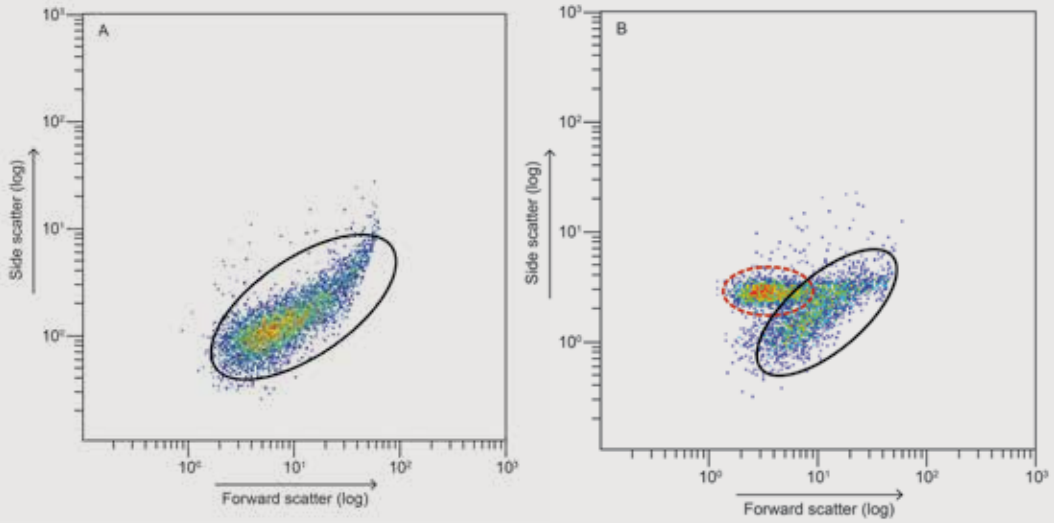
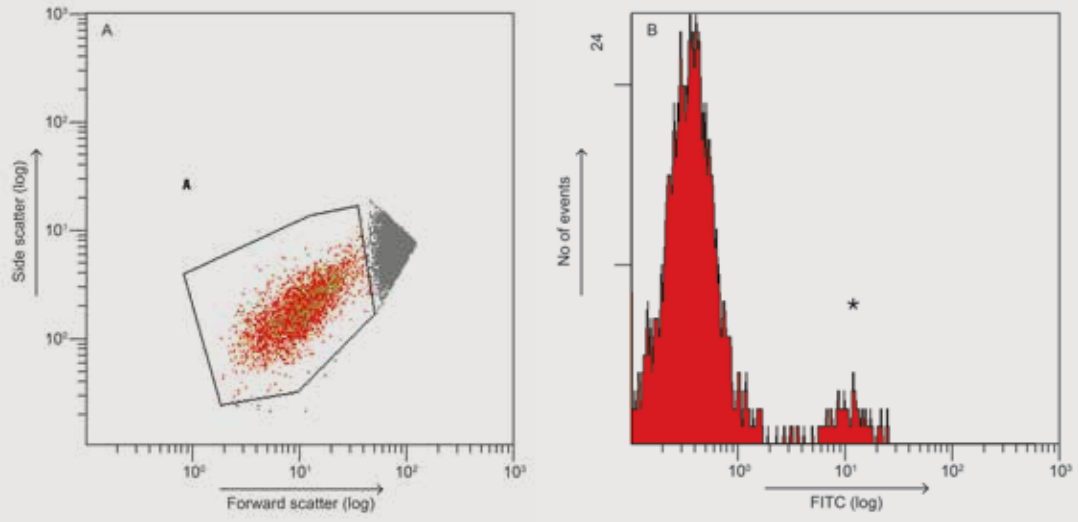


Figure 2 (above, left and right). Aberrant forward and side scatter profiles due to problems with a fixative. (A) Normal forward and side scatter profile, (B) Additional population with decreased forward scatter. (Normal populations are shown within black ovals, while unexpected populations are within red ovals).



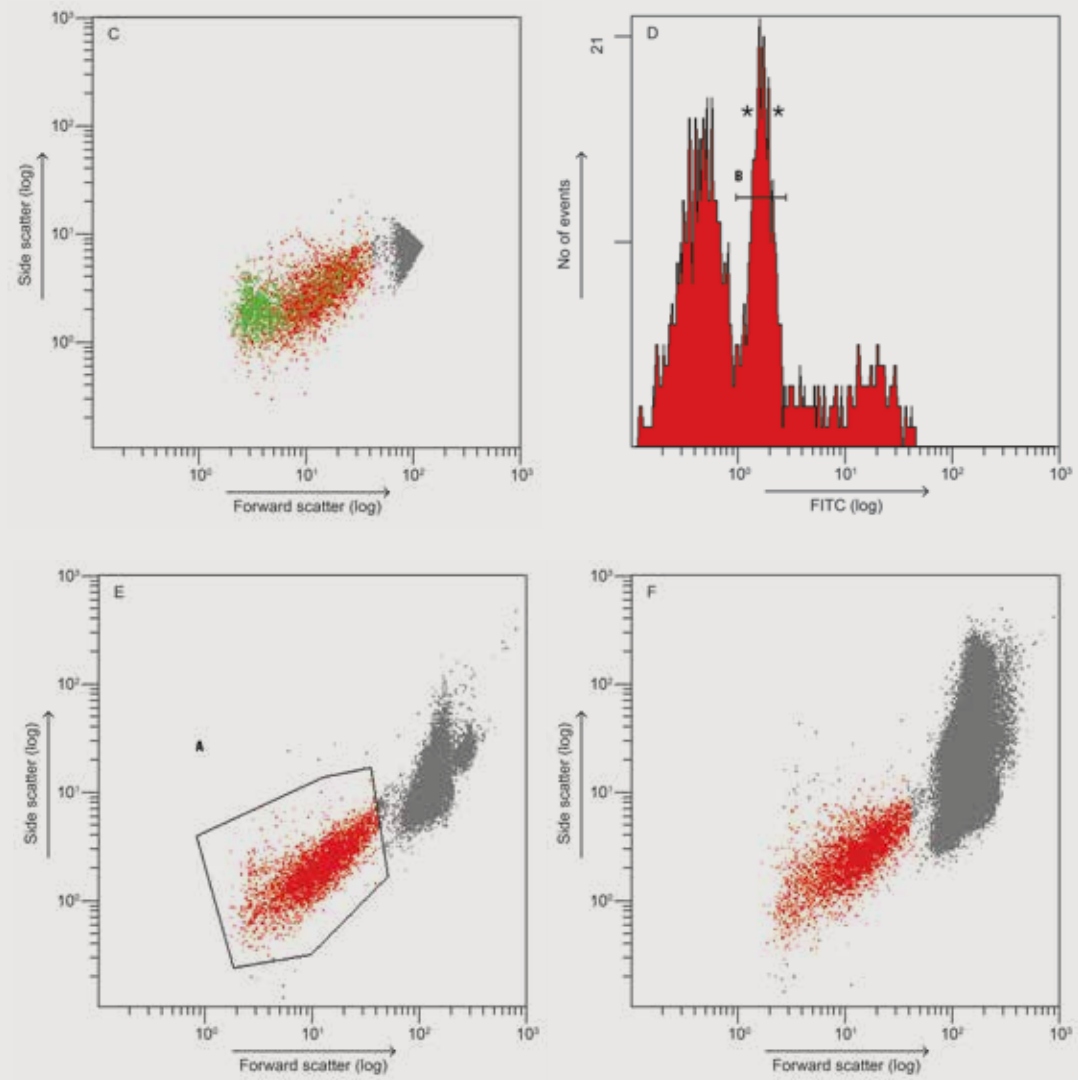


Figure 3 (following page). Aberrant forward and side scatter and platelet activation profiles due to buffer problems. (A) Non-activated sample. Normal forward and side scatter profile, showing platelets in red, and a sub-population of red cells in grey, (B) Non-activated sample. Fluorescence histogram of activation marker shows an unexpected positive population (★), (C) Activated sample. Abnormal forward and side scatter population shown in green, (D) Activated sample. Histogram of activation marker shows an unexpected middle peak (★★), (E) Forward and side scatter profile using the original sample suspension buffer, showing platelets in red, and the entire red cell population in grey. The red cells have a decreased forward and side scatter profile when compared with (F) which was suspended in a new batch of buffer.

side scatter properties. The source of the problem was eventually found to be a faulty batch of fixative causing red cell shrinkage, and the unexpected events were thought to be red cell fragments. As a general principle all data should be collected even if it is initially thought to be irrelevant. Monitoring the red cell population should be an essential part of quality control in whole blood platelet assays.

A further example of buffer induced problems is seen in Figure 3. Figure 3A shows the expected forward and side scatter plot of platelets, shown in red, and a small portion of the red cells, with higher forward scatter visible through a live gate and shown in grey. The histogram in Figure 3B representing binding of a fluorescently (FITC) labelled antibody to an activation marker however shows an unexpected positive population (★) in what should have been a completely negative, non-activated sample.

When the platelets were activated an unexpected tail appeared on the scatter plot (Figure 3C, green). Furthermore, the histogram of the activation marker showed an unexpected middle peak (Figure 3D,★★)

Analysis of the FSC and SSC plots without a live gate and therefore showing the entire red and white cell populations revealed the source of the problem. The original batch of HBS used to dilute the whole blood sample was found to have caused the platelet activation and shrinking of the red cells (Figure 3E, grey) compared with a new batch of HBS (Figure 3F, grey). Such red cell damage may not only result in red cell fragments appearing as aberrant events with similar light scatter properties to platelets, but may also directly result in platelet activation. The data again illustrate the pitfalls of using live gates to exclude apparently unwanted events.

Even with a clean flow cytometer combined with optimal buffers and data acquisition platelet flow cytometery still has technical challenges. There are for example fewer readily available platelet-specific monoclonal antibody specificities compared with the range available for leucocyte work, and those that are available tend to be conjugated to a relatively small range of fluorochromes, factors which limit multi-colour analysis. In addition, most platelet-specific CD markers are expressed on all normal platelets, so it is not possible to use antigen-negative platelets as negative controls, meaning that isotype controls may still have a role in platelet work. One should however remember that within platelet activation work one is often looking at the binding of specific proteins rather than antibodies, and it may be possible to generate a negative control by inhibiting protein binding. For example, annexin V binding is often measured as a marker of platelet activation; as binding is calcium dependant a test without added calcium serves as an ideal negative control.

The fact that many glycoproteins exist as complexes on the platelet surface also leads to technical challenges. For example, glycoprotein (GP) IIb, also known as CD41 and GP IIIa (CD61) exist as a GPIIbIIIa (CD41/CD61) complex, but it is not always clear whether CD41 and CD61 antibodies recognise epitopes expressed on the GPs alone, or as part of the complex. Hence results with different antibodies may vary. Furthermore, certain anticoagulants such as EDTA may denature the GPIIbIIIa complex, leading to aberrant antibody binding depending on the true epitope specificity of the antibody. Other platelet specific proteins however, such as GPVI may require EDTA for optimal analysis as its presence inhibits shedding of the protein.

It is therefore clear that although platelet flow cytometry is a relatively simple and quick technique there are numerous potential pitfalls that can result in aberrant, misleading results. However, with careful attention to detail at every stage from sample collection to final data analysis valuable results can be obtained for both diagnostic and research applications.

References

Michelson AD. (1996) Flow Cytometry: A Clinical Test of Platelet Function. Blood 87. 4925-36.

Schmitz G, Rothe G, Ruf A, Barlage S, Tschope D, Clemetson KJ, Goodall AH, Michelson AD, Nurden AT, Shankey TV. (1998) European Working Group on Clinical Cell Analysis: Consensus Protocol for the Flow Cytometric Characterisation of Platelet Function. Thrombosis and Haemostasis 79. 885-96.

Stephen F. Garner

National Health Service Blood and Transplant Cambridge & Department Of Haematology, University of Cambridge, Long Road, Cambridge, CB2 0PT
sfg21@cam.ac.uk

Stephen has a background in haematology and blood transfusion, moving from clinical diagnostic laboratories to his current position in research. His flow cytometry work has explored aspects of peripheral blood cells and his current studies relate to understanding the genetics underlying variation in platelet functional responses between individuals. He promotes flow cytometry education by working with colleagues including RMS members and the Mid-Anglia Cytometry Club.