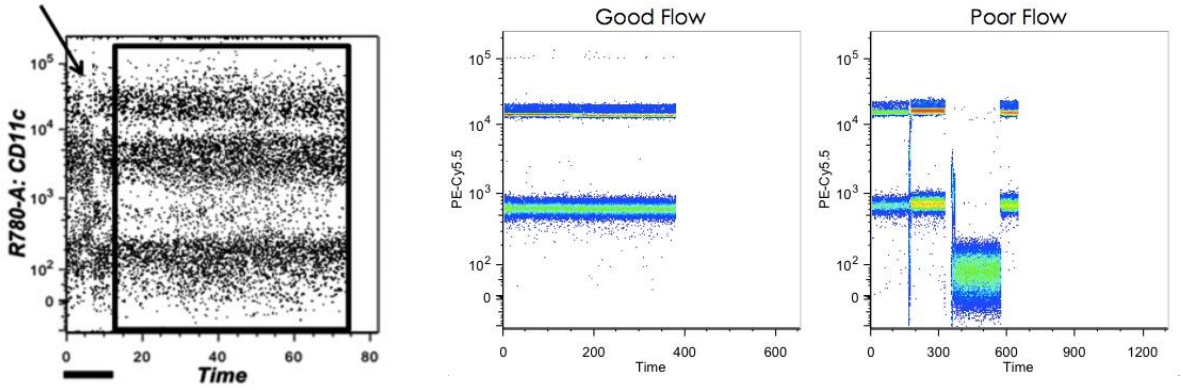
Quality data generation

Eliminating artifacts

Time parameter

If fluidic instability occurs during sample acquisition, either during long acquisitions or in a multiwell plate assay, there can be an observed loss of scatter and fluorescence sensitivity. If not detected, this instability could generate false populations with diminished fluorescence or scatter. Visualization of time versus scatter and/or fluorescence as part of the data analysis scheme is used to track stability of the data over the time period of the file acquisition. If aberrations are detected, a gate can then be set to exclude the part of the data file that is invalid. There are automated algorithms in the statistical programming language R that can do this (*Fletez-Brant, Spidlen, Brinkman, Roederer, & Chattopadhyay, 2016*).



A. **B.**

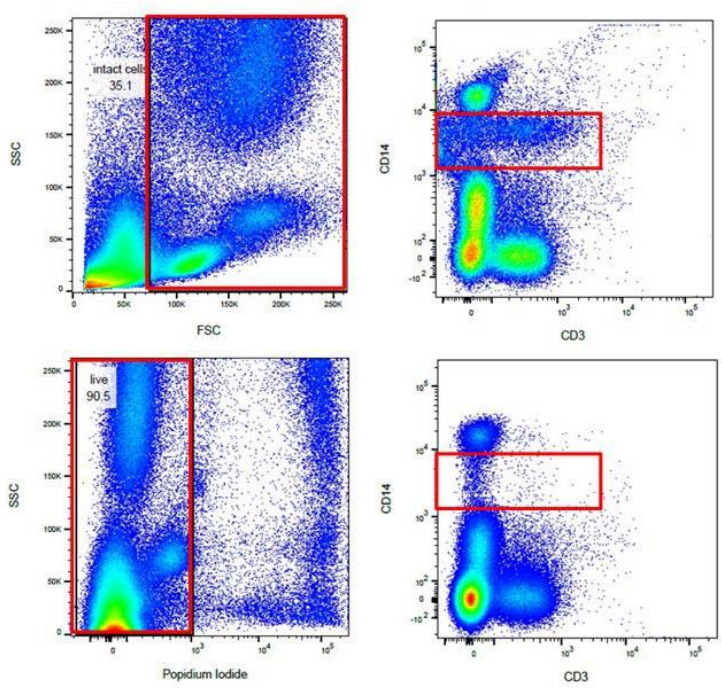
Identifying problematic sample acquisition: A. Time gate - Data aberrations caused by changes in fluidics during acquisition can be visualized by looking at the scatter or fluorescence parameters versus time. It can be seen here that data is compromised at the very beginning of the acquisition period (arrow). A gate is set to exclude these initial data points from subsequent analysis. **B. Good Flow vs Bad Flow** – the plot illustrating "poor flow" highlights a few different issues. The start of the acquisition is good, before it is followed by an increase in flow rate resulting in a disruption of data before the second section starts. The third section where there is a gap in acquisition is due to a blockage (from the increased flow rate) resulting in a loss of PE-Cy5.5 signal, the fourth section illustrates resolution of the blockage and the return of the PE-Cy5.5 signal.

Gating out false positives

Dead cells

Dead cells are sticky and can masquerade as false positives due to nonspecific binding of antibodies. A live/dead cell marker should be included in all experiments to identify and eliminate dead cells from analysis. Dead cells are typically identified by the permeability of their cell membranes, whereby these stains diffuse freely into the cell and attach to intracellular components, often resulting in dead cells staining positive for the marker. In unfixed cells, the most commonly used stains are 4',6-diamidino-2-phenylindole (DAPI), Propidium Iodide (PI), or 7-Aminoactinomycin D (7-AAD). Alternative fixable live/dead stains that bind amine or thiol residues,

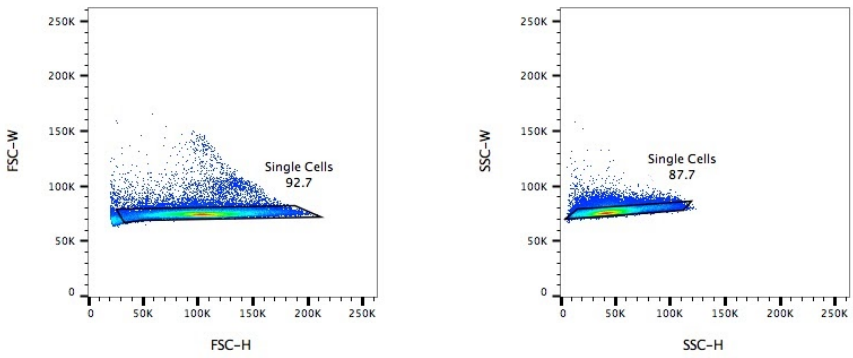
are used to identify initially viable cells before their fixation and permeabilisation, and subsequent staining for intracellular antigens.



Data with and without Live/Dead Discrimination – Top row show data gated on FSC vs SSC showing an additional intermediate CD14 and CD3+ populations (indicated); these are dead cells which have non-specifically bound the antibodies and are not real. The second row uses PI as a live/dead discrimination dye to exclude positively-stained dead cells, subsequently illustrating the real CD14 and CD3 populations.

Doublet discrimination

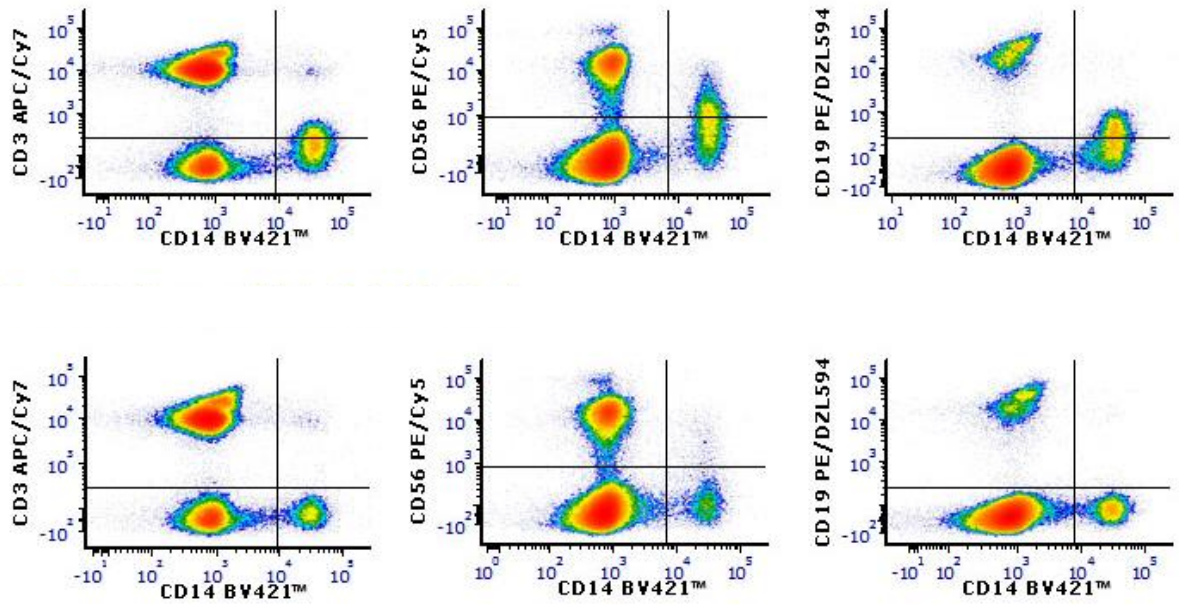
Another source of false positivity is the presence of doublets or cell aggregates: two cells stuck together that can often possess uncharacteristic staining whose passage past the laser are seen as one cell. When a doublet passes the laser, the voltage pulse generated by the detectors has a lower height and a larger width than that of a single positive cell with the same pulse area. The doublets can thus be eliminated by plotting pulse SSC and FSC versus pulse width and height and gating out the cells with higher width or lower height. It is never possible to eliminate all of the doublets, as only those that pass the laser in the correct proportion are distinguishable.



Doublet Discrimination – Gating using FSC-W vs FSC-H and then followed by SSC-W and SSC-H removes doublets, cleaning up your data for analysis.

Fc Block

Cells with Fc receptors (e.g. monocytes and macrophages) are able to bind the antibody/fluorochrome via the Fc portion of the antibody. These cells will appear as positive. The staining is specific for the Fc receptor, but is undesirable; only the Fab binding specifically to the epitope of interest is desirable. The Fc-mediated binding can be blocked using a commercially available Fc block or by adding serum from the same species as the antibody source. For example, for commonly used monoclonal antibodies raised in mice, this would be mouse serum. The serum contains antibodies that will block the Fc receptors on the cells, leaving the antibody of interest to bind only through the Fab domains specific for the antigen of interest. An isotype control at the panel development stage can indicate if this is a potential problem.



No Fc Block vs Fc Blocking – The top row illustrates a 4 colour panel without using Fc Block; showing a clear skewing of the CD14+ populations, unconventional staining profile, and increased numbers due to non-specific Fc binding. In comparison, the lower row displays the same sample after treatment with Fc-block before their staining, reducing the artefacts seen above.

Other nonspecific staining

Some fluorochrome entities, the Cyanine (Cy) dyes for example, are known to bind non-specifically to certain cells. An isotype control can also be useful in this case.

Annotated from:

Maciorowski, Z., Chattopadhyay, P.K., & Jain, P. (2017). Basic multicolor flow cytometry. Current Protocols in Immunology, 117, 5.4.1–5.4.38. doi: 10.1002/cpim.26