

Best practices for multiparameter flow cytometry

Experimental setup and panel design for accurate results.

Flow cytometry is an elegant quantitative technology, allowing the interrogation of single cells among tens of thousands or even millions of cells in minutes. Advantages of multiparameter flow cytometry include the ability to probe single cells with multiple functional markers, to correlate protein expression levels using multiple antibodies, and ultimately to more accurately define cell populations. Increasing the number of targets and fluorophores, however, also increases the complexity of the experiment and requires greater attention to detector optimization, panel design, controls, and other setup details. Here we describe a few best practices for designing a multiparameter flow cytometry experiment. While not comprehensive, they encompass some of the most important features of good experimental setup and panel design [1,2].

Voltage walk for highest-quality data

Cytometer manufacturers provide a performance test that certifies the instrument is performing optimally with respect to a precise set of specifications. Detector optimization takes this process a step further, enabling the highest-quality data to be obtained in each flow cytometer channel. For each detector, the voltage (or gain) chosen must provide the best separation between positive and negative signals and ensure all measurements are within the detector's linear range.

Typically, the voltage walk method (Figure 1) is used to determine the minimum voltage requirement (MVR) that allows clear resolution of dim fluorescent signals from the background noise of the instrument. In this method, dimly fluorescent beads are run using a series of increasing voltage settings, and the spread of the signal (or the coefficient of variation, CV) is plotted against the voltages. Decreasing the voltage for a detector below its MVR can result in the loss of resolution of dim populations, and increasing the voltage above its MVR gives no advantage for population resolution. Because this method does not ensure that the brightest signals do not exceed the upper limit of the detector's range, alternative methods have been developed in which both unstained and brightly stained beads or cells are used to determine MVR [3,4].

Antibody titration in panel design

Antibody titration is also an important optimization technique for multiparameter flow cytometry and is the best way to minimize non-specific binding and increase signal detection. It can also be used

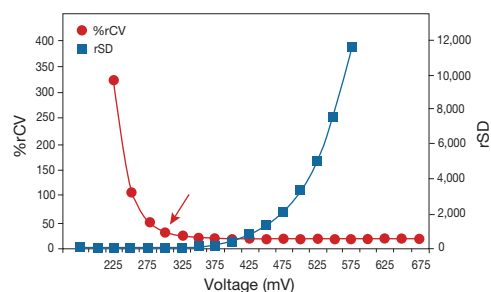


Figure 1. Determination of the optimal voltage setting for a flow cytometer detector using a voltage walk. The voltage walk method shown uses a single dimly fluorescent hard-dyed bead. Data are acquired at each voltage setting increment in a given detector, and the percent robust coefficient of variation (%rCV) and robust standard deviation (rSD) are exported and plotted vs. voltage to visualize the point of inflection. The lowest voltage on the %rCV curve before the increase in the rSD should be used for the detector. In this example, the MVR is determined to be 300 mV (arrow).

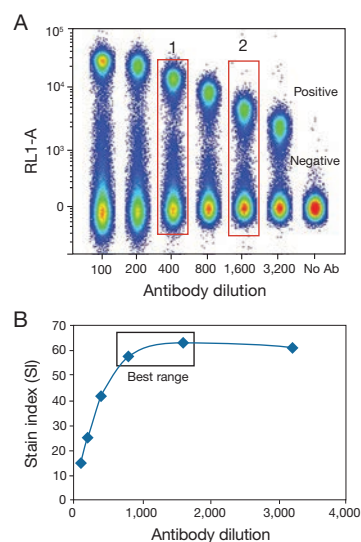


Figure 2. Antibody titration example. (A) Cells were incubated with serial dilutions of an APC-conjugated mouse anti-CD8 antibody, run on the Invitrogen™ Attune™ NxT Flow Cytometer, and analyzed using FlowJo™ software. Data are shown on a single graph for easier comparison. Note that the sample highlighted in box 1 is the saturation concentration of antibody, whereas the box 2 sample is the separation concentration. **(B)** The stain index (SI) was calculated for the data in (A) using the equation: $(\text{Mean}(\text{positive cells}) - \text{Mean}(\text{negative cells})) / (2 \times \text{SD}(\text{negative cells}))$, and plotted as a function of the antibody dilution.

to minimize spillover spreading, which occurs when the signal from dyes that emit fluorescence over a broad range of wavelengths is captured in multiple detectors, complicating data interpretation. To perform a simple antibody titration, start with the manufacturer's →

recommended concentration, perform serial 2-fold dilutions, and plot the stain index (SI), which is a measure of the relative brightness of a fluorophore-conjugated antibody [5]. The SI for a specific antibody–dye conjugate and its spillover spreading will help to determine if a separating concentration (at which negative and positive cells display the greatest difference in fluorescence), or a saturating concentration (at which the antibody has saturated the antigen available in the cells) of antibody should be used (Figure 2). A separating concentration provides good separation of labeled vs. unlabeled cells (e.g., when identifying percent-positive populations in immunophenotyping experiments), reduces spreading error, and conserves antibody. Saturating antibody concentrations—sometimes required for the detection of low-abundance antigens—can lead to increased spillover spreading and difficulty detecting dim signals in other detectors.

Fluorophore selection and allocation

One of the biggest challenges in multiparameter flow cytometry is selecting the combinations of fluorophores and antibody conjugates that minimize the need for compensation and spillover adjustments without compromising data quality. The more dyes included in a flow cytometry panel, the more likely that spillover spreading will reduce the ability to distinguish the specific signal of one fluorophore in the presence of others. When choosing fluorescent labels:

- Use bright fluorophores with antibodies for low-abundance targets and dim fluorophores with antibodies for highly expressed antigens
- Minimize the spectral overlap of fluorophores to reduce spillover
- Use fluorophores that are spectrally distinct for the detection of coexpressed markers

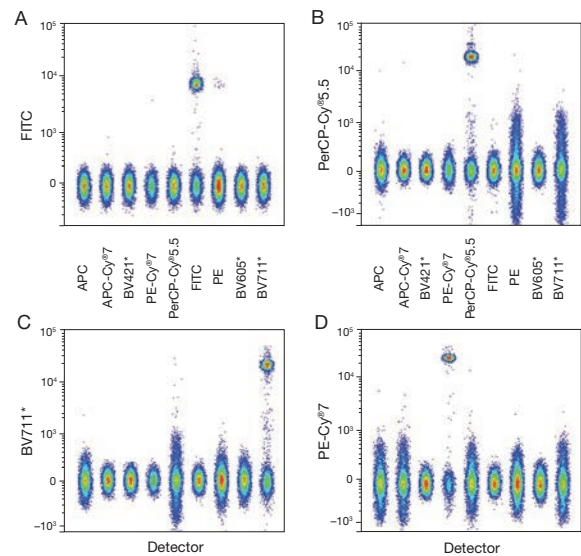


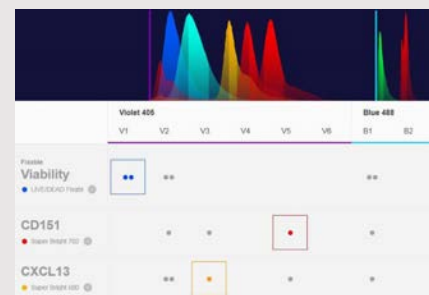
Figure 3. Spreading error visualization. Single-stained samples were run on the Invitrogen™ Attune™ NxT Flow Cytometer and analyzed using FlowJo™ software. Data from each detector were combined into a single plot. (A) Staining with the FITC antibody conjugate appears robust when analyzed in the FITC detector; minimal spreading error is observed in other channels. (B) Staining with PerCP-Cy[®]5.5 contributes high spreading error into the PE and BV711 channels. (C) Staining with BD Horizon Brilliant™ Violet 711 (BV711) contributes noticeable spread into the PerCP-Cy[®]5.5, APC, and PE channels. (D) Staining with PE-Cy[®]7 demonstrates extensive spreading error in multiple channels. *BD Horizon Brilliant™ Violet dyes (Becton, Dickinson and Company).

- Use fluorophores that are spectrally similar for different cell sub-populations that will be gated separately

Figure 3 shows a method for visualizing spillover spreading error due to spectral overlap. Although commonly used, the tandem fluorophore PE-Cy[®]7 exhibits significant spreading due to low-energy

Flow Cytometry Panel Builder—A tool for all flow cytometrists

Whether you are a novice or an expert, designing a panel for flow cytometry is a highly complex process. If you are a beginner, let the Invitrogen™ Flow Cytometry Panel Builder lessen your anxiety over panel building by making the pairing of markers and fluorophores quick and simple using a highly visual format. Are you an expert? Then you will appreciate using the Flow Cytometry Panel Builder to easily review the spectral signals and filters per laser line and check fluorophore spillover values per channel. With access to information on over 13,000 antibodies for flow cytometry, this tool allows quick selection of antibodies for flow cytometry panels. Get started building your panel today at thermofisher.com/flowpanel.



(long-wavelength) photons, which in turn negatively impacts the resolution of fluorescent labels in other channels, especially those associated with poorly expressed antigens. Use of a spillover spread matrix is another way to visualize the spread into all other detectors for a given fluorophore [6].

Controls, controls, controls

Controls—e.g., fluorescence minus one (FMO) controls, compensation controls, and viability controls—are critical for evaluating multiparameter flow cytometry data. FMO controls are required for setting gates when multiple fluorophores are used together and when markers are expressed on a continuum. They help to account for the signal introduced from all other fluorescent labels in the channel being gated. FMO controls, which contain all markers except the one of interest, can provide clarity for low-density or smeared populations and can help to delineate two populations that are not easily resolved.

Also required in every multiparameter flow cytometry panel is a viability control, a fluorescent probe that specifically identifies dead cells so that they can be properly excluded from data analysis [7]. Dead cells are sticky and can nonspecifically bind antibodies and other probes, complicating the analysis (Figure 4).

Guidelines for flow cytometry

Follow these best practices for multiparameter flow cytometry:

- Optimize the voltage settings for each flow cytometer detector
- Titrate each antibody for optimal performance in the panel
- Carefully consider the pairing of dyes with targets and minimize spillover spreading
- Use FMO and viability controls to set gates correctly

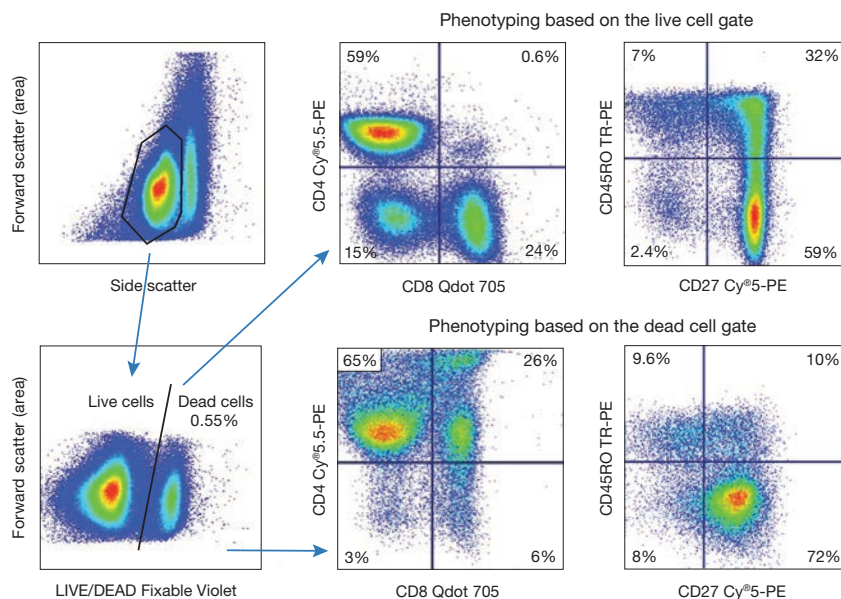


Figure 4. Effects of viability gating on population statistics. The inclusion or exclusion of a viability dye can drastically affect population statistics obtained from an experiment, and discriminating live and dead cells using only scatter parameters can be subjective and inaccurate [7]. In this example from Perfetto et al. [7], after application of a lymphocyte gate (forward scatter vs. side scatter), live and dead cells were discriminated using the Invitrogen™ LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Cat. No. L34955); note the significant number of dead cells despite a scatter gate. Subsequent analysis of live cells and dead cells shows the dramatic difference in apparent phenotypes between the two cell populations. Reprinted from Perfetto SP, Chattopadhyay PK, Lamoreaux L, Nguyen R, Ambrozak D, Koup RA, Roederer M (2006) *J Immunol Methods* 313:199–208, with permission from Elsevier.

Panel design is an iterative process that requires testing all combinations and reviewing the spillover spread matrix at each iteration. Resources—such as webinars, eLearning courses, instrument information, and a library of application notes and protocols, as well as a link to the Invitrogen™ Flow Cytometry Panel Builder—are available at the Flow Cytometry Learning Center at thermofisher.com/flowlearning.

For more information

For a more in-depth discussion of best practices for multiparameter flow cytometry, watch the 1-hour webinar “Panel design for multiparameter flow cytometry”, which expands on the topics included in this article. Find it at thermofisher.com/flowwebinars. ■

References

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