Technical Primer

Selecting Fluorochrome Conjugates for Maximum Sensitivity

Holden T. Maecker, Tom Frey, Laurel E. Nomura, and Joe Trotter
BD Biosciences, San Jose, California

Received 26 March 2004; Revision Received 13 August 2004; Accepted 18 August 2004

Most users of flow cytometry are aware that, to maximize sensitivity for an antibody whose staining is dim, a bright fluorochrome such as phycoerythrin (PE) should be selected for that conjugate. What is often less well appreciated is the role that spectral overlap plays in determining the sensitivity of a given fluorescence channel. This has become more of an issue with the advent of polychromatic flow cytometry (up to 12 colors) (1) and with the introduction of digital electronics and postacquisition compensation, which can affect resolution, as described below. In this article, the pitfalls of selecting suboptimal conjugate combinations are highlighted, in addition to general rules that should guide conjugate selection. We start from first principles to describe how spectral overlap contributes to the observed background in a given fluorescence channel.

VARIANCE OF A POPULATION INCREASES PROPORTIONALLY TO THE MEAN

What this implies for flow cytometry is that brightly fluorescent populations tend to have greater standard deviation (S.D.) than dimly fluorescent populations (once the population is well above electronic noise). The use of a logarithmic scale visually compensates for this tendency by compressing the data at the high end of the fluorescence scale. Thus, populations tend to keep a similar shape on a log scale, whether they are bright or dim, although the bright populations have much greater S.D. It is also for this reason that the coefficient of variation rather than S.D. is commonly used as a measure of dispersion of a flow cytometric population. Because the coefficient of variation is adjusted for the population mean, it tends to be similar for bright and dim fluorescent populations.

RELATION OF MEAN AND VARIANCE APPLIES TO ALL PARAMETERS IN WHICH A POPULATION OF STAINED CELLS FLUORESCES

As we all know, cells stained with an antibody labeled with fluorescein isothiocyanate (FITC) will fluoresce primarily in the FITC channel and secondarily in the PE channel. This is due to the spectral overlap of the dyes and the resultant spillover of FITC signal into the PE detector. Thus, when a FITC conjugate is very bright, it has a large S.D. not only in the FITC channel but also in the PE channel.

COMPENSATION CORRECTS FOR SPECTRAL OVERLAP BUT DOES NOT REMOVE ANY ERROR IN THE MEASUREMENT

We “compensate” for the spectral overlap of FITC into PE by measuring spillover and applying a correction to the PE signal for each data point such that a FITC-stained population, on average, shows no PE fluorescence after compensation (Fig. 1). The greater the spillover of FITC into PE, the higher the compensation required, and the higher the measured S.D. of that population in the PE channel. One way to think of this is that by compensating we are moving a population from an area of the display where the S.D. is relatively large into an area where the S.D. is expected to be relatively small. However, the compensated population’s S.D. remains relatively large, so we are left with “data spread,” which refers to the fact that bright fluorescent signals tend to spread in the dimensions against which they are compensated.

RESOLUTION SENSITIVITY IN A COMPENSATED CHANNEL IS AFFECTED BY DATA SPREAD

Because of data spread of a FITC-positive population after compensation against PE, it will occupy more space in the PE dimension than will a FITC-negative population (Fig. 1). In general, the greater the spillover, the greater the spread of the compensated positive population. Because bright fluorescent populations have higher variance than dim ones, they will exhibit more data spread. This in turn affects the
resolution of dim signals in the second channel (e.g., PE). Simply stated, you will not be able to resolve very dim events above background in the PE channel when those events also have a very bright signal in the FITC channel.

**A PRACTICAL EXAMPLE: CD8 APC-CY7 AND ANTI-INFNγ APC, A POTENTIALLY PROBLEMATIC COMBINATION**

In antigen-specific cytokine flow cytometry, high sensitivity is required to detect potentially rare populations of cytokine-positive cells. Many users select PE or allophycocyanin (APC) conjugates for their anti-cytokine antibodies because of the relatively high intensity of these fluorochromes. However, the following example demonstrates that this may not always produce the desired result. When a relatively bright antibody (to CD8) is used in the APC-Cy7 channel, the resulting data spread in the APC channel can mask the ability to detect dim interferon-γ (IFNγ) APC-positive cells (Fig. 2A). The same is true of the PE channel, where CD4 PE-Cy7-positive cells cause data spread that makes detecting dim IL-2 PE-positive events difficult. Note that the data spread is restricted to populations positive for APC-Cy7 or PE-Cy7, respectively. For example, the CD8 APC-Cy7-positive cells, negative for CD4 PE-Cy7, have a much tighter distribution in the PE channel than does the CD4 PE-Cy7-positive population. To demonstrate that this data spread is dependent on the presence of the APC-Cy7 or PE-Cy7 antibody, the same experiment was performed without CD8 APC-Cy7 (Fig. 2B). This reduces the data spread in the APC channel but does not affect the PE channel.

**ANOTHER ISSUE—FALSE POSITIVES ASSOCIATED WITH THE USE OF TANDEM DYES**

The example shown in Figure 2 highlights one of the potential problems of tandem dyes (e.g., PE-Cy7 or APC-Cy7): they produce fluorescence spillover in the channel of the parent fluorochrome (i.e., PE or APC). Precise conjugation chemistry and careful storage (avoiding heat and light) can minimize such spillover, usually to levels below than 10%, which makes the tandems quite useful nonetheless. However, there is also a potential for false-positive events in the parent fluorochrome channel.

In Figure 2, data spread decreases sensitivity to dim-positive events in PE or APC, but as long as population boundaries are drawn appropriately, there are few, if any, nonspecific positive events. This is not always the case. In the example shown in Figure 3A, dim PE-positive events are seen in the cell population positive for PE-Cy7, and dim APC-positive events in the population positive for APC-Cy7. These do not appear to be true cytokine-producing cells because (a) the sample was not incubated with a secretion inhibitor to allow for intracellular detection of cytokines, (b) the pattern of expression observed is not characteristic of spontaneous cytokine-producing cells, and (c) the positive events remain, to some extent, even in the absence of any cytokine-specific antibody (not shown). The cells also are not a compensation artifact because manipulation of compensation of all channels that spill over into APC or PE did not significantly decrease these events (data not shown). However, these dim-positive events can be shown to be directly dependent on the presence of the respective tandem dye. In the absence of APC-Cy7, a similarly gated population of cells displays few, if any, APC-positive cells (Fig. 3B). Likewise, in the absence of PE-Cy7, the corresponding PE-positive population is greatly decreased (Fig. 3C). Most likely, selective breakdown of the tandem dye leads to fluorescence of certain cells in the parent fluorochrome channel. The mechanism for such breakdown on selected cells is not currently understood. However, users of these assays would be advised to avoid using PE or APC for detection of rare events on a population of cells positive for PE-Cy7 or APC-Cy7, respectively.
IS THIS PROBLEM RESTRICTED TO DIGITAL CYTOMETERS?

Some users, although they appreciate the phenomena of data spread and false positive events, will no doubt report that they never see these problems, or not to the same extent, on their older cytometers. Why is that? First, some definition of terms is in order. We refer to the most commonly used cytometers as having “analog” electronics, in that compensation is performed by pulse subtraction, before the signal is digitized. This is in contrast to the newer “digital” systems, in which raw pulses are digitized, and compensation is performed afterward by application of a spillover matrix.

One reason that data spread may be less apparent on instruments with analog electronics is that they do not allow fluorescence values below a preset baseline, which essentially clips near zero (2). They also usually report signals based on pulse height, which at low levels can be noisy. In addition, noise can be introduced during analog compensation (3–6). The clipping at zero and added noise can cause very low and negative events to be scored as positive after compensation, thus increasing the observed mean or median, which leads to overcompensation. Further, users frequently compensate visually on these systems by aligning the tops of populations rather than the medians (7). This leads to further overcompensation. The overcompensated populations continue to look “reasonable” because the lower values are artificially increased, giving a symmetrical cluster. In contrast, fully digital instruments retain compensated values that are low or neg-
FIG. 3. Demonstration of false positives in PE or APC channels.

A. With CD8 APC-Cy7 and CD4 PE-Cy7:

False positives are present only in cells positive for the respective tandem dye. APC false positives are dependent on the presence of the APC-Cy7 reagent. PE false positives are dependent on the presence of the PE-Cy7 reagent.

B. Without CD8 APC-Cy7:

False positives in APC channel reduced in absence of APC-Cy7 False positives in PE channel remain

C. Without CD4 PE-Cy7:

False positives in APC channel remain False positives in PE channel reduced in absence of PE-Cy7

Fig. 3. Demonstration of false positives in PE or APC channels. A: False positives are present only in cells positive for the respective tandem dye. B: APC false positives are dependent on the presence of the APC-Cy7 reagent. C: PE false positives are dependent on the presence of the PE-Cy7 reagent.
ative. The result is that the true spread of the data is exposed as compensation is applied. Therefore, digital electronics preserve the actual data values more accurately than do analog electronics. This and the fact that tandem dyes are much more commonly used on digital systems mean that problems of data spread and false positive events become more apparent, although they are not unique to these systems.

**HOW TO QUANTITATE BRIGHTNESS OVER BACKGROUND**

Recently, Bigos et al. (8) suggested the use of an algorithm that normalizes the relative brightness of a positive fluorescence signal with the degree of spread in the negative population to which it is to be compared. A simplified formula that accomplishes this is given by:

\[
\frac{\text{mean}_{\text{positive}} - \text{mean}_{\text{background}}}{2 \times \text{S.D.}_{\text{background}}}
\]

In certain systems, median may be more appropriate than mean. The use of this or similar algorithms that take into account data spread and the difference in positive and negative population means are encouraged for all multicolor applications where resolution sensitivity is important.

**A SET OF RULES FOR SELECTING ANTIBODY-FLUOROCHROME CONJUGATES IN MULTICOLOR APPLICATIONS**

All of the above leads us to propose a set of caveats for selecting which antibodies to use with which fluorochromes in multicolor staining. The process should start with determining which markers require the most sensitivity. This could be because they identify populations that are dimly positive, and/or because positive cells are very rare. Examples of the former include markers such as CCR5, CCR7, CD25, and CD5 on B cells. Examples of the latter include cytokines and MHC-peptide tetramers. In the case of dim markers, these need to go into the brightest fluorescence channels, usually PE, PE tandems, or APC. In the case of rare event markers, there is more flexibility, unless the positive events are both rare and dim. However, in either case, the next step is to ensure that antibodies to bright markers are not used in channels that spill over significantly into the channel chosen for high sensitivity. Thus, in the case of PE, the critical channels are FITC and any PE tandems being used. In the case of APC, APC-Cy7 contributes the most spillover. PE and APC tandems can also contribute false-positive events to the PE and APC channels, as described above. An exception is if the bright marker is present on an entirely different subset of cells than the subset that expresses the dim or rare marker. In this case, the data spread will not affect the cells for which high sensitivity is required. Thus, it would be acceptable to use CD3 FITC with CD19 PE or CD14 APC-Cy7 with CD56 APC. In each case, the second marker is relatively dim, but it is present on a completely distinct subset of cells from the first (bright) marker.

How can one determine whether data spread or false positives are a significant problem in a particular experimental setup? In addition to using the formula given above, one can perform a “fluorescence minus one” control (3), i.e., a control sample that has all but one of the fluorescent conjugates. If high sensitivity in the PE channel is desired, one would use a control containing all fluorescent conjugates except PE. In this way, the inherent spread of the data and degree of false positives in the PE dimension can be visualized. It is also a good idea when examining data to look at bivariate displays of parameters that have significant spectral overlap with each other (e.g., APC vs. APC-Cy7 and FITC vs. PE). This will help to determine whether compensation has been set properly, and whether or not data spread is a significant problem in a given type of experiment.

**LITERATURE CITED**