



Selecting reagents for multicolor flow cytometry with BD™ LSR II and BD FACSCanto™ systems

Flow cytometers, such as the BD™ LSR II and BD FACSCanto™ systems, which detect six, eight and more colors, have spurred the development of new fluorochromes and antibody conjugates to take advantage of these capabilities. However, choosing optimal antibody-fluorochrome combinations is a complex process. Here we provide some simple guidelines for the selection of reagent panels for multicolor flow cytometry.

Reagent selection starts with the instrument configuration. The lasers and detectors dictate whether a given fluorochrome can be excited and whether there are enough detectors to properly detect a given combination of fluorochromes. The optical system design also affects the efficiency with which particular dyes are detected, as do the instrument settings, including photomultiplier voltages. Finally, the choice of optical filters greatly influences the effective brightness of one fluorochrome versus another. Virtually testing filter combinations using a web tool, such as the viewer at <http://bdbiosciences.com/spectra/>, may be useful.

Given the variety of instrument configurations, it is impossible to define the best fluorochrome combinations for six, eight or more colors. However, for a particular cytometer, such as the BD LSR II instrument, it is possible to rank available dyes according to their brightness. How is brightness defined? A good functional definition should include the effects of background. In a particular detector, background is influenced by signal intensity, autofluorescence, nonspecific staining, electronic noise and optical background from other fluorochromes (spillover), all of which can increase the width of a negative population (Fig. 1). A good normalized functional measure of reagent brightness is the stain index¹, defined as D/W , where D is the difference between positive and negative populations and W is equal to 2 s.d. of the negative population.

When the same antibody is conjugated to various dyes, their stain indices can be compared to give an idea of their relative brightness on a particular instrument. We have done this for various fluorochrome conjugates run on both a BD LSR II and a BD FACSCanto II flow cytometer (Table 1). This leads to the first rule of reagent selection: pick the brightest fluorochromes. For example, in a four-color panel using fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP)-Cy™5.5 and allophycocyanin (APC), an obvious choice for a fifth color is PE-Cy™7 because it is the brightest fluorochrome not already in the panel. (Cy™ is a trademark of Amersham Biosciences

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Brightness on its own only goes so far. The more colors to be resolved, the more spillover between those colors becomes an issue. Compensation can be used to correct spillover for the cell population as a whole. However, signals of individual cells above or below the mean will result in data spread, which is higher when spectral overlap introduces additional noise that compensation cannot correct¹. Data spread reduces the resolution sensitivity and, therefore, the stain index in a fluorescence detector that receives spillover from other detector(s). Thus, a second rule of reagent selection is to minimize spectral overlap when choosing a reagent combination. This rule can conflict with choosing the brightest fluorochromes. For example, PE-Cy5 has a very bright stain index but has considerable spillover into the APC detector. The resolution sensitivity in APC will be reduced compared with that achieved

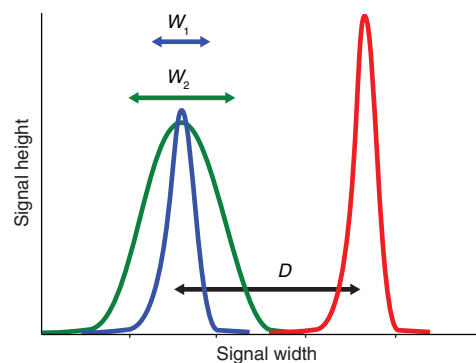


Figure 1 | Stain index. The effective brightness of a reagent depends on the difference (D) between the positive (red) and the negative (blue, green) populations and the spread of the negative population (W). The stain index is a useful metric for normalized signal over background.

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Table 1 | Average stain indices on BD LSR II and BD FACSCanto II

Fluorochrome ^a	Stain index
¹ PE-Cy5	353
PE	302
² APC	278
² Alexa Fluor [®] 647	214
PE-Cy7	139
¹ PerCP-Cy5.5	107
³ BD Horizon™ V450	85
³ Pacific Blue™	80
⁴ Alexa Fluor [®] 488	73
Alexa Fluor [®] 700	61
⁴ FITC	56
⁵ APC-Cy7	37
¹ PerCP	37
AmCyan	25
⁵ APC-H7	24

Average stain index of each fluorochrome conjugate run on both a BD LSR II and a BD FACSCanto II flow cytometer. All fluorochromes were conjugated to BD monoclonal antibody RPA-T4. Fluorochromes listed with the same superscript number are read in the same detector and thus would not normally be used in combination.

^aAlexa Fluor[®] and Texas Red[®] are registered trademarks, and Pacific Blue™ is a trademark, of Molecular Probes, Inc. The Alexa Fluor, Pacific Blue, and Texas Red dyes are licensed from Molecular Probes.

using, for example, a combination of PerCP-Cy5.5 and APC. One might wish to sacrifice a certain amount of brightness in one detector to avoid spillover (and loss of resolution sensitivity) in another.

Taking these two rules into account, we selected sets of fluorochromes that are reasonable choices for experiments requiring six, eight or more colors (Table 2).

Once the fluorochromes have been defined, antibody specificities can be matched to particular fluorochromes. Brightness and spillover remain key issues.

As an example, we will discuss CD62L staining on CD8⁺T cells. CD8, an abundant protein, stains very brightly. CD62L is relatively 'dim' (the protein is not abundant on the cell surface and/or the antibodies are of low affinity). One might consider using the brightest fluorochrome, PE, for CD62L, while using a dimmer fluorochrome, such as FITC, for CD8. But FITC has considerable spillover into the PE detector, compromising the resolution sensitivity in PE to the point that the CD62L resolution might be suboptimal. Possible solutions include using for CD8 a fluorochrome with less spectral overlap with PE or, for CD62L, using a fluorochrome that is still relatively bright but does not overlap with FITC.

This example illustrates two additional rules: reserve the brightest fluorochromes for dim antibodies and vice versa, but avoid spillover from bright cell populations into detectors requiring high sensitivity for those populations.

One final consideration is the potential for tandem dye degradation. APC-Cy7, and to a lesser extent PE-Cy7, can degrade in the presence of light, fixation and elevated temperatures so that they emit in the parent dye detector (APC or PE), leading to false positive events¹. By minimizing the exposure of samples to these conditions, this problem can be largely avoided. Additionally, BD Biosciences has developed APC-H7, which displays enhanced stability under these conditions. Still, for some

Table 2 | Choices for six-, eight- and ten-color experiments

Six-color	Eight-color	Ten-color
FITC or Alexa Fluor [®] 488	FITC or Alexa Fluor [®] 488	FITC or Alexa Fluor [®] 488
PE	PE	PE
		PE-Texas Red [®] or PE-Alexa Fluor [®] 610
PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5
PE-Cy7	PE-Cy7	PE-Cy7
APC or Alexa Fluor [®] 647	APC or Alexa Fluor [®] 647	APC or Alexa Fluor [®] 647 Alexa Fluor [®] 680 or 700
APC-Cy7 or APC-H7	APC-Cy7 or APC-H7	APC-Cy7 or APC-H7
	AmCyan	AmCyan
	BD Horizon V450	BD Horizon V450

applications, there is an additional rule: consider the consequences of degradation of tandem dyes and whether this will compromise sensitive readouts in the APC or PE detectors. If so, a different reagent configuration might be in order.

In testing a multicolor reagent cocktail, it is wise to include fidelity and fluorescence-minus-one controls in the initial testing². Fidelity controls use an antibody by itself (or with minimal additional gating reagents) and compare the results with those obtained using the antibody in a complete cocktail. From this, the effect of additional reagents on the readout can be seen, ensuring that the other reagents are not compromising that readout. Fluorescence-minus-one controls combine all the reagents in a given cocktail except for one, to gauge the sensitivity of particular detectors in the context of the other reagents. They can also be used for routine gating of those detectors for which other means of setting gates are not possible or practical. Usually, once a reagent panel has been well validated, it is not necessary to run all of these controls on a day-to-day basis.

Summary

Here we have outlined rules for selecting reagents for multicolor flow cytometry. These rules need to be balanced to achieve the best possible results. First, choose the brightest fluorochromes for a particular instrument configuration. Second, choose fluorochromes that minimize the potential for spectral overlap. Third, reserve the brightest fluorochromes for dim antibodies and vice versa. Fourth, avoid spillover from bright cell populations into detectors requiring high sensitivity for those populations. Finally, take steps to avoid tandem dye degradation, and consider its impact on results.

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1. Maecker, H.T., Frey, T., Nomura, L.E. & Trotter, J. Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry A* **62**, 169–173 (2004).
2. Baumgarth, N. & Roederer, M. A practical approach to multicolor flow cytometry for immunophenotyping. *J. Immunol. Methods* **243**, 77–97 (2000).

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