Selecting Reagents for Multicolor Flow Cytometry

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Application Note

Flow cytometers that detect six, eight, and more colors have spurred the development of new fluorochromes and antibody conjugates that take advantage of these capabilities. However, choosing the optimal combination of fluorochromes for your particular antibody specificities is a complex process. This application note provides some simple guidelines to help you select reagent panels for multicolor flow cytometry, avoid rounds of trial and error, and increase your chances of a successful experiment.

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The basics: Know your instrument

Reagent selection starts with your instrument configuration. The type and number of lasers and detectors dictate whether the optical system can excite a given fluorochrome and properly detect a given combination of fluorochromes. The design of the optical system also impacts the efficiency with which particular dyes are detected, as do the instrument settings, including PMT voltages. (See the new BD Cytometer Setup and Tracking [CS&T] feature in BD FACSDiva™ 6.x software at **bdbiosciences.com/facsdiva**). Finally, the choice of optical filters that are used with each detector greatly influences the effective brightness of one fluorochrome versus another. Filter selection is a give-and-take process: using a wider bandpass filter can increase the ability to detect a given fluorochrome, but may also increase the amount of spillover background contributed into that detector from other neighboring fluorochromes. A good way to visualize these effects is by virtual testing of filter combinations using a web tool such as the viewer at **bdbiosciences.com/spectra**. This tool illustrates predicted spillovers* for particular fluorochromes and filter combinations.

* The predicted "leakage" in normalized percentage of emitted fluorescence, which might not necessarily correspond to the percentage used for compensation, due to the effects of varying detector gains.

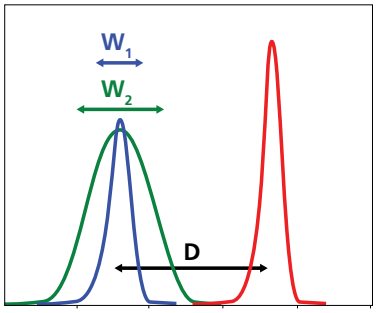

Figure 1. The effective brightness of a reagent depends upon the difference between the positive and the negative (D) and the spread of the negative population (W). The stain index is a useful metric for normalized signal over background.

Fluorochromes: Go for the bright...

Given the many differences in instrument configuration, it is impossible to universally state the "best" fluorochromes to use in combinations of 6, 8, 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 on that instrument (when configured with a specified set of lasers and filters). But how exactly do we define and measure brightness? A good functional definition of reagent brightness should include a term to weigh the effects of background contributions, or how resolvable the signal is over the unstained cell populations. Background in a particular detector is influenced by signal levels, cell autofluorescence, non-specific staining, electronic noise, and optical background from other fluorochromes (spillover). To the extent that these factors each contribute noise and/or signal, they increase the width (the variance or SD^2) of a negative population (see **Figure 1**). As such, a good normalized functional measure of reagent brightness is the stain index,[1] defined as D/W, where D is the difference between positive and negative populations, and W is equal to 2 SD of the negative population.

When the same antibody is conjugated to various dyes, their stain indexes can be compared to get an idea of the relative brightness of the dyes on a particular instrument. This assumes that the conjugation chemistries for all of the reagents have been optimized. Using this assumption, **Table 1** shows the stain index for a number of different CD4 conjugates, using the specified filters on a standard BD LSR II flow cytometer.

The information in this table provides an idea of the relative brightness of different fluorochromes on this platform. This leads to the first rule of reagent selection, which is to pick the brightest available fluorochromes. Suppose you have a four-color panel consisting of reagents in FITC, PE, PerCP-Cy™5.5, and APC, and you want to add a fifth color. PE-Cy™7 is an obvious choice, since it is the brightest fluorochrome not already in your panel.

Fluorochrome	Clone	Stain Index
[1]PE-Cy5	RPA-T4	**353**
PE	RPA-T4	**302**
[2]APC	RPA-T4	**278**
[2]Alexa Fluor® 647	RPA-T4	**214**
PE-Cy7	RPA-T4	**139**
[1]PerCP-Cy5.5	RPA-T4	**107**
[3]BD Horizon™ V450	RPA-T4	**85**
[3]Pacific Blue™	RPA-T4	**80**
[4]Alexa Fluor® 488	RPA-T4	**73**
Alexa Fluor® 700	RPA-T4	**61**
[4]FITC	RPA-T4	**56**
[5]APC-Cy7	RPA-T4	**37**
[1]PerCP	RPA-T4	**37**
AmCyan	RPA-T4	**25**
[5]APC-H7	RPA-T4	**24**

Table 1. Average stain index of various fluorochrome conjugates run on both a BD LSR II and a BD FACSCanto II flow cytometer

[1,2,3,4,5] Fluorochromes listed with the same superscript number are read in the same detector, and thus would not normally be used in combination.

...but minimize spillover

Brightness, on its own, only goes so far. The stain indexes listed in **Table 1** are calculated with the reagents run singly, not as part of a cocktail. As soon as other reagents are added, spectral overlap (or spillover) becomes an issue. The more colors one attempts to resolve, the more spillover between those colors that will have to be dealt with. We deal with spillover by applying "compensation," which applies a correction to all signals such that, for example, a cell population fluorescing only in PE will show no FITC fluorescence, on average. While this is true for the population on average, individual cells will fall above or below the mean, and this "data spread" is higher when spectral overlap introduces additional noise.[1] Compensation unfortunately does not remove this noise even though the median is corrected since the data spread (variance) from photoelectron statistics remains. The effect of data spread is thus to reduce the resolution sensitivity, and therefore the stain index, in a fluorescence detector that receives spillover from other detectors. Thus, the second rule of reagent selection is to minimize the potential for spectral overlap when choosing a reagent combination. This can conflict with the first rule, which was to choose the brightest fluorochromes. For example, PE-Cy5 is very bright with regard to stain index (**Table 1**), but it has considerable spillover (ie, adds background) into the APC detector. While these two fluorochromes can be used together, the resolution sensitivity in APC will be reduced compared to, for example, a combination of PerCP-Cy5.5 and APC. This is a case when you might wish to sacrifice a certain amount of brightness in one detector to avoid spillover (and loss of resolution sensitivity) in another.

Colors and specificities: Define winning combinations

Taking the two rules into account, we generated a set of fluorochromes that are reasonable choices to use for experiments requiring 6, 8, or more colors (**Table 2**). Note that these choices are based upon BD instruments (BD LSR II, BD FACSAria™ II cell sorter, or BD FACSCanto™ II flow cytometer), and upon reagents that usually can be purchased from a catalog as antibody conjugates.

Once the fluorochromes to be used have been defined, you can begin to match antibody specificities to particular fluorochromes to select the actual conjugates to be used. For this purpose, brightness and spillover remain key issues, as the following example illustrates.

6-color	8-color	10-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

Table 2. Common choices for 6-, 8-, and 10-color experiments

Imagine that you want to look at CD62L staining on CD8+ T cells. While CD8 is an abundant protein, and antibodies to it stain cells very brightly, CD62L is relatively "dim" (the protein is not abundant on the cell surface, and/or the available antibodies are of low affinity). Initially, you might consider using the brightest available fluorochrome, PE, for CD62L, while using a dimmer fluorochrome, like FITC, for CD8. But FITC has considerable spillover into the PE detector (see **Figure 2**). The result is that, for CD8+ cells that are highly stained in the FITC detector, resolution sensitivity in PE is compromised, to the point that CD62L resolution might be suboptimal. There are multiple possible solutions to improve this, including:

1) Move CD8 to a fluorochrome that has less spectral overlap with PE (such as PerCP-Cy5.5 or APC).

2) Move CD62L to a detector that is still relatively bright, but does not overlap with FITC (such as APC or PE-Cy5). Note that in this example, only the CD8+ cells are highly stained in the FITC detector, so only CD8+ cells contribute to data spread in the PE detector. If you were interested in CD62L staining only on CD4+ (CD8–) cells, the original reagent combination (CD8 FITC, CD62L PE) would be fine.

This example illustrates two additional rules in reagent selection: 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.

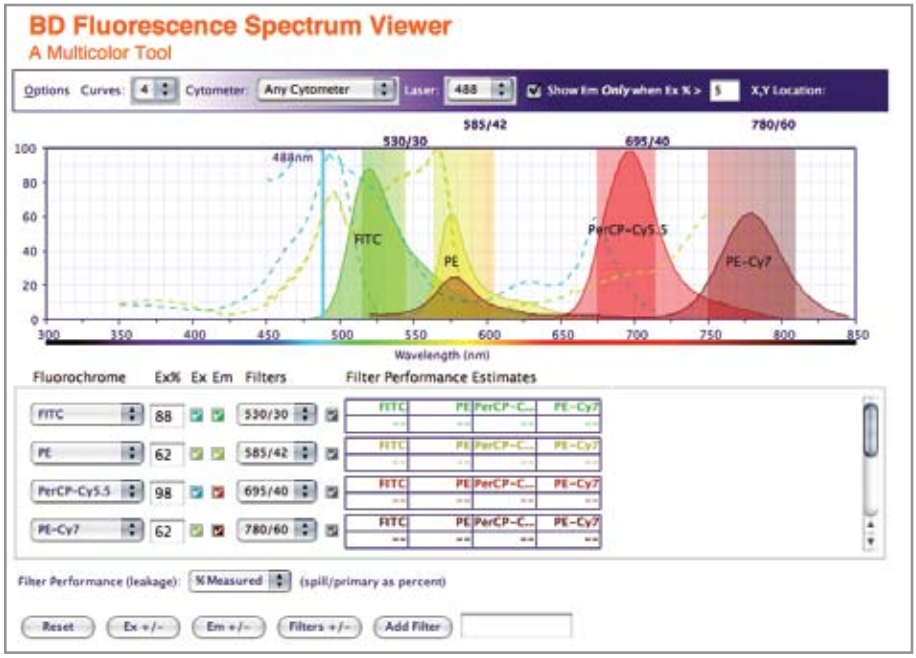

Figure 2. Spectral overlap of FITC into the PE detector. Taken from **bdbiosciences.com/spectra**

Tandem dyes: Watch out for degradation

One final topic to consider 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). This process often starts with a small subpopulation of cells, leading to false positive events in APC or PE.[1] By minimizing the

exposure of samples to light, heat, and formaldehyde-based fixatives, this problem can be largely avoided. Additionally, BD Biosciences has developed APC-H7, an analog of APC-Cy7 with enhanced stability in light, heat, and formaldehyde-based fixatives. Still, for some 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. For situations in which final fixation of samples is required (eg, biohazardous samples), there is a stabilizing fixative available that helps prevent degradation of APC-Cy7 while still fixing cells (BD Biosciences Cat. No. 338036). For more stable tandem dyes, BD now offers APC-H7 conjugated antibodies.

Controls to validate your panel

Once all of these factors have been considered, you are ready to test a multicolor reagent cocktail. In so doing, there are two types of controls that you might wish to include in your initial testing: fidelity controls and fluorescence-minus-one (FMO) controls.[2] Fidelity controls use a given antibody by itself (or with minimal additional gating reagents), and they compare the results to the use of that antibody in a complete cocktail. From this, you can see the effect of additional reagents on the readout of interest, to be sure that the other reagents are not compromising that readout. FMO controls combine all the reagents in a given cocktail, except for one reagent of interest. They are useful to gauge the sensitivity of particular detectors in the context of the other reagents. They also may be used for routine gating of those detectors for which other means of setting gates are not possible or practical. In general, though, once a reagent panel has been well validated, it is not necessary to run all of these controls on a day-to-day basis.

To summarize, this article has set forth rules for selecting reagents for multicolor flow cytometry. These rules need to be balanced to achieve the best possible results:

Rule 1: Choose the brightest set of fluorochromes for your particular instrument configuration.
Rule 2: Choose fluorochromes to minimize the potential for spectral overlap.
Rule 3: Reserve the brightest fluorochromes for dim antibodies, and vice versa.
Rule 4: Avoid spillover from bright cell populations into detectors requiring high sensitivity for those populations.
Rule 5: Take steps to avoid tandem dye degradation, and consider its impact upon results.

With time, proven panels of multicolor reagents will become available, both from literature sources and from manufacturers. Many users will not have to start from scratch in designing reagent panels. Nevertheless, given the huge variety of applications of multicolor flow cytometry, most researchers sometimes will find themselves having to select their own multicolor reagents. We hope that these rules will prove helpful.

Please visit **bdbiosciences.com/colors** for more details about our multicolor flow cytometry reagents.

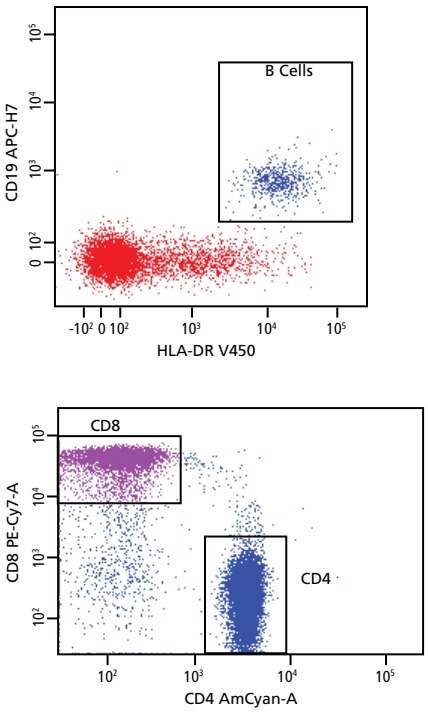

Figure 3. Example of the use of gating reagents (CD4, CD8, CD19) using non-traditional fluorochrome conjugates excited by blue, red, and violet lasers.

Multicolor flow cytometry reagents

Expand your multicolor flow with options beyond your typical five colors.

- BD Biosciences continues to increase our portfolio of multicolor reagents to support your cutting-edge research using the expanded fluorescent detection capabilities of our flow cytometers.
- We offer directly conjugated antibodies to fluorochromes that go beyond your typical five colors by providing colors for violet, red, and blue lasers. These include AmCyan, BD Horizon V450, Alexa Fluor® 700, and PE-Cy7 conjugates to support up to nine-color staining with off-the-shelf reagents.
- BD Biosciences Custom Conjugation Program* offers more choices for your multicolor experiment design. This program offers multiple colors, as shown in **Table 3**.

* Please contact your BD sales representative for more information about the Custom Conjugation Program.

Fluorochrome	Ex Max (nm)
Alexa Fluor® 488	495
Alexa Fluor® 594	590
Alexa Fluor® 647	650
Alexa Fluor® 680	679
Alexa Fluor® 700	696
AmCyan	457
APC	650
APC-Cy7	650
BD APC-H7	650
BD Horizon V450	404
FITC	494
Pacific Blue™	401
PE-Cy5	496, 564
PE-Cy7	496, 564
PerCP	482
PerCP-Cy5.5	482
PE-Texas Red®	496, 564
Texas Red®	595

Table 3. Custom conjugates available from BD Biosciences

References

1. Maecker HT, Frey T, Nomura LE, Trotter J. Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry A*. 2004;62:169.

2. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. *J. Immunol. Met.* 2000;243:77.

Table 4. BD Biosciences fluorochrome specifications

Fluorochrome	Fluoresence Emission Color	Ex-Max (nm)	Excitation Laser Line (nm)	Em-Max (nm)	BD FACScan™	BD FACSCalibur™	BD FACSCanto™	BD FACSCanto™ II	BD FACSVantage™ SE	BD™ LSR	BD™ LSR II	BD FACSAria™ family	BD FACSArray™	BD Influx™
BD Horizon V450	Blue	404	405, 407	448				●	●		●	●		●
Pacific Blue™	Blue	401	405, 407	452				●	●		●	●		●
AmCyan	Green	457	405, 407	491				●	●		●	●		●
Alexa Fluor® 488	Green	495	488	519	●	●	●	●	●	●	●	●		●
FITC	Green	494	488	519	●	●	●	●	●	●	●	●		●
PE	Yellow	496, 564	488, 532	578	●	●	●	●	●	●	●	●	●	●
PE-Texas Red®	Orange	496, 564	488, 532	615	●			●	●	●	●	●		●
Texas Red®†	Orange	595	595	615					●					
APC*	Red	650	595, 633, 635, 647	660		●	●	●	●	●	●	●	●	●
Alexa Fluor® 647	Red	650	595, 633, 635, 647	668		●	●	●	●	●	●	●	●	●
PE-Cy5*	Red	496, 564	488, 532	667	●	●			●	●	●	●		●
PerCP	Red	482	488, 532	678	●	●	●	●		●	●	●		●
PerCP-Cy5.5	Far Red	482	488, 532	695	●	●	●	●	●	●	●	●	●	●
Alexa Fluor® 700	Far Red	696	633, 635	719				●	●		●	●		●
PE-Cy7	Infrared	496, 564	488, 532	785	●	●	●	●	●	●	●	●	●	●
APC-Cy7	Infrared	650	595, 633, 635, 647	785			●	●	●	●	●	●	●	●
BD APC-H7	Infrared	650	595, 633, 635, 647	785			●	●	●	●	●	●	●	●

** APC and PE-Cy5 may be used together on instruments with cross-beam compensation.*
† Texas Red® detection requires a dye laser for 595–600 nm excitation.

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