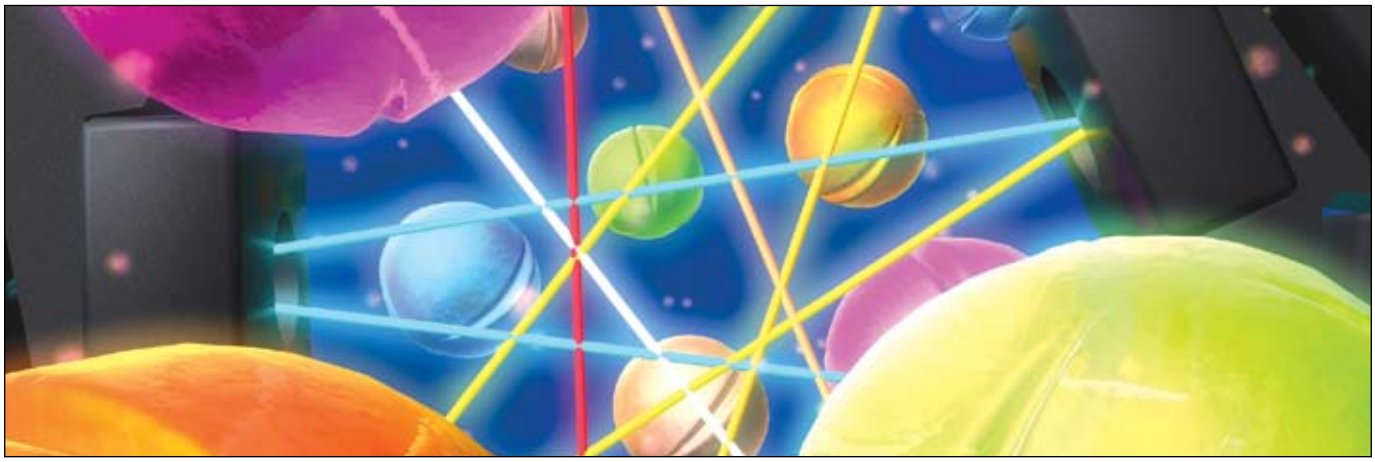
Selecting Reagents for Multicolor Flow Cytometry



By Holden Maecker and Joe Trotter

The availability of flow cytometers capable of detecting 6, 8, and more colors has spurred the discovery of new fluorochromes and the development of antibody conjugates to take advantage of these capabilities. However, the complexities of choosing antibody combinations are such that simply using a random combination of fluorochromes for a particular set of antibody specificities is unlikely to provide optimal results. On the other hand, with a bit of forethought, one can usually avoid many rounds of trial and error. The goal of this article is to provide some simple guidelines to aid in the selection of reagent panels for multicolor flow cytometry, which should result in more successes than failures.

The basics: Know your instrument

Reagent selection starts with your instrument configuration. The lasers and detectors that you have available dictate whether or not you can excite a given fluorochrome, and whether or not you have enough detectors to read out 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 BD Application Note, *Establishing Optimum Baseline PMT Gains to Maximize Resolution on BD Biosciences Digital Flow Cytometers*, **bdbiosciences.com/baselineoptimization**). Finally, the choice of optical filters that are used with each detector greatly influences the brightness of one fluorochrome versus another. One can think of filter selection as 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 detected in 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 **bdbiosciences.com/spectra**. Here you can get an idea of predicted spillovers* for particular fluorochromes and filter combinations.

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, if one considers 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 definition of brightness should probably start with *resolution sensitivity*; the ability to discriminate a dim positive signal from background. Background in a particular detector is influenced by electronic noise, cell autofluorescence, and spillover from other detectors. To the extent that these factors introduce noise, they also increase the width (or standard deviation, SD) of a negative fluorescence peak (see **Figure 1**). As such, a good measure of resolution sensitivity is the stain index[1], defined as D/W, where D is the difference between means of a positive and negative peak, and W is equal to 2 SD of the negative peak.

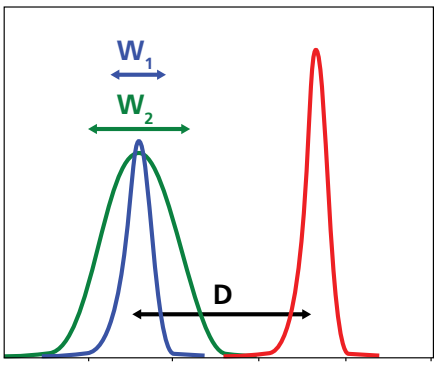


Figure 1. Resolution sensitivity (the ability to resolve a dim positive signal from background) depends upon the difference between positive and background peak means **(D)** and the spread of the background peak **(W)**. The stain index is a metric that captures both of these factors.

* The predicted spillovers in normalized percentage of omitted fluorescence, which may not necessarily correspond to the percentage used for compensation due to the effects of varying detector gains.

Table 1. Stain index of various fluorochrome conjugates on a BD LSR II

Reagent	Clone	Filter	Stain Index
PE	RPA-T4	585/40	356.3
[1]Alexa Fluor® 647	RPA-T4	660/20	313.1
[1]APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
[2]PE-Cy5	RPA-T4	695/40	222.1
[2]PerCP-Cy5.5	Leu-3a	695/40	92.7
PE-Alexa Fluor® 610	RPA-T4	610/20	80.4
[3]Alexa Fluor® 488	RPA-T4	530/30	75.4
[3]FITC	RPA-T4	530/30	68.9
[2]PerCP	Leu-3a	695/40	64.4
APC-Cy7	RPA-T4	7801/60	42.2
Alexa Fluor® 700	RPA-T4	720/45	39.9
Pacific Blue™	RPA-T4	440/40	22.5
AmCyan	RPA-T4	525/50	20.2

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

When the same antibody is conjugated to various dyes, one can compare the stain index for those conjugates to get an idea of the relative brightness of the dyes on a particular instrument. This assumes, of course, that the conjugation chemistries for all of the reagents have been optimized. With this as a caveat, **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.

From this table, one can get 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 4-color panel consisting of reagents in FITC, PE, PerCP-Cy5.5, and APC, and you want to add a fifth color. PE-Cy7 is an obvious choice, since it is the brightest fluorochrome not already in your panel.

...But minimize spillover

But brightness, on its own, only goes so far. The stain indexes listed in **Table 1** are calculated with the reagents run on their own, not as part of a cocktail. As soon as other reagents are added, spectral overlap (or spillover) becomes an issue. Simply stated, 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 results in a correction being applied to all signals such that a cell population fluorescing only in PE, for example, will show no FITC fluorescence, on average. But 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. The effect of data spread is thus to reduce the resolution sensitivity, and therefore the stain index, for a fluorescence detector that receives spillover from other detector(s). As a result, we can state a second rule of reagent selection, which is to minimize the potential for spectral overlap when choosing a reagent combination. This can be in 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 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 where one might wish to sacrifice a certain amount of brightness in one detector to avoid spillover (and loss of resolution sensitivity) in another.

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

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	APC-Cy7	APC-Cy7
	AmCyan	AmCyan
	Pacific Blue™	Pacific Blue™

Colors and specificities: Define winning combinations

Taking the two rules above into account, one can generalize 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™ cell sorter, or BD FACSCanto™ flow cytometer), and upon reagents that can be generally purchased as catalog antibody conjugates.

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

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

Selecting Reagents for Multicolor Flow Cytometry *(continued)*

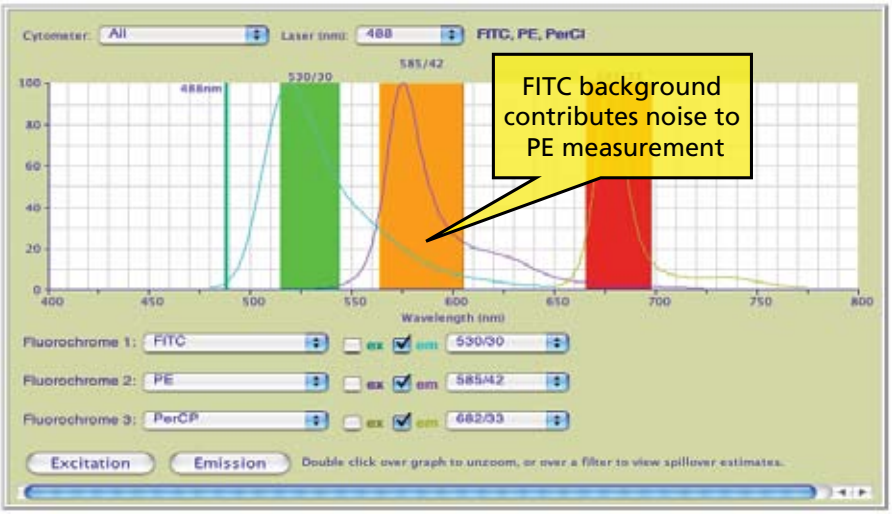


Figure 2. Spectral overlap of FITC into the PE detector. Taken from **bdbiosciences.com/spectra.**

on the cell surface, and/or the available antibodies are of low affinity). Initially, one would be tempted to use 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 may be suboptimal. There are multiple possible solutions to improve this, including:

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

2) Move CD62L to a detector that is still relatively bright, but does not overlap with FITC (like 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 one 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.

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. Still, for some applications, it may be desirable to think about 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 may 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).

Controls to validate your panel

Once all of these considerations have been taken into account, you are ready to test a multicolor reagent cocktail. In so doing, there are two types of controls that you may wish to include in your initial testing: fidelity controls and fluorescence-minus-one (FMO) controls.[2] Fidelity controls are those that 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, one 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 are those that combine all the reagents in a given cocktail, except for one reagent of interest. These are useful to gauge the sensitivity of particular detectors in the context of the other reagents. They may also 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, although sometimes in conflict with each other, 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 so as 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 in the literature and from manufacturers, such that 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 will at least sometimes find themselves having to select their own multicolor reagents, we hope that these rules will prove helpful.

References

1. Maecker HT, Frey T, Nomura LE, and Trotter J. Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry A.* 2004;62:169.
2. Baumgarth N and Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. *J. Immunol. Methods.* 2000;243:77.

Multicolor Flow Cytometry Reagents

Expand your multicolor flow with options beyond your typical 5 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 5 colors by providing colors off the Violet, Red, and Blue lasers. These include **AmCyan, Pacific Blue™, Alexa Fluor® 700**, and **PE-Cy7** conjugates to support up to 9-color staining with off-the-shelf reagents.
- BD Biosciences Custom Conjugation Program* offers even more choices for your multicolor experiment design. This program offers multiple colors, as featured in the table at right.

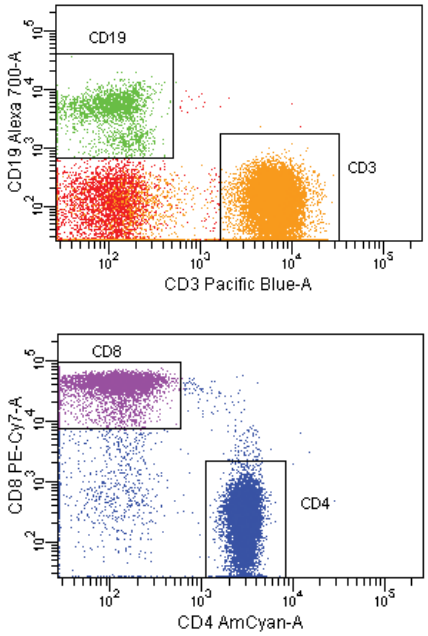


Figure 3. Example of the use of gating reagents (CD3, CD4, and CD19) using non-traditional fluorochrome conjugates excited by blue, red, and violet lasers.

Custom conjugates available from BD Biosciences

Fluorochrome	Ex Max (nm)
Alexa Fluor® 405	401
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
FITC	494
Pacific Blue™	405
PE-Cy5	496, 564
PE-Cy7	496, 564
PerCP	482
PerCP-Cy5.5	482
PE-Texas Red®	496, 564
Texas Red®	595

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

Fluorochrome chart on page 30.

*Please contact the Technical Service Department for more information about the Custom Conjugation Program.

Fluorochromes for combinations beyond the typical 5-color experiment

		Blue laser (488 nm)	Red laser (633 nm)	Violet laser (407 nm)	
Name	**Reactivity**	**PE-Cy7** Ex Max (nm): 496, 564	**Alexa Fluor® 700** Ex Max (nm): 696	**Pacific Blue™** Ex Max (nm): 405	**AmCyan** Ex Max (nm): 457
CCR4	Hu	557864			
CCR6	Hu	557993			
CCR7	Hu	557648			
CD2	Hu	335786			
CD3	Hu	557851, 341091	557943	558117	339186
CD3	Bab, Cyno, Rhe	557749	557917	558124	
CD3e	Ms	552774		558214	
CD4	Hu	557852, 348789	557922	558116	339187
CD4	Ms	552775	557956	558107	
CD5	Hu	348790			
CD8	Hu	557746, 335787	557945	558207	339188
CD8	Bab, Cyno, Rhe	557750			
CD8a	Ms	552877	557959	558106	
CD10	Hu	341092			
CD11a	Ms	558191			
CD11b	Hu	557743		558123	
CD11b	Ms	552850	557960		
CD13	Hu	338425			
CD14	Hu	557742	557923	558121	
CD16	Hu	557744, 335788	557920	558122	
CD19	Ms	552854	557958		
CD19	Hu	557835, 341093	557921		339190
CD20	Hu	335793			
CD25	Hu	557741			
CD25	Ms	557744, 335789			
CD33	Hu	333946			
CD34	Hu	348791			
CD38	Hu	335790			
CD45	Hu	557748			339192
CD45	Ms	552848			
CD45R/B220	Ms	552772	557957	558108	
CD45RA	Hu	337167			
CD45RO	Hu	337168			
CD56	Hu	557747, 335791	557919		
CD69	Hu	557745			
CD69	Ms	552879			
CD95	Ms	557653			
CD117	Hu	339195			
CD195	Hu	557752			
CD274	Hu	558017			
HLA-DR	Hu	335795			
IFN-γ	Hu	557844, 557643			
IFN-γ	Ms	557649			
IgG_1, κ	Hu	348788			339185
IgG_1, κ	Ham	552811			
IgG_1, κ	Ms	557646, 557872	557882	558120	
IgG_1, κ	Rat	557645			
IgG_1, λ	Ham	557798			
IgG_1, λ	Rat	552869			
IgG_2, λ	Ham	557652			
IgG_{2a}, κ	Rat	552784	557963	558109	
IgG_{2a}, κ (specific for TNP)	Ms	552868		558118	
IgG_{2b}, κ	Rat	552849			
IgM	Ms	552867			
IL-4	Hu	557989			
Ly-6G and Ly-6C	Ms	552985	557979		
NK-1.1	Ms	552878			
PD-1	Hu	558016			
PD-L1	Hu	558017			
TNF	Hu	557647			
TNF	Ms	557644			

Applicable Patents: APC: US 4,520,110; 4,859,582; 5,055,556; Europe 76,695; Canada 1,179,942; PerCP: US 4,876,190; PE-Cy7: US 4,542,104; APC-Cy7: US 5,714,386