

An Introduction to Compensation for Multicolor Assays on Digital Flow Cytometers

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Technical Bulletin

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Multicolor flow cytometry rapidly reveals a large amount of biological information from a single sample. It often is the only means to adequately identify or functionally characterize complex populations of interest within the immune system. Over the past few years, the number of parameters (and consequently colors) simultaneously analyzed in typical flow cytometry experiments has increased.¹ This is enabled by the availability of high-performance instrumentation with additional laser and detector options and data computational power, along with advances in biochemistry that have led to more fluorochrome options. Not only has this increased the usefulness of flow cytometry, but it has increased the importance of proper experiment setup to ensure accurate and meaningful results.

Compensation is an essential part of proper experimental setup for multicolor assays. However, it is also a subject that many flow cytometry users find difficult and confusing. Here we discuss some of the basics of spillover and compensation, tips for setting up compensation controls, and the tools that BD offers to help you easily plan correct compensation for multicolor assays.

Introduction to compensation

What are spillover and compensation?

When using multiple fluorochromes in an experiment, there are many factors that can impact the accuracy and quality of the data. The most critical factor is determining which color should be matched to each antibody in the reagent panel. This is due to a very large range of intrinsic brightness among the fluorochromes commonly used, some antigens being dimly expressed while others brightly expressed, and signals from one reagent optically interfering with signals from another. These choices of color and antibody must also be made in the context of which markers might be coexpressed on the same cells. Whenever more than one marker is expressed on a single cell, the presence of the other fluorescent reagents can contribute significant optical background in proportion to their brightness. This phenomenon is called spillover.

- Spillover is due to the physical overlap among the emission spectra of certain commonly used fluorochromes.
- Spillover occurs whenever the fluorescence emission of one fluorochrome is detected in a detector designed to measure signal from another fluorochrome (Figure 1).
- The amount of spillover is a linear function, so the measured average signal levels can be corrected (ie, the population medians aligned) by the process called compensation.

With proper compensation setup, complex data sets can then be properly visualized and analyzed if a well chosen immunofluorescent reagent panel is used. If the compensation is incorrect, interpreting the data can become extremely difficult or impossible.

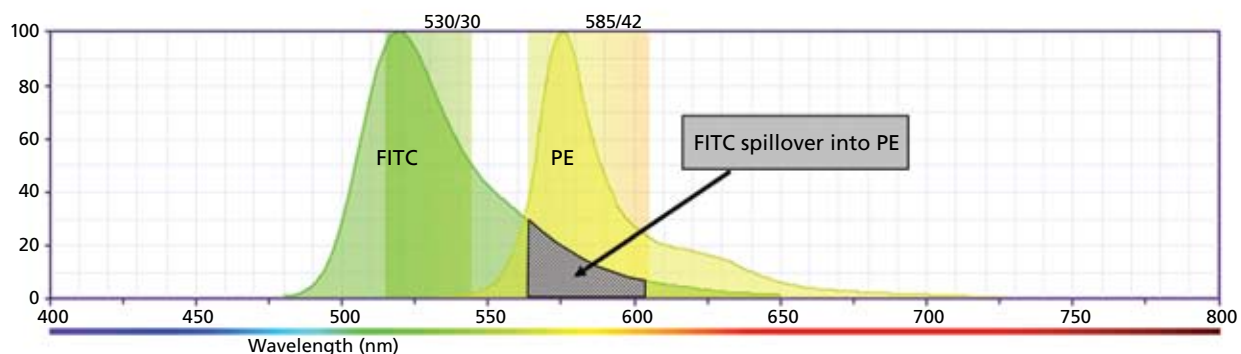


Figure 1. Example of FITC spillover into the PE channel.

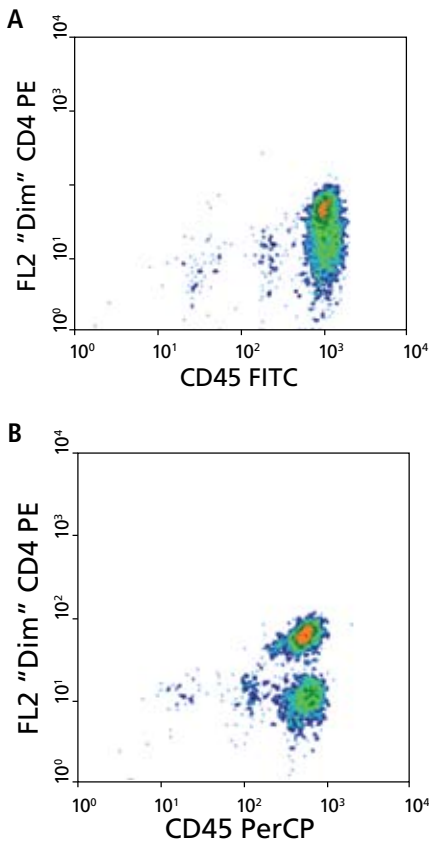


Figure 2. Example of the importance of reagent selection and resolution sensitivity, especially when trying to resolve dim populations.²

Why are reagent selection and compensation important to accurate data interpretation?

Beyond sample quality and cytometer setup, reagent selection and compensation are usually the most important factors affecting proper interpretation of the data. It is important that all relevant populations are resolved as best as possible. Compensation correctly aligns populations affected by spillover, but it does not fix all its undesirable effects. Even with proper compensation, if a large amount of unwanted signal spills over into a neighboring detector where a dim signal is to be detected, resolution sensitivity might be lowered and the populations might not be able to be accurately resolved.

To illustrate this, a comparison was made of a population of dim CD4-PE expression (CD4-PE in the presence of excess unlabeled antibody to create a dim CD4 population) measured on cells that also express CD45. When the highly expressed anti-CD45 is labeled with FITC, the spillover into the PE detector provides a sufficiently high optical background in the PE detector such that, even after proper compensation, the dim CD4-PE population is still not resolvable over the CD4 negative population (Figure 2A). When CD45-PerCP is used rather than CD45-FITC, the populations are easily resolved because there is essentially no significant spillover from the CD45-PerCP detector into the PE detector (Figure 2B).

How does compensation correct for spillover?

The primary goal of compensation is to remove the signal from a given fluorochrome from all neighboring channels where it is also detected, a laborious task to perform manually in a large multicolor experiment.

- To correct for spillover, spectral overlap values are measured for all fluorophores and in all detectors, via single-color controls. The spillover values are then placed in a symmetrical matrix.
- Then, to determine the actual compensation values to use to correct the data, the measured spectral overlap values (of all colors in all detectors) are inverted by matrix algebra to yield compensation values.^{3,4} This matrix algebra operation essentially calculates the simultaneous solution of the equations for the contributions of the spectral overlaps of each of the colors into every detector.
- Finally, the compensation values (not spectral overlap values) are used by the flow cytometer to correct the contributions of other colors overlapping into a given detector.

BD FACSDiva™ software is very useful because it collects data from each compensation control tube and automatically calculates accurate compensation values for each fluorochrome combination.

Resolution Sensitivity: the degree to which a flow cytometer can differentiate dimly stained cells from unstained cells in a mixture.

Correct vs incorrect compensation

When compensation is correct, the median fluorescence intensities (MFIs) of the positive and negative populations of the compensation control are aligned in the neighboring channels. Figure 3 shows an example of FITC, correctly and incorrectly compensated out of the PE channel. These graphs are used to visually demonstrate how BD FACSDiva software calculated the spillover to properly compensate. Attempting to set compensation visually in a complex multiparameter experiment (compensation by eye) amounts to guesswork and is not recommended.

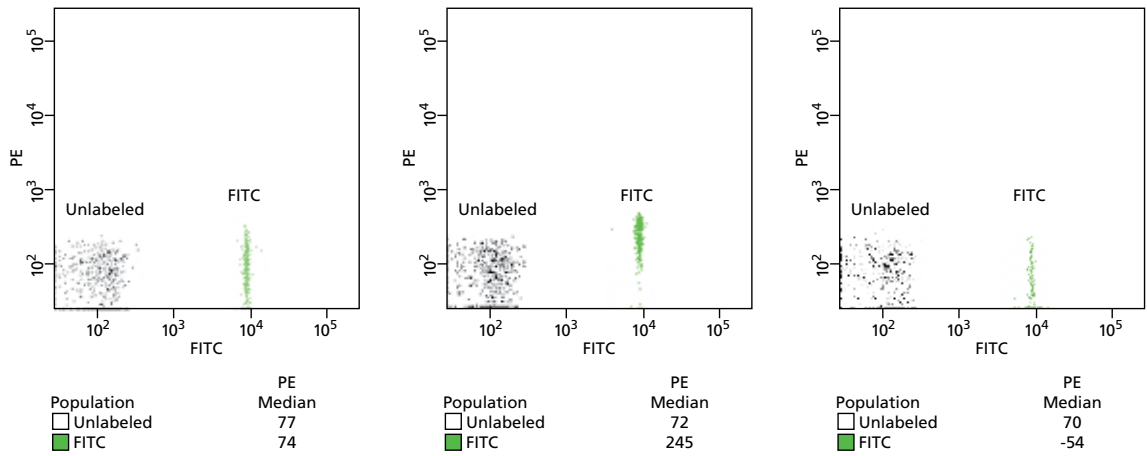


Figure 3.

Compensated properly

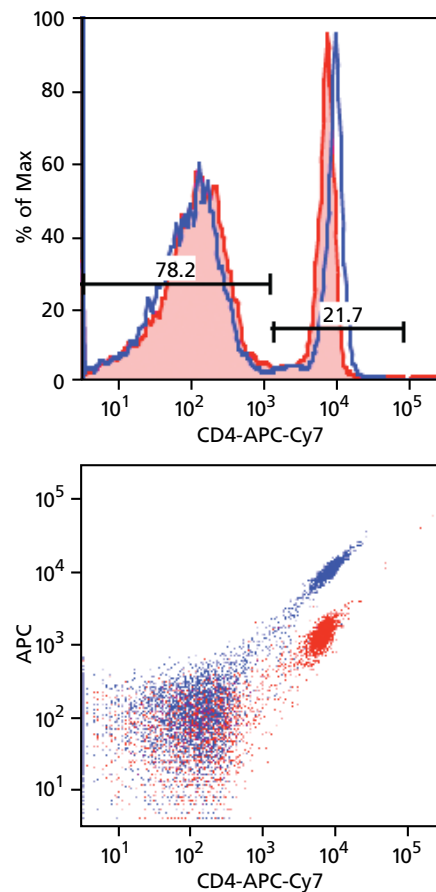
The medians of the positive and negative FITC populations are equal in the PE channel.

Undercompensated

Not enough fluorescence subtraction. The PE MFI of the positive FITC population is greater than that of the negative FITC population.

Overcompensated

Too much fluorescence subtraction. The PE MFI of the negative FITC population is greater than that of the positive FITC population.



	S/N	Spillover %
BD Biosciences	126	19
Competitor	295	115

Figure 4. Two lots of the same tandem reagent might perform similarly yet have very different spillover values. (Red represents BD reagents and blue represents a different brand.) Both CD4 APC-Cy7TM reagents were run on a BDTM LSR II system.

Tips for setting up compensation

Compensation when using tandem dyes

When working with tandem dyes, it is important to understand some of the variables that affect compensation. The photon transfer efficiency from donor to acceptor in tandem pairs tends to be different each time the conjugation chemistry is performed, thus resulting in spectral overlap values that can vary from lot to lot.⁷ Compensation controls should have spectral characteristics identical to the reagents used in the experiment, so each antibody conjugate lot of a particular tandem dye will need its own compensation control. One of the useful features of BD FACSDiva software is that it can accommodate two or more different controls for the same color.⁸

Note: Guidelines on setting up label-specific compensation controls appear in the Typical workflow for setting up compensation on a BD flow cytometer section of this document. For more information, see the BD FACSDiva training module at bdbiosciences.com/support/training/self_paced.jsp.

Tandem dye reagent handling tips

Tandem dyes often require special consideration because they are subject to degradation with prolonged exposure to light, elevated temperatures, and fixatives. When a tandem dye starts to degrade, an increase in spillover into the channel to the donor fluorophore is observed. This complicates compensation and can cause false positives in the channel of the donor fluorophore. To achieve the best results from an assay, samples should be protected from the elements that contribute to degradation.

- Analyze samples as soon as possible after staining.
- Minimize exposure to light. This can be as simple as covering samples with aluminum foil, storing samples in a light-protected area, and working in a dimly lit room.
- Minimize exposure to formaldehyde-based fixatives. The stability of tandem dyes in fixatives can be extended by using BDTM Stabilizing Fixative (Cat. No. 338036).
- If the tandem dye has degraded, BD FACSDiva software may give a warning message that the spectral overlap is greater than 100% when the compensation is calculated.

FRET: When the donor fluorophore of a tandem dye is excited, it transfers energy to an acceptor fluorophore through a mechanism called Fluorescence Resonance Energy Transfer (FRET), thus causing it to emit at the wavelength of the acceptor fluorophore.

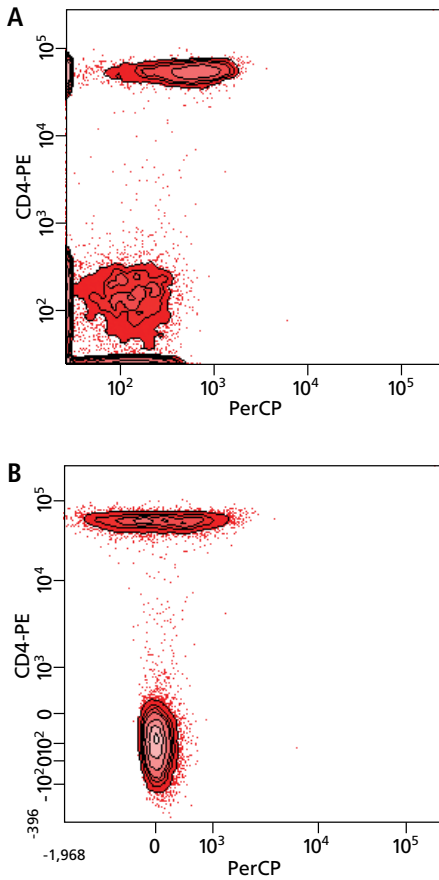


Figure 5. Example of human CD4-PE compensated out of the PerCP channel by BD FACSDiva software. In Plot A, viewed on a standard Log vs Log scale, the CD4-PE-labeled population appears to be undercompensated. When the same plot is viewed on a biexponential scale (Plot B), it is clear that BD FACSDiva software has correctly compensated the sample.

Biexponential scale: scale that is close to log at the upper end and close to linear at the low end, allowing events at or below zero to be displayed.

Practices to avoid: compensation by eye

BD FACSDiva software was designed to allow flow cytometry users to easily and accurately compensate multicolor assays. Besides being more labor intensive, manual compensation and using visual cues to compensate (compensating by eye) will most likely lead to improper compensation. It can be next to impossible to determine the median of a population just by looking at it. This is especially apparent when using a traditional log scale, on which values at or near zero are often piled up on the axis, making it extremely difficult to determine the median without the use of statistics. Although manually aligning populations based on median statistics might be better than compensating by eye, it also is not as accurate as software compensation. Allowing BD FACSDiva software to compensate the assay is much more accurate because the matrix algebra operation solves all colors simultaneously, while the serial subtraction process used in manual compensation does not.

It is also important to note the significance of biexponential display when visualizing compensated data. Versions of BD FACSDiva software 6.0 and later have biexponential display,^{5,6} which allows the best visualization of compensated data and errors in compensation to be more easily detected. While BD FACSDiva software automatically calculates accurate compensation values, errors in compensation can occur if the wrong compensation controls are used, if there is contamination from a small amount of fluorophore carried over from the previous sample, or if the compensation controls are not gated properly. Additionally, when displayed with the traditional log scale, data might appear to be improperly compensated, when in fact it has been properly compensated by BD FACSDiva software (Figure 5).

Tips for setting up compensation controls

Although BD FACSDiva software will accurately set the compensation values, proper compensation relies on setting up the right compensation controls. The following are some tips for setting up proper compensation controls.

Compensation controls must consist of a negative and a positive population for each single color. Single-stained cellular controls and/or single-stained BD™ CompBead particles can be used for this purpose.

- Single-stained cellular controls contain cells that are unstained and cells stained with a single color.
- BD CompBeads are beads that have been coupled to an antibody specific for the kappa light chain of Ig, from mouse, rat, or rat/hamster. In this case, the compensation control tube contains unstained beads and beads stained with a single color.

Set up a single-stained compensation control tube for each color and each individual tandem reagent used in the experiment.

- Similar fluorochromes should be compensated separately (ie, FITC/ Alexa Fluor® 488) due to different spectral characteristics.
- If necessary, adjust the PMT voltages if the stained cells are off scale or if the negative population is too high. It is important to keep the compensation controls within the linear range of each detector.

The negative and positive populations should have the same spectral characteristics (autofluorescence).

Compensation is dependent on the fluorochrome, not the cell type, so it is not necessary to compensate on the same tissue that will be analyzed in the experiment. However, it is important that the positive and negative populations have the same autofluorescence. If using CD3⁺ lymphocytes for the positive population, use CD3⁻ lymphocytes for the negative population, not CD3⁻ monocytes. The autofluorescence of the BD CompBeads does not need to be an exact match to the cell type analyzed.

Reagents used for compensation controls should be bright enough to resolve a positive and negative population.

This ensures that the values in the spillover channel are above background and provides greater accuracy for calculating low (but real) spillovers. Since BD CompBeads have a robust signal regardless of the antibody conjugate, they provide a great advantage over single-stained cellular controls for which the fluorescent signal could be dim or the antigen could be expressed on a rare cell type, resulting in inaccurate values.

Set up compensation controls and run compensation for each experiment.

Spillover values are affected by fluorescence intensity, which can be changed by adjusting the PMT voltages. If the PMT voltages are adjusted between experiments in a way that affects the fluorescence intensity in that channel, compensation settings should not be applied from one experiment to another.

Example of proper compensation controls

If the following experiment is being run, the following compensation controls should be prepared.

Experiment		Compensation Controls for the experiment	
Tube 1	Tube 2	Single-Stained Compensation Control	Reasoning
CD4 APC lot A	CD3 Alexa Fluor® 647 lot E	CD4 APC lot A	Since APC and Alexa Fluor® 647 have different spectral characteristics, a separate compensation control is needed for each.
		CD3 Alexa Fluor® 647 lot E	
CD3 BD Horizon™ V450 lot B	CD8 BD Horizon™ V450 lot G	CD3 or CD8 BD Horizon™ V450	In general, it is acceptable to run one compensation control for non-tandem dyes of the same color.
CD25 APC-Cy7 lot C	CD25 APC-Cy7 lot F	CD25 APC-Cy7 lot C	Different lots of tandem dyes have different spectral characteristics. Since different lots of tandem dyes might have been used to make each of these conjugates, it is necessary to run a compensation control for each.
		CD25 APC-Cy7 lot F	
FoxP3 PE lot D	FoxP3 PE lot D	FoxP3 PE lot D	FoxP3 is expressed only on a small subset of cells. BD CompBeads eliminate biological factors that may affect compensation when using cells, so using BD CompBeads would be the ideal way to compensate this reagent.

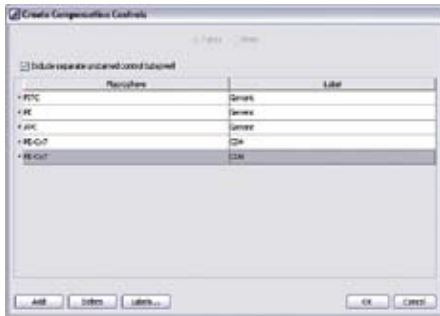


Figure 6. Window for creating compensation controls in BD FACSDiva software, showing two conjugates from the same tandem.

Typical workflow for setting up compensation on a BD flow cytometer

Start up System -> Check Performance Using CS&T -> Set up Experiment -> Set up Compensation Controls -> Record Data -> Analyze Data -> Shut down System

Start up the system

1. Start up the cytometer and computer.
2. Start BD FACSDiva software.
3. Check fluid levels in the Cytometer window and prepare tanks as needed.
4. Check the flow cell for air bubbles.
5. Verify that the detectors and optical filters are appropriate for your experiment.
6. Check that laser warmup has finished.

Check cytometer performance

1. Select **Cytometer > CST**.
2. Run the BD Cytometer Setup and Tracking beads.
3. View the Cytometer Performance Report.
4. Close the Cytometer Setup and Tracking window.

Set up the experiment

1. Create an experiment in the Browser.
2. Apply application settings to the cytometer settings.

Set up Compensation (using BD CompBeads or single-stained sample control tubes)

1. Select **Experiment > Compensation Setup > Create Compensation Controls**.
2. Create label specific controls if necessary (ie, the experiment contains more than one conjugate for a particular tandem) (Figure 6).
 - a. Click **Add**.
 - b. Select the fluorophore from the list.
 - c. Enter the name.
3. Install and run the unstained control tube.
4. Verify that settings are appropriate.
5. Install and run the stained control tubes.
6. Ensure that the positive cells are on scale for each detector.
7. Record and view the data, and gate the positive populations.
8. Select **Experiment > Compensation Setup > Calculate Compensation**.
9. Rename the compensation setup and apply it to the experiment.

Record experimental data

1. Create Browser elements.
2. Create the plots, gates, and statistics needed for recording.
3. Enter information in the Experiment Layout as needed.
4. Record the data.

Analyze the data

Shut down the system

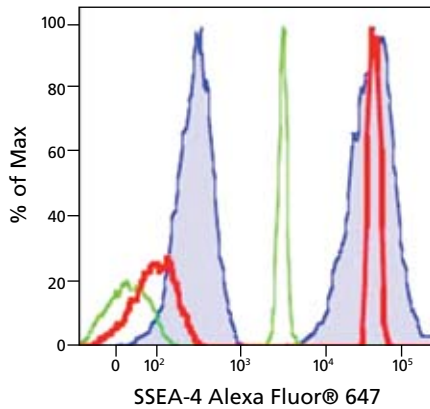


Figure 7. Live H9 hESCs (blue), standard BD CompBeads (3 µm) (green), or BD CompBeads Plus (7.5 µm) (red) stained with SSEA-4 Alexa Fluor® 647.

Tools for easy, accurate compensation for multicolor assays

BD CompBeads

- BD CompBeads are polystyrene particles that have been coupled to an antibody specific for the IgG, κ light chain of mouse, rat, or rat/hamster. Each set also contains a negative control consisting of particles labeled with FBS that have no binding capacity. When mixed together with a fluorochrome-conjugated rat, rat/hamster or mouse antibody, BD CompBeads provide distinct positive and negative stained populations that can be used to set compensation levels.
- BD CompBeads are ideal for dimly expressed antigens, antibodies that have low affinity for receptors on the cell surface, or when the positive population is rare.
- Since cells are not used, BD CompBeads are ideal when trying to conserve cells.
- BD now offers BD CompBead Plus particles, which are optimal for compensating experiments with larger cell types, for example, human embryonic stem cells (hESCs). These 7.5-µm beads have forward scatter properties better suited for compensating larger cell types than the standard BD CompBeads (Figure 7).
- **Ordering Information:** Anti-Mouse Ig, kappa (Cat. No. 552843), Anti-Rat Ig, κ (Cat. No. 552844), Anti-Rat/Hamster Ig, κ (Cat. No. 552845), Anti-Mouse Ig, κ CompBead Plus (Cat. No. 560497), Anti-Rat Ig, κ CompBead Plus (Cat. No. 560499).

BD Cytometer Setup and Tracking (CS&T) Beads

- CS&T beads, used with BD FACSDiva v6.0 and later software, automate cytometer setup and optimize all the key parameters for superior multicolor performance.
- CS&T software functions incorporate daily performance tracking and graphical selection and automated baseline definition of cytometer configurations.
- The system ensures that the cytometer is set up with the optimal PMT voltages so that the dimmest populations can be resolved above electronic noise.
- **Ordering Information:** CS&T beads, 50 tests (Cat. No. 641319) and 150 tests (Cat. No. 642412).

BD FACSDiva software

BD FACSDiva software provides the ability to simultaneously set up and acquire flow cytometry data from 20 different parameters, flexible experiment and analysis designs and templates, and powerful tools, including biexponential scaling for dynamic visualization and automated compensation.

- The software reads the spectral overlap values from single-color compensation controls in sequential acquisitions. Spectral overlap values are automatically calculated by the software and applied to the experiment. All of the spectral overlaps are stored as a setup file for immediate use or for later recall.

- BD FACSDiva software can accommodate two or more different controls for the same color, making it very easy to ensure that the proper compensation settings are applied to each tube.
- BD FACSDiva v6.0 and later software has biexponential display to allow the best visualization of data so that errors in compensation can be easily detected.

Flow cytometers

BD cytometers have a long history of spatially separated laser beams, which helps minimize spillover. Opposed to coaxial laser excitation, spatial separation of laser beams allows only one laser to excite a cell at a time. Electronics hold the signal of the cell as it passes through each laser. Once the cell passes through all lasers, the signals are correlated and the data can be visualized using BD FACSDiva software. With spatially separated laser beams, there is no interference of signals from the other lasers, and the optics design and detection are more streamlined. This helps minimize spectral overlap and unwanted noise, which in turn, simplifies and minimizes compensation.

Reagents

As part of our effort to make multicolor flow cytometry more accessible, BD continues to lead the way by developing dyes optimal for multicolor assays.

- BD has engineered a new organic violet laser excitable dye, BD Horizon™ V500, to address the limitations of existing fluorochromes in this spectral range. Specifically, BD Horizon V500 has significantly less spillover into the FITC channel when compared to AmCyan and is brighter than Pacific Orange™. This makes BD Horizon V500 an optimal accompaniment to V450 as the second violet reagent in this channel and allows more choice and flexibility when developing multicolor panels.
- BD has developed the BD™ APC-H7 dye to have less spillover into the APC channel than APC-Cy7, thus simplifying compensation. Additionally, BD APC-H7 is more stable in light and paraformaldehyde-based fixatives, making it easier to handle overall.

For more information about the fluorochromes, visit bdbiosciences.com/colors.

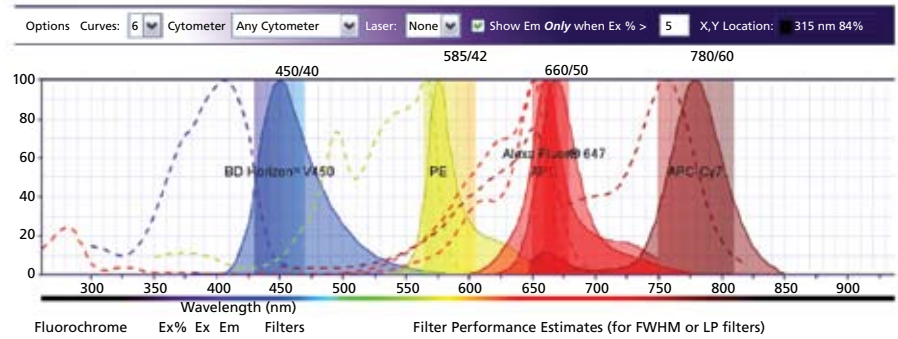
Web tools and literature

One of the guidelines for optimal multicolor panel design is to select reagents that will give minimal spillover. The interactive Fluorescence Spectrum Viewer web tool allows you to view the estimated performance of the filters on the cytometer (bdbiosciences.com/spectra). Following is an example of how this tool can be used to show potential spectral overlap for the reagents used in the Table on page 8.

- For more information on selecting the optimal reagents for multicolor panels, see the BD Biosciences application note *Selecting reagents for multicolor flow cytometry*.

BD Fluorescence Spectrum Viewer

A Multicolor Tool



- Visit bdbiosciences.com/colors for more tools and literature to assist you in designing multicolor experiments.

Technical support and customer training

- Our experts are here to help you with questions you may have regarding compensation or any other aspect of multicolor flow cytometry. Please contact our experienced support staff at 877.232.8995 or by email at ResearchApplications@bd.com.
- BD training courses offer the highest quality education in cell analysis using state-of-the-art flow cytometry equipment and methodologies. Find out more about our training courses, eLearning courses, and training tools at bdbiosciences.com/support/training.

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APC-Cy7: US patent 5,714,386

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