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Multiparameter Conventional Flow Cytometry

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Abstract

Multicolor flow cytometry is a useful technique when examining mixed populations of cells, such as blood and tissue cells in human and animal samples. The ability to use multiple fluorescent markers simultaneously allows for the identification of multiple cell types, as well as functional markers that further characterize each sample. The introduction of instruments capable of measuring 12-plus colors and new reagents has made this type of flow cytometry both popular and problematic. Adapting a typical staining panel from 4 to 6 color tubes to more than 12 colors is not simply a matter of “plug and play”, but must be approached in a systematic manner to achieve a successful multi-parameter staining panel. This chapter will examine the considerations and methods needed to successfully perform multicolor flow cytometry.

Keywords

Multicolor flow cytometry; Staining; Flow cytometry; Multi-parameter; Immunology

1 Introduction

One of the hallmarks of flow cytometric analysis is its ability to simultaneously and rapidly measure multiple parameters on mixed populations of cells. This technique has become indispensable in numerous fields of study including immunology, virology, cancer biology, and infectious disease monitoring. In the early years of flow cytometry, staining panels were limited to 3–4 colors requiring multiple tubes of cells for each experimental sample. This proved to be problematic when dealing with small samples sizes such as tissue biopsies, pediatric samples or samples from small animal models because there were insufficient cells available for analysis. For example, when many cell populations are defined by multiple markers, such as T_{reg} cells, which require a minimum of 4 markers (CD3, CD4, CD25, and FoxP3) for identification before any other marker such as memory or activation can be considered, utilization of 3–4 colors is only partially helpful. In these early analysis panels, not only were large numbers of cells required, but also large amounts of antibody since each staining tube had to include identifying markers for each cell population under consideration. Even with the introduction of staining reagents and instruments capable of six colors, this requirement for large numbers of cells and reagents persisted. It was not until the introduction of instruments and reagents capable of analyzing more than 12 that the need for

large numbers of cells and amounts of antibody has eased. These larger color staining panels also had the advantage of allowing for more detailed analysis of each cell population and sub population.

New flow cytometers can detect more fluorochromes on each sample by using tandem dyes, multiple laser configurations, digital electronics, and software generated compensation matrices. There can be up to eight fluorochromes detected off a single laser and the separation between fluorochomes is narrower than on older 4–6 color instruments. In addition, Quantum Dots (Qdots) and tandem dyes can also be excited by multiple lasers making compensation more complicated. Designing and implementing a staining panel with these new challenges requires a more systematic approach that considers the instrument configuration (lasers, detectors), reagents (availability, clone selection, fluorochrome brightness), spectral overlap (amount of compensation needed) and appropriate controls (Fluorescence Minus One, experimental).

Instrument configurations on digital flow cytometers manufactured by BD Biosciences (San Jose, CA) can be obtained using FACSDiva CS&T software. The instrument configuration is available under the "*Cytometer—View Configurations"* tabs. Proper maintenance and quality control should be standard procedures for all flow cytometers. Quality control protocols for multicolor flow cytometry have been published elsewhere [1, 2] and should be followed on a regular basis along with daily quality control such as using FACSDiva CS&T software and beads. An example of instrument configurations and recommended fluorochromes for a BD LSRII and BD FACSymphony is shown in Fig. 1.

Reagent selection is a critical part of multicolor panel design. For larger multicolor panels, antigen density and relative fluorochrome brightness must be considered when designing a staining panel. Specifically, low density antigens (IL-4, IL-12, CXCR5, CCR7, etc.) should be placed on bright fluorochromes for maximum resolution. Higher density antigens (CD3, CD4, CD8, CD20, CD45, etc.) can be placed on dim fluorochromes [3]. In general, fluorochrome brightness is dependent on laser wavelength, laser power and detector configuration; Table 1 contains examples of flurochrome brightness [4]. Another consideration when selecting reagents is the spectral overlap of each fluorochrome and the amount of compensation needed. Whenever possible, the fluorochromes in an experimental panel should be spread out on multiple lasers to minimize compensation. For example, the combination of FITC, BV605, PE, APC, and BUV395 is a better choice than using FITC, PE, PE-TxRED, PE-CY5, and PE-Cy7 together. Unfortunately, even with advances in fluorochrome design, tandem dyes will still have to be used to get to more than 12 colors in any multicolor staining panel. This creates an additional challenge because tandem dyes can be excited by multiple lasers further complicating compensation. None of these challenges are insurmountable, but they need to be considered when designing a multicolor staining panel. Further reagent preparation such as antibody titration will be discussed in Subheading 3.1.

Controls for a multicolor flow cytometry experiment need to consider autofluorescence, background staining of antibodies and spectral overlap of fluorochromes. Autofluorescence can be addressed by examining unstained cells in each fluorescence detector during panel

optimization. Isotype controls can be used to check background staining of antibodies and single color staining controls (either cells or beads) should be used to set compensation. Background antibody staining and spectral overlap of fluorochromes can cause issues with the determination of positive and negative signal for each marker. When working with large numbers of fluorescent dyes in each sample several considerations need to be addressed. Compensation can cause different distributions of signal in each channel so that a single set threshold for positivity is not practical. To combat this, fluorescence minus one (FMO) controls should be used whenever possible [5]. Briefly a FMO control is made for each fluorochrome-conjugated antibody by staining with all the other antibodies in the panel except the specific reagent being tested. Table 2 gives an example of a 12-color panel with FMO controls. Finally, the use of a viability dye such as one of the fixable amine-binding dyes is strongly recommended since dead cells will pick up antibody and introduce staining artifacts.

2 Materials

2.1 Reagents

1. Test samples: PBMC (fresh or thawed from frozen), tissue samples (processed and ficolled), etc.
2. Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca^{2+} or Mg^{2+}.
3. Fixable amine-binding viability dye: for example, Live/Dead (ThermoFisher, Waltham, MA), Zombie (Biolegend, San Diego, CA), Fixable Viability (BD Biosciences).
4. Fluorochrome-conjugated monoclonal antibodies.
5. Antibody capture beads for compensation: for example, Comp-Bead Anti-Mouse Ig beads (BD Biosciences), COMPtrol beads (Spherotech, Lake Forest, IL), UltraComp beads (eBioscience, San Diego, CA).
6. ArC Amine Reactive Compensation Beads (ThermoFisher) for compensation of viability dye. Cells can be substituted for this compensation control (*see* Note 1).
7. Brilliant Stain Buffer (BD Biosciences) if using Brilliant Violet, Ultraviolet or Blue reagents.
8. FACS Wash Buffer for PBMC: D-PBS, 1% BSA or FBS, penicillin-streptomycin.
9. FACS Tissue Wash Buffer for tissue samples or PBMC: D-PBS, 1% FBS, 15 mM HEPES, 2 mM EDTA, penicillin-streptomycin.
10. Fixation Buffer: 1% Paraformaldehyde or ultrapure (methanol-free) formaldehyde in D-PBS.

[1]·The ArC beads do not always stain well. Cells are a more consistent control. Briefly, cells are microwaved for a few seconds to induce cell death and then stained with the viability dye.

2.2 Equipment and Supplies

1. 12 × 75 mm polystyrene tubes and caps.
2. 96-well plates: deep-well plates are recommended, standard U-bottomed plates can be used but require more washes.
3. Adhesive plate lids.
4. Multichannel pipette.
5. 12-channel vacuum manifold and length adjuster for aspiration (V&P Scientific, San Diego, CA).
6. Centrifuge with swinging bucket rotor and tube or plate holders.
7. Flow cytometer with multiple lasers and detectors.
8. (Optional) 96-well plate loader for flow cytometer.

3 Methods

3.1 Titration of Antibodies

All antibodies in the staining panel should be titrated for optimal concentration using the stain index (SI). The SI is measurement of relative brightness for a reagent that considers the difference between positive and negative signals in the fluorescence channel and the relative spread of the negative signal [3, 6].

1. Use each fluorochrome-conjugated antibody in a single color experiment to stain a test sample at multiple concentrations or volumes and analyze the data to generate the SI [6] as shown in Fig. 2.

   SI = D/W.

   D = Difference in Medians between the positive and negative peaks.

   W = Width of negative signal calculated as 2 × robust Standard Deviation (rSD)

2. Once the optimal concentration for each individual antibody has been determined, stain a test sample with the complete panel of antibodies to see if any adjustments need to be made. For example, antibody concentrations sometimes need to be adjusted to reduce compensation because of spectral overlap in other fluorescence channels (PE-Cy5/APC, PerCP-Cy5.5/BV711, PE-CF594/BV605, etc.).

3.2 FMO Controls

1. Create control samples for each fluorescent channel by staining a representative sample with the complete panel of antibodies minus the specific antibody being tested (*see* Table 2). Ideally, the same type of cells or tissue samples should be used for these controls. However, if cell numbers are low, then an alternate sample such as PBMC can be substituted, or the FMO controls for well established markers such as lineage markers (CD3, CD19 etc.) can be eliminated.

2. Set up the flow cytometer with appropriate instrument settings and run compensation controls as described in Subheading 3.5.
3. Run the FMO controls and determine background and positivity threshold for each antibody.
4. Optimally this should be a part of every experiment, but low cell numbers may prohibit this control from being performed at every time point. However, this is a critical part of staining panel optimization.

3.3 Tube Staining Procedure

1. Prepare samples by counting cells, washing cells out of media, and resuspending cells at 1×10^7 cells/mL in D-PBS.
2. Put 100 μL of cells into each labeled 12 × 75 mm tube.
3. Add a working concentration of amine-binding fixable viability dye to each tube and incubate for 15 min at room temperature. Buffers containing protein or sodium azide should be avoided during staining with amine-binding fixable viability dyes. Live/Dead Aqua (ThermoFisher #L34966) will be used as an example:
    a. Add 50 μL of DMSO to vial to dissolve dye.
    b. Add 50 μL of D-PBS to vial to create working concentration.
    c. Use 1 μL of working concentration to stain each sample.
4. Resuspend cells in 2 mL of D-PBS and centrifuge at 300 × *g* for 5 min. Decant supernatant and resuspend in 50 μL D-PBS.
5. Prepare a cocktail or master mix of antibodies by calculating how many samples will be stained and adding extra antibodies for 2 additional samples. For example, if 10 samples will be stained, add an amount of each antibody to the master mix that is enough for 12 samples.
6. Add the master mix of antibodies to each sample tube and incubate for 30 min at 4 °C in the dark. The final staining volume is dependent on the number of antibodies and the volume of each antibody (i.e., 12 antibodies/5 μL per antibody will give an antibody cocktail of 60 μL per sample). If using Brilliant Violet, Ultraviolet or Blue reagents, also add 50–100 μL of Brilliant Stain Buffer to the tube in addition to the antibody cocktail.
7. Add 3 mL of FACS Wash Buffer or FACS Tissue Wash Buffer and centrifuge at 300 × *g* for 5 min.
8. Decant supernatant and resuspend in 300–400 μL of Fixation Buffer.
9. Stain single color compensation tubes using either cell controls or antibody capture beads according to manufacturer’s instructions.
10. Stain FMO controls if there are sufficient cells.

11. Acquire samples on flow cytometer within 24 h.

3.4 96-Well Plate Staining Procedure

1. Perform **steps 1–6** as in Subheading 3.3 but increase cell concentration to 2×10^7 cells/mL so that 50 μL of cells can be added to each well instead of 100 μL.
2. For 96-well deep-well plates, add 500 μL of FACS Wash Buffer or FACS Tissue Wash Buffer; for standard 96-well U-bottomed plates, add 100 μL.
3. Centrifuge at 300 × *g* for 5 min.
4. Aspirate the supernatant off with vacuum manifold and repeat **steps 2–3** (*see* Note 2). For standard 96-well U-bottomed plates, increase the wash volume to 200 μL and repeat the wash step one additional time for a total of three washes.
5. Aspirate the supernatant off with vacuum manifold and resuspend cells in 200 μL of Fixation Buffer.
6. FMO and compensation controls can be stained in the plate as well.
7. Acquire samples on flow cytometer within 24 h. If using the HTS 96-well plate loader (BD Biosciences), transfer samples to standard 96-well U-bottomed plate before acquisition.

3.5 Instrument Setup and Compensation (See Note 3)

1. Confirm that the instrument is working properly by performing daily quality control. For example, use FACSDiva CS&T software and beads.
2. To reduce data file size and optimize instrument performance, delete any detector that is not being used in this panel. In addition, the threshold should also be increased to reduce the amount of debris included in the final data file.
3. Create compensation control samples in the experimental document.
4. In general, compensation values are related to PMT voltage settings. To keep compensation values low, PMT detector settings within a single laser should be kept within 150–200 V of each other. CS&T generated instrument settings are not optimal for flow cytometric analysis of more than 12 colors and should not be used. If there are no available application settings for the staining panel, set each detector at 500 V as a starting point and then adjust accordingly.
5. Stained compensation controls should be used to optimize instrument settings. Use an acquisition template that displays all the fluorescent channels (the template for *"Unstained Control"* in FACSDiva works well). Run each single

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2. Set up the aspirating manifold using a length adjuster so that it leaves a little space at the bottom of the well and does not disturb the pellet following centrifugation. This reduces cell loss when staining in 96-well plates. Using an aspiration manifold is preferable to decanting following centrifugation when staining in 96-well plates.
3. The instrument setup in this procedure is based on using a LSRII or FACSSymphony flow cytometer. Other manufacturers make instruments capable of analyzing multiparameter flow cytometry samples. For those instruments, follow the normal quality control procedures. However, the PMT voltage set up should be universal and compensation can be effectively calculated using analysis software such as FlowJo.

color compensation control, making sure that the signal for each fluorochrome is highest in its detector with lower signals in other detectors. If there is an overlapping signal in a different detector, then adjust PMT voltage settings until the conflict is resolved. For example, PerCP-Cy5.5 should have its highest signal in that detector and lower signals in the PE-Cy5, Qdot 700 and AlexaFluor 700 detectors (*see* Fig. 3).

6. Acquire the compensation controls and then calculate compensation using the acquisition software. The software will indicate if there are compensation problems that can be addressed before sample acquisition. Apply compensation to the samples or acquire the samples uncompensated for later compensation using analysis software.

7. Create and label sample files in the experimental document.

8. Acquire samples and collect large numbers of events. Optimally a stopping gate should be placed on the population of interest so that the data file will contain enough events of that population to give an accurate analysis.

3.6 Sample Staining Panels

Sample staining panels for 14 colors on a LSRII and 23 colors on a FACSymphony are shown in Fig. 4. The 14-color panel has been used to phenotype blood and tissues from humanized BLT mice infected with HTLV-1 on a LSRII flow cytometer. The 23-color panel is suitable for a deep profile of T cells and NK cells, including memory, activation, T_{reg} cells, T_H1 cells, T_H2 cells, T_{FH} cells, T_{H17} cells, CTL responses, and NK responses. It includes intracellular markers and cytokines. The same panel setup and instrument setup discussed in this chapter are appropriate for intracellular staining, but the staining method described in the “Multiparameter Intracellular Cytokine Staining” chapter in this edition should be used for intracellular staining.

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LSR II Configuration

Laser	Dichroic	BP Filter	Fluorochromes
488 nm	505LP	525/50	FITC, Alexa 488, BB515, CFSE, GFP
488 nm	690LP	710/50	PerCP, PerCP-Cy5.5, PerCP-eFluor710, BB700
532 nm		575/26	PE
532 nm	600LP	610/20	PE-TxRed, ECD, PE-CF594, PE-eFluor610, PI
532 nm	635LP	660/20	PE-Cy5
532 nm	685LP	710/50	PE-Cy5.5 (not with PerCP-Cy5.5)
532 nm	755LP	780/60	PE-Cy7
628 nm		670/30	APC, Alexa 647, eFluor660
628 nm	685LP	730/45	Alexa 700, APC-R700
628 nm	755LP	780/60	APC-Cy7, APC-H7, APC-eFluor780, Alexa 750
405 nm		450/50	Pacific Blue, v450, BV421, eFluor450, Live/Dead Violet Dye
405 nm	505LP	525/50	AmCyan, v500, Live/Dead Aqua Dye, BV510, BV480
405 nm	557LP	560/20	Qdot 565, Live/Dead Yellow, BV570
405 nm	570LP	585/42	Qdot 585
405 nm	600LP	610/20	Qdot 605, BV605, eVolve605
405 nm	635LP	670/30	Qdot 655, BV650, eVolve655
405 nm	690LP	710/50	Qdot 705, BV711
405 nm	750LP	780/60	Qdot 800, BV786

FACSymphony Configuration

Laser	Dichroic	BP Filter	Fluorochromes
488 nm	505LP	515/20	FITC, Alexa 488, BB515, CFSE, GFP
488 nm	595LP	610/20	BB630
488 nm	635LP	660/40	PerCP, BB660
488 nm	690LP	695/40	PerCP, PerCP-Cy5.5, PerCP-eFluor710, BB700
488 nm	750LP	780/60	BB790
532 nm		586/15	PE
532 nm	595LP	610/20	PE-TxRed, ECD, PE-CF594, PE-eFluor610, PI
532 nm	635LP	670/30	PE-Cy5
532 nm	690LP	710/50	PE-Cy5.5 (not with PerCP-Cy5.5)
532 nm	750LP	780/60	PE-Cy7
532 nm	770LP	820/60	BYG790
628 nm		670/30	APC, Alexa 647, eFluor660
628 nm	690LP	730/45	Alexa 700, APC-R700
628 nm	750LP	780/60	APC-Cy7, APC-H7, APC-eFluor780, Alexa 750
405 nm		450/50	Pacific Blue, v450, BV421, eFluor450, Live/Dead Violet Dye
405 nm	505LP	515/20	AmCyan, v500, Live/Dead Aqua Dye, BV510, BV480
405 nm	595LP	610/20	Qdot 605, BV605, eVolve605
405 nm	635LP	670/30	Qdot 655, BV650, eVolve655
405 nm	690LP	710/50	Qdot 705, BV711
405 nm	730LP	750/30	BV750
405 nm	750LP	780/60	Qdot 800, BV786
355 nm		379/28	BUV395
355 nm	410LP	450/50	DAPI, Live/Dead Blue Dye
355 nm	450LP	515/30	BUV496
355 nm	525LP	540/30	BUV563
355 nm	635LP	670/30	BUV661
355 nm	690LP	740/35	BUV737
355 nm	770LP	820/60	BUV805

Fig. 1.

Instrument configuration for LSRII (BD Biosciences) and FACSymphony (BD Biosciences) flow cytometers in the NCI Vaccine Branch Flow Cytometry Core Facility. The tables include dichroic and bandpass filters for each detector. Recommended dyes are also given for each detector from multiple manufacturers. When designing a staining panel, one fluorochrome should be chosen for each detector

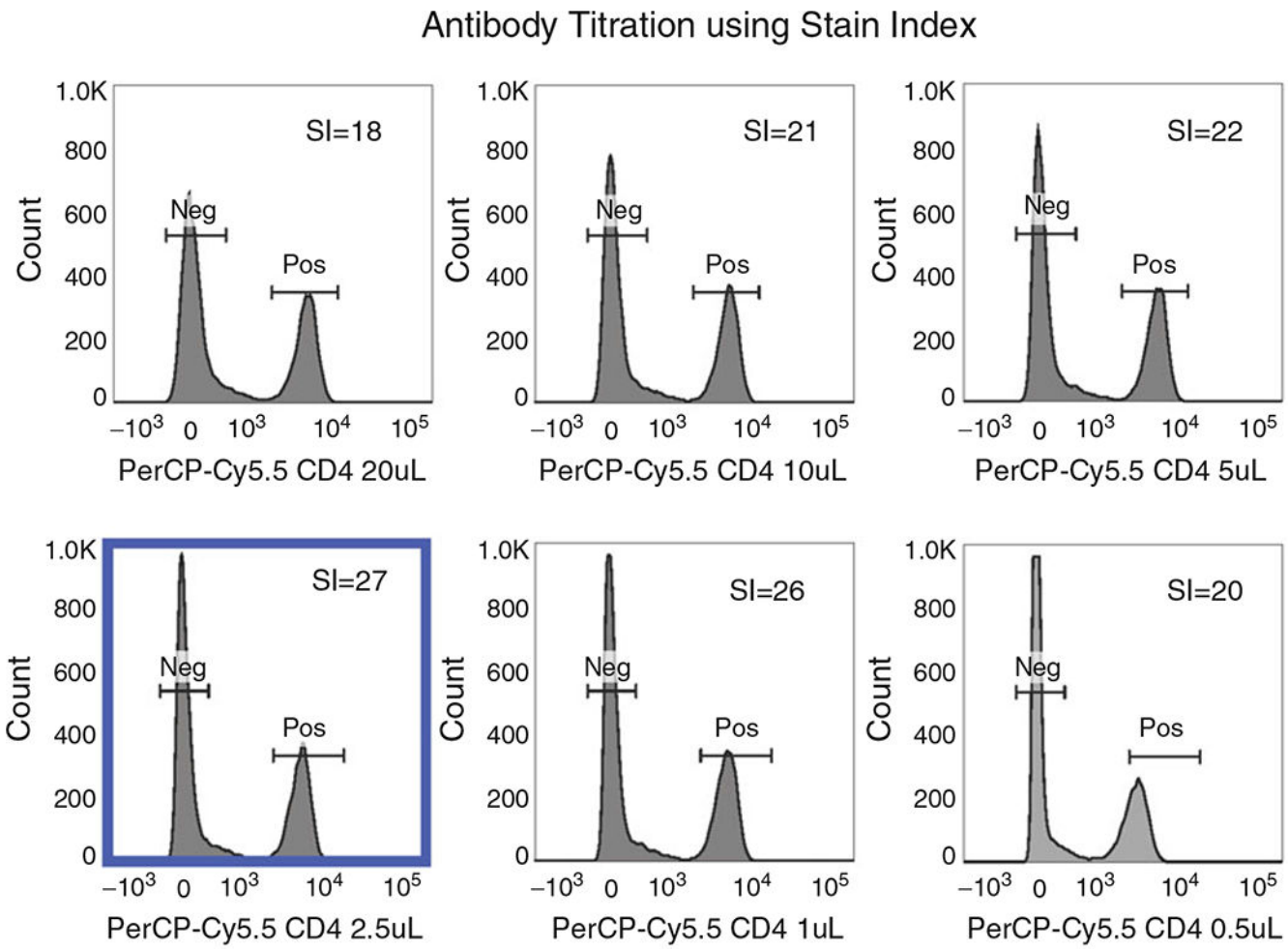

Fig. 2.

Example of antibody titration using Stain Index (SI). Non-human primate PBMC were stained with different volumes of CD4 PerCP-Cy5.5 (clone L200, BD Biosciences) and then acquired using a LSRII flow cytometer. Data was analyzed in FlowJo 10.2 software (FlowJo, LLC, Ashland, OR) and the stain index was calculated using the formula SI = D/W

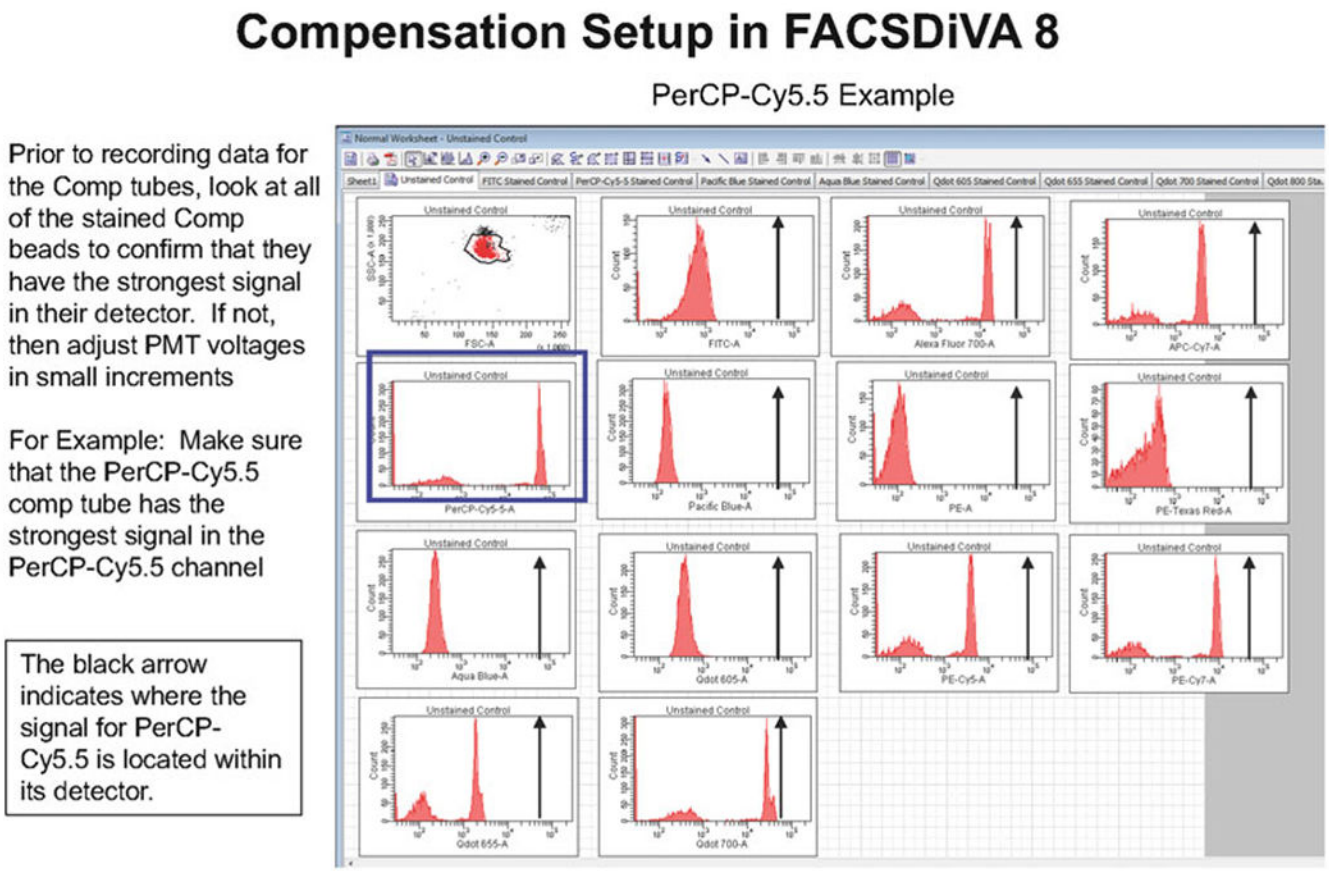

Fig. 3.

Example of compensation setup using FACSDiva 8 software. COMPTrol beads were stained with PerCP-Cy5.5 and then examined on a LSRII flow cytometer for spill-over into other fluorescent channels. Since the fluorescence signal was strongest in the PerCP-Cy5.5 channel, the detector settings were considered correct

14-color Staining Panel - LSRII

Laser	Dichroic	BP Filter	Antibodies and Fluorochromes
488 nm	505LP	525/50	CD14 FITC
488 nm	690LP	710/50	CD4 PerCP-Cy5.5
532 nm		575/26	CD25 PE
532 nm	600LP	610/20	PD-1 PE-CF594
532 nm	635LP	660/20	CD7 PE-Cy5
532 nm	685LP	710/50	
532 nm	755LP	780/60	CD45RA PE-Cy7
628 nm		670/30	CD45RO APC
628 nm	685LP	730/45	CD3 Alexa 700
628 nm	755LP	780/60	CD45 APC-Cy7
405 nm		450/50	CD16 BV421
405 nm	505LP	525/50	Live/Dead Aqua Dye
405 nm	557LP	560/20	
405 nm	570LP	585/42	
405 nm	600LP	610/20	CD19 BV605
405 nm	635LP	670/30	CD8 BV650
405 nm	690LP	710/50	
405 nm	750LP	780/60	Anti Mouse CD45 BV786

23-color Staining Panel - FACSymphony

Laser	Dichroic	BP Filter	Antibody and Fluorochrome
488 nm	505LP	515/20	CXCR5 FITC
488 nm	595LP	610/20	
488 nm	635LP	660/40	
488 nm	690LP	695/40	TGF-B PerCP-Cy5.5
488 nm	750LP	780/60	
532 nm		586/15	Granzyme B PE
532 nm	595LP	610/20	FoxP3 PE-CF594
532 nm	635LP	670/30	CD45RA PE-Cy5
532 nm	690LP	710/50	
532 nm	750LP	780/60	TNF-a PE-Cy7
532 nm	770LP	820/60	
628 nm		670/30	Perforin Alexa 647
628 nm	690LP	730/45	IL-17 APC-R700
628 nm	750LP	780/60	CD4 APC-H7
405 nm		450/50	IL-6 BV421
405 nm	505LP	515/20	CD45RO BV480
405 nm	595LP	610/20	IL-2 BV605
405 nm	635LP	670/30	IL-10 BV650
405 nm	690LP	710/50	IL-4 BV711
405 nm	730LP	750/30	IFN-g BV750
405 nm	750LP	780/60	CD127 BV786
355 nm		379/28	CD8 BUV395
355 nm	410LP	450/50	Live/Dead Blue Dye
355 nm	450LP	515/30	CD16 BUV496
355 nm	525LP	540/30	CD25 BUV563
355 nm	635LP	670/30	CD3 BUV661
355 nm	690LP	740/35	CD56 BUV737
355 nm	770LP	820/60	CD45 BUV805

Fig. 4.
Sample staining panels for LSRII and FACSymphony

Table 1

Relative fluorochrome brightness

Laser	Dim	Moderate	Bright
355 nm	BUV805 BUV496	BUV395 BUV563	BUV661 BUV737 BV421
405 nm	Pacific Blue V450 V500 eFluor 450 Pacific Orange AmCyan	BV480 BV510 Qdot 705	BV605 BV650 BV711 BV786 Qdot 605 Qdot 655 Qdot 800 eVolve 605 eVolve 655
488 nm	PerCP	FITC Ax488 PerCP-Cy5.5 PerCP-eFluor 710	BB515 BB700 PE PE-CF594 PE-TxRed ECD PE-Cy5 PE-Cy5.5 PE-Cy7
532 nm			PE PE-CF594 PE-TxRed ECD PE-Cy5 PE-Cy5.5 PE-Cy7
640 nm	APC-Cy7 APC-H7	Ax700 APC-eFluor 780 APC-Fire 750	APC Ax647 APC-R700

Table 2

Example of FMO Controls for a 12-color Experiment

Fluorochrome	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
FITC	**FMO FITC**	CD45	CD45	CD45	CD45	CD45	CD45	CD45	CD45	CD45	CD45	CD45
PerCP-Cy5.5	CD4	**FMO PerCPCy5.5**	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4
Pac blue	CD8	CD8	**FMO PacBlue**	CD8	CD8	CD8	CD8	CD8	CD8	CD8	CD8	CD8
Aqua live/dead	Aqua	Aqua	Aqua	**FMO Aqua**	Aqua	Aqua	Aqua	Aqua	Aqua	Aqua	Aqua	Aqua
BV 605	CD69	CD69	CD69	CD69	**FMO BV605**	CD69	CD69	CD69	CD69	CD69	CD69	CD69
BV650	CD19	CD19	CD19	CD19	CD19	**FMO BV650**	CD19	CD19	CD19	CD19	CD19	CD19
APC	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g	**FMO APC**	IFN-g	IFN-g	IFN-g	IFN-g	IFN-g
Alexa 700	CD3	CD3	CD3	CD3	CD3	CD3	CD3	**FMO Ax700**	CD	CD3	CD3	CD3
APC-Cy7	CD27	CD27	CD27	CD27	CD27	CD27	CD27	CD27	**FMO APCCy7**	CD27	CD27	CD27
PE	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	IL-2	**FMO PE**	IL-2	IL-2
PE-Cy5	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	TNF-a	**FMO PECy5**	TNF-a
PE-Cy7	CD28	CD28	CD28	CD28	CD28	CD28	CD28	CD28	CD28	CD28	CD28	**FMO PECy7**

The FMO control for each fluorochrome is indicated in **Bold**. For those tubes, the samples will be stained with every fluorochrome except the fluorochrome indicated in **Bold**