Basic Multicolor Flow Cytometry

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Multicolor flow cytometry is a rapidly evolving technology that uses multiple fluorescent markers to identify and characterize cellular subpopulations of interest, allowing rapid analysis on tens of thousands of cells per second, with the possibility of isolating pure, viable populations by cell sorting for further experimentation. This unit covers the tools needed by the beginning immunologist to plan and run multicolor experiments, with information on fluorochromes and their characteristics, spectral spillover, compensation and spread, instrument and reagent variables, and the basic elements of multicolor panel design. Protocols to quantify and maximize sensitivity by titration of reagents and optimization of instrument settings, as well as basic surface and intracellular cell staining, are included.

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INTRODUCTION

Flow cytometry is a rapidly evolving field that allows the measurement of multiple parameters using fluorescent markers, cell by cell, on thousands to millions of cells, as well as the ability to sort pure subpopulations of these cells for further experimentation.

This unit is intended to introduce the basic theory and protocols needed for the immunologist new to flow cytometry to plan and run simple to complex multicolor experiments.

Variables in cell and reagent preparation, fluorochrome choice, cytometer function, data acquisition parameters, and data analysis will all affect the experimental outcome in terms of accurate identification of desired populations of cells and quantification of their characteristics.

A basic understanding of fluorochromes and their characteristics, spectral overlap and spillover, compensation and spread, as well as cytometer variables that can affect the sensitivity of the measurements is necessary. Protocols and exercises are included to help the beginner perform and understand crucial elements of good multicolor practice. These tools include how to calculate stain index, how to determine correct detector gain settings, how to perform antibody titration, and how to determine compensation and spread, along with basic generic staining protocols. Rules and recommendations for the choice of fluorochrome combinations for multicolor panel design will be discussed in detail, as well as the pros and cons of different controls.

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Before beginning, it is recommended that those completely new to flow cytometry read the basic flow cytometry units to understand what goes on inside the black box and how that can affect your data. The unit in Chapter 5 on advanced multicolor experiments is targeted toward those using more than 10 colors, and will provide the next steps for the optimum utilization of high-end multiparameter flow cytometry.

BASIC PROTOCOL 1

STAIN INDEX

The stain index calculation (Bigos, 2007) is used to quantify the effective brightness of a fluorochrome, and is affected by intrinsic fluorochrome brightness, antigen density, antibody affinity, and cytometer characteristics and settings. The stain index calculates the separation of medians of the positive (signal) and negative (noise or background) populations, normalized to the width, or robust Standard Deviation (rSD), of the negative population.

The stain index can be used to determine the optimal detector gain setting, usually photomultiplier tube (PMT) voltage, for each detector (Basic Protocol 2) or to determine optimal antibody concentration by titration (Basic Protocol 3).

A common way to characterize the sensitivity of a cytometer for effective brightness of each fluorochrome is to compare the stain index for each parameter at its best gain setting, using reagents in which the fluorochromes are all coupled to the same antibody clone. Spillover into other channels is easily visualized, and this data also allows the calculation of the spillover spread matrix for each cytometer, either manually (Nguyen et al., 2013) or with Flowjo v9 SSM in the compensation wizard. Spread index calculation on your instrument provides valuable information for choosing fluorochromes in panel design (see Commentary).

Materials

Compensation beads appropriate to your antibody; some compensation beads are generic and will bind any species of antibody, others are species-specific and will bind mouse, rat, or hamster antibodies
Staining buffer: phosphate-buffered saline (PBS; Gibco, cat. no. 14190-094) without Ca or Mg, containing 1% bovine serum albumin (BSA; Sigma-Aldrich)
Fluorochrome-labeled antibody

12 × 15 mm round-bottom tubes
Centrifuge
Flow cytometer (see Chapter 5 in this manual)

Additional reagents and equipment for flow cytometry (see Chapter 5 in this manual)

Bead or cell staining

1. Add 1 drop of positive and 1 drop of negative compensation beads to a 12 × 15–mm round-bottom tube.

   Cells can also be used (1–5 × 10^6 cells/ml), but must contain a mix of cells that are positive for the epitope and cells negative for the epitope. If using cells, add 100 µl of cells.

2. Add 100 µl of staining buffer.
3. Add antibody to the appropriate concentration as determined by titration (Basic Protocol 3) or as recommended by manufacturer.

4. Incubate 30 min at room temperature in the dark, or under the recommended conditions for your antibody.
5. Wash by adding 2 ml staining buffer, centrifuging 5 min at 300 × *g*, 4°C, then removing the supernatant, vortexing the pellet, and resuspending in 200 µl staining buffer.

Data acquisition on cytometer

6. Ensure cytometer is functioning correctly by running daily quality-control procedure.
7. Acquire data on 5000 beads/cells at correct gain and threshold settings (see Basic Protocol 2).

Data analysis

8. Set a forward scatter/side scatter gate on the cells of interest.
9. Create fluorescence histograms gated on forward/side scatter.
10. Create gates on negative and positive populations.
11. Create statistics view with median fluorescent intensity and rSD (robust standard deviation).
12. Calculate stain index according to the following formula:

$$\frac{\text{Median positive} - \text{median negative}}{2 \times \text{rSD negative}}$$

DETERMINATION OF BEST GAIN OR PMT VOLTAGE SETTINGS (VOLTRATION)

BASIC PROTOCOL 2

The detector gain, or photomultiplier tube (PMT) voltage setting for each parameter, will affect the sensitivity of that detector in terms both of separation of positive and negative populations and the width of the negative population. The optimal gain setting for each detector will bring the negative population out of the detector's electronic noise range so that the measurement variations in the negative population are not due to electronic background. Also of importance is that the gain setting ensure that the positive population is not off the top of the scale or above the linearity range of the detector.

There are several ways to determine the best gain settings (Maecker & Trotter, 2006; Perfetto, Ambrozak, Nguyen, Chattopadhyay, & Roederer, 2006). This protocol uses stain index calculation with compensation beads or cells stained with an antibody/fluorochrome appropriate for each detector. The data is acquired over a range of gain or voltage settings for each detector, and the stain index is calculated as in Basic Protocol 1 for each setting. The optimal gain is the lowest setting that gives the maximum stain index.

Once determined, these gain settings are valid for all samples run on that cytometer. If, however, adjustments are made to the cytometer, for instance laser change or realignment or detector or filter replacement, the best-gain settings will need to be recalculated.

If samples are so bright that they are above or close to the top of the scale, there are several options. The gain setting can be reduced, a neutral density filter can be inserted in front of the detector, or the staining protocol can be revised to reduce brightness on the problematic parameter.

Materials are as described in Basic Protocol 1.

Bead or cell staining is performed as in Basic Protocol 1.

Data acquisition on the cytometer are performed as in Basic Protocol 1.

If doing this on all parameters, it saves time to create a generic template with a tube for each gain setting, with the same gain on all the parameters. For example, tube 'gain 400' would have a setting of 400 V on all parameters. This also gives a good idea of how much spillover you are seeing in the other detectors at equivalent gain settings.

Data analysis

Analyze and calculate stain index as in Basic Protocol 1. Make a plot of stain index versus gain or PMT voltage setting. The optimal gain is the lowest gain that gives a maximal stain index, where it reaches a plateau.

If the positive population goes off the top of the scale before a maximum stain index plateau, for example at a medium gain setting, then your sample is too bright to test the full range of gain settings. Restain the beads/cells either with less antibody or diminish staining by adding unlabeled antibody.

BASIC PROTOCOL 3

ANTIBODY TITRATION

Determination of the correct antibody concentration is necessary for each experimental system and each lot of antibody. Titration should be carried out before using the antibody in a multicolor experiment.

The optimal antibody concentration to maximize sensitivity, the separation of positive from negative cells, is particularly important in the case of dimly stained populations—too little antibody and the positive population will not be visible; too much antibody and the background will be high or spread out. The cell preparation conditions—whether cells are unfixed, fixed, or fixed and permeabilized—will affect the antibody binding and background; thus, titrations must be carried out under the same conditions. This protocol details the procedure for testing a series of antibody concentrations on a cell preparation of interest, and includes the laboratory protocol, instrument setup, and data acquisition and analysis steps. This is an 8-point doubling dilution series starting at 2× the manufacturer's recommendation; more points can be added or the dilution factors changed if needed.

Materials

Antibody of interest labeled with fluorochrome: at a concentration 4× the manufacturer's recommendation, can be higher or lower if desired
Staining buffer: phosphate-buffered saline (PBS; Gibco, cat. no. 14190-094) without Ca or Mg, containing 1% bovine serum albumin (BSA; Sigma Aldrich)
Sample: cell suspension (1–5 × 10^6 cells/ml); the cells should be prepared, unfixed or fixed, in the same way as they will for the multicolor experiment
12 × 15–mm round-bottom tubes or 96-well u-bottom plates

Centrifuge
Flow cytometer (see Chapter 5 in this manual)

Additional reagents and equipment for data acqusition by flow cytometry and data analysis (Basic Protocol 1)

Prepare antibody serial dilutions

1. See Table 5.4.1 for antibody-dilution schema.
2. Label nine tubes or wells as 1 through 9.
3. Add 50 µl of staining buffer to each tube or well.

Table 5.4.1 Serial Dilution: Schema for Preparation of Serial Dilutions for Titration Experiment

Tube	Stain buffer	Transfer from previous tube	Temporary volume	Transfer to next tube	Final Ab volume	Antibody concentration	Stain Index
1	50 µl		100 µl	50 µl	50 µl		
2	50 µl	50 µl	100 µl	50 µl	50 µl		
3	50 µl	50 µl	100 µl	50 µl	50 µl		
4	50 µl	50 µl	100 µl	50 µl	50 µl		
5	50 µl	50 µl	100 µl	50 µl	50 µl		
6	50 µl	50 µl	100 µl	50 µl	50 µl		
7	50 µl	50 µl	100 µl	discard 50 µl	50 µl		
8	50 µl	0 µl (no antibody)	50 µl	NA	50 µl		

4. Add 50 µl of antibody at 4× the manufacturer's recommendation to the first tube or well.

   The final concentration of the first tube/well will thus be at 2× the manufacturer's recommendation.

5. Mix well and transfer 50 µl to the next tube/well.
6. Repeat step 5 until tube 7.
7. Discard the excess 50 µl from tube 7.

   All tubes should now have 50 µl of antibody, except tube 8, which is a no-antibody control with staining buffer only.

Add cells

The cell preparation must contain a mix of cells that are positive for the epitope and cells negative for the epitope. An Fc block step will also have been performed if necessary, or the appropriate serum will have been included in the staining buffer (see Commentary).

8. Add 100 µl of the 1–5 × 10^6 cell/ml suspension to all tubes and mix well.
9. Incubate 30 min at room temperature in the dark, or the recommended conditions for your antibody.
10. Wash by adding 2 ml (if using tubes) or 150 µl (if using microtiter wells) of staining buffer, centrifuging 5 min at 300 × *g*, 4°C, then removing the supernatant, vortexing the pellet, and resuspending in 200 µl staining buffer.

Data acquisition

11. Acquire data by flow cytometry as in Basic Protocol 1.

Data analysis

12. Analyze data and calculate stain index as in Basic Protocol 1.
13. Plot stain index against antibody dilution/concentration.
14. Choose the lowest antibody concentration that gives the highest stain index.

COMPENSATION

While compensation is best calculated using the automated cytometer acquisition or third-party software compensation algorithms, those still using older analog cytometers, such as a FACSCalibur, may find themselves obliged to calculate the compensation using a manual procedure. It is possible, however, on those cytometers to record the single-color controls and multicolor samples uncompensated, or undercompensated, and perform or perfect the compensation on the recorded files using third-party software.

A simple procedure is given below for 2-color compensation, using a FITC and PE example. Remember that good-quality single-color controls are key to correct compensation. Compensation should always be set on the single colors by matching the medians of the positive and negative populations using statistics, and never visually. Compensation is dependent on detector gain or voltage setting. The gains must be exactly the same in all single colors and the multicolor samples. If they are not, you have to start over.

If you must do more than two colors manually—an unfortunate situation—you will have to compensate all colors against all other colors. It is preferable, if possible, to use third-party software on the uncompensated data: the automated algorithms use matrix algebra for the calculations, compensating all colors simultaneously, more accurately than the sequential procedure done manually.

For materials, see Basic Protocol 1. Perform bead or cell staining as described in Basic Protocol 1.

Data acquisition on cytometer

1. Ensure cytometer is functioning correctly by running the daily quality-control procedure.
2. Set appropriate gain (see Basic Protocol 2) and threshold.
3. Set a forward scatter/side scatter gate on the cells of interest.
4. Create a dot plot of fluorochrome 1 (FITC) versus fluorochrome 2 (PE) gated on forward/side scatter.
5. Create gates on negative and positive populations.
6. Create a statistics view to show median fluorescent intensity for the positive and negative populations in both colors.
7. Open the compensation matrix.
8. Start acquiring the first single-color control, in this case FITC, increasing the PE-FITC (PE minus % FITC) compensation value until the median of the positive population is the same as the median of the negative in the PE channel statistics.

   Here we are correcting for the spillover of FITC into PE. There is no PE in this tube; therefore, in the PE channel we should see no positive signal when it is correctly compensated.

9. Record 5000 events on this FITC single-color control.
10. Now we will compensate in the other direction, using the PE single color. While acquiring the PE single color, increase the FITC-PE (FITC minus % PE) compensation value until the the median of the positive population is the same as the median of the negative in the FITC channel statistics. Here we are correcting for the spillover of PE into FITC. There is no FITC in this tube; therefore, in the FITC channel, we should see no positive signal when correctly compensated.

11. Record 5000 events on this PE single-color control.
12. Save this compensation matrix and apply this (and the same detector gains or PMT voltages!) when you record your multicolor samples.

SURFACE STAINING

BASIC PROTOCOL 5

This basic surface staining protocol for unfixed cells gives a simple sequence of steps for cell preparation, data acquisition, and analysis. A dead cell exclusion marker and doublet discrimination are used to exclude false positives. The protocol assumes that you have titrated your antibodies and know the best gain settings for your instrument.

Preparation of an FMO (fluorescence minus one) control is included for reference—you may not need one, or you may need several. The FMO control is needed in situations where determination of the cutoff between positive and negative is difficult; thus, the FMO will contain all the antibodies except for (minus) the difficult marker (see Commentary).

Be sure to use the same antibodies in the single-color controls as you use in your mix.

This is a generic protocol that will need to be adapted to your cells, antibodies, and cytometer.

Materials

Staining buffer: phosphate-buffered saline (PBS; Gibco, cat. no. 14190-094) without Ca or Mg, containing 1% bovine serum albumin (BSA; Sigma-Aldrich)
Sample: single-cell suspension (1–5 $\times$ 10^6 cells/ml in staining buffer; use more cells if looking for rare populations)
Fluorochrome-labeled antibodies, titrated as described in Basic Protocol 3
Compensation beads appropriate to your antibody; some compensation beads are generic and will bind any species of antibody, others are species-specific and will bind mouse, rat, or hamster antibodies
Live/dead cell marker (PI, DAPI, 7-AAD) appropriate to your fluorochrome panel, usually ~2 to 10 μg/ml final concentration

12 $\times$ 15–mm round bottom tubes
Centrifuge
Flow cytometer (also see Chapter 5 in this manual)

Experimental setup

1. Prepare tubes as in the example of the experiment schema in Table 5.4.2:

   Unstained cells
   Single color compensation controls (one for each color)
   FMO (fully stained minus one fluorochrome)
   Fully stained cells.

   Be sure to use the same antibodies in the single-color controls as you do in your mix.

Cell staining

Single-cell preparations will have undergone red blood cell lysis procedure or Ficoll enrichment if necessary. An Fc block step will also have been performed if necessary, or the appropriate serum will have been included in the staining buffer (see Commentary).

Table 5.4.2 Experiment Layout: Schema for Experiment Layout for 4 Antibodies, Unstained Control, Compensation Controls, FMO Example, and Fully Stained Cells

Column 1	Column 2	Add	Antibody 1	Antibody 2	Antibody 3	Antibody 4
Unstained		Cells				
Compensation controls						
	Single color 1	Cells or beads	+			
	Single color 2	Cells or beads		+		
	Single color 3	Cells or beads			+	
	Single color 4	Cells or beads				+
FMO Ab 1	Example for Ab 1	Cells	No antibody 1	+	+	+
Fully stained sample		Cells	+	+	+	+

2. Add 100 µl staining buffer to tubes.
3. Add antibodies to tubes at appropriate final concentrations as determined by titration (Basic Protocol 3).
4. Add cells or beads to appropriate tubes.

   For compensation controls, add 1 drop of positive and 1 drop of negative compensation beads to the tube. Cells can also be used (1–5 × 10^6 cells/ml) but must contain a mix of cells that are positive for the epitope and cells negative for the epitope. If using cells, add 100 µl of cells

5. Incubate 30 min at room temperature in the dark, or under the recommended conditions for your antibody.
6. Perform the first wash by adding 2 ml staining buffer, centrifuging 5 min at 300 × *g*, 4°C, then removing the supernatant and vortexing the pellet. Resuspend the pellet in 2 ml staining buffer.
7. Perform a second wash by repeating the centrifugation in step 6. Resuspend in 200 µl staining buffer with live/dead cell marker at appropriate concentration.

Data acquisition on cytometer

8. Ensure cytometer is functioning correctly by running daily quality-control procedure.
9. Set up correct gain or voltage settings for each parameter (see Basic Protocol 2).
10. Set threshold on forward scatter height parameter. If unsure about forward scatter gain setting, backgate on positive cells of interest, for example CD3, to make sure they are not on the edge of the threshold. Increase forward scatter until these cells are well above threshold.
11. Make sure forward and side scatter height and width parameters for doublet exclusion are checked for acquisition.
12. Set up acquisition histogram and gating schema, including dead cell exclusion and doublet exclusion gates.
13. Spot check bright, fully stained sample to make sure no events are off the top end of the scale. Reduce gain if necessary.

14. Run single-color controls. Acquire data on 5000 beads/cells.

    No positives should be off the top end of the scale.

15. Calculate compensation now using the cytometer's automatic compensation software, or later using third-party software.

16. Run FMO and all multicolor samples. Acquire enough events to detect rare populations if necessary.

Data analysis

17. Create a dot plot of forward scatter vs time, and fluorescence versus time. Verify that there are no variations over time. If aberrations are seen, gate out the bad data (See Commentary).

18. Set a gate on FSC versus the live/dead marker to eliminate dead cells.

19. Set a Forward Scatter/Side Scatter gate on the cells of interest.

    Doublet exclusion gates can be set immediately after the FSC/SSC gate. It is sometimes more effective when working with mixed populations of cells to set several doublet exclusion gates farther down in the hierarchy, each on a different subpopulation of interest.

20. Remember to use bi-exponential scaling on all fluorescence dot plots.

21. Set positive/negative discrimination gates on difficult markers using the FMO control for that color, then apply to the multicolor samples.

SURFACE AND INTRACELLULAR STAINING

BASIC PROTOCOL 6

This basic surface and intracellular staining protocol for fixed and permeabilized cells includes steps for initial surface staining, then fixation, permeabilization, and intracellular staining. The dead cell exclusion marker is added before staining, but it must be fixable. There are a number of "live/dead fixable" stains available on the market, with different excitation and emission spectra. This protocol assumes that you have titrated your antibodies under the fix/perm conditions that you are using in the experiment and that you know the best gain settings for your instrument. Preparation of a single FMO (fluorescence minus one) control is included for reference—you may not need one, or you may need several.

This is a generic protocol that will need to be adapted to your cells, antibodies, and cytometer. In particular, you will need to ensure that your fix/perm procedure retains the surface antigen staining and fluorochromes intact, yet enables antibody access to the cytoplasmic and nuclear targets. This will require preliminary testing of the best fixation and permeabilization procedures, as well as optimization of buffers for difficult antigens. Also remember that your controls, including single-color compensation beads or cells, should be treated with the same fix and perm procedures as your samples.

Materials

Staining buffer (optimized for your system: see annotation to step 9):
phosphate-buffered saline (PBS; Gibco, cat. no. 14190-094) without Ca or Mg, containing 1% bovine serum albumin (BSA; Sigma-Aldrich)
Sample: single-cell suspension (1–5 $\times$ 10^6 cells/ml in staining buffer; use more cells if looking for rare populations)
Fluorochrome-labeled antibodies, titrated as described in Basic Protocol 3
Live/dead cell fixable marker appropriate to your fluorochrome panel

Compensation beads appropriate to your antibody; some compensation beads are generic and will bind any species of antibody, others are species-specific and will bind mouse, rat, or hamster antibodies
Fixation and permeabilization buffers optimized for your system

12 × 15 –mm round-bottom tubes
Centrifuge
Flow cytometer (also see Chapter 5 in this manual)

Cell staining

Single cell preparations of cells will have undergone red blood cell lysis procedure or Ficoll enrichment if necessary. An Fc block step will also have been performed if necessary, or the appropriate serum will have been included in the staining buffer (see Commentary).

1. Prepare tubes (see example in Table 5.4.2) for single-color compensation (one for each color), FMO, and fully stained cells. Be sure to use the same antibodies in the single-color controls as you use in your mix.
2. Add 100 µl staining buffer to tubes.
3. Add cells or beads to appropriate tubes.
4. For the single color compensation controls, add 1 drop of positive and 1 drop of negative compensation beads to the tube.

   Cells can also be used (1–5 × 10^6 cells/ml) but must contain a mix of cells that are positive for the epitope and cells negative for the epitope. If using cells, add 100 µl of cells

5. Where indicated, add the fixable live/dead cell marker to the cells, following the manufacturer's staining and washing instructions.
6. Add antibodies for surface markers to tubes at appropriate final concentrations as determined by titration (Basic Protocol 3) or as recommended by manufacturer.
7. Incubate all tubes 30 min at room temperature in the dark, or under the recommended conditions for your antibody.
8. Wash by adding 2 ml staining buffer, centrifuging 5 min at 300 × *g*, 4°C, then removing the supernatant and vortexing the pellet.
9. Fix and permeabilize the cells.

   You will need to determine the appropriate fixation and permeabilization procedure (see Chapter 5 for cell preparation) adapted to your antigens of interest. Paraformaldehyde fixation is often used, followed by permeabilization with something like saponin or Tween, or using commercial buffers specifically adapted to your application. You must be sure that your surface staining survives the procedure and that the intracellular antigens are preserved and accessible to the antibodies. The single colors, particularly for the surface antigens, should be treated with the same fixation and permeabilization conditions as the multistained cell sample.

10. If required after the perm step, repeat the wash described in step 8.
11. Add antibodies for cytoplasmic and nuclear markers at appropriate concentrations.
12. Incubate all tubes 30 min at room temperature in the dark, or according to the manufacturer's recommendation.
13. Repeat the wash described in step 8 twice, then resuspend in 200 µl staining buffer.

Flow cytometry and data analysis

14. Perform data acquisition on cytometer and data analysis as described in Basic Protocol 5.

COMMENTARY
Background Information

Light and fluorochromes

Flow cytometry makes use of light in the visible wavelengths, from 355 nm ultraviolet to 800 nm far-red light, passing through all the colors of the rainbow. The shorter violet/blue wavelengths are higher energy and the longer orange/red wavelengths are lower energy. Figure 5.4.1A shows the visible spectrum with the laser lines most often used on flow cytometers to excite the commonly used fluorochromes. Many of these laser lines, particularly the 405, 488, and 640 nm, are standard on the current cytometers.

Fluorochromes are molecules that can absorb light energy at specific wavelengths and then re-emit this energy as light at higher wavelengths. Fluorochrome structures generally include a series of aromatic rings; examples of two common fluorochromes are shown in Figure 5.4.1B. In the very simplified schema shown in Figure 5.4.1C, the fluorochrome, in this case FITC, has an electron in the ground energy state which absorbs the energy of blue 488-nm laser light. The electron jumps to a higher, but unstable, energy level. Within nanoseconds (the fluorescence lifetime), the unstable electron loses some of this energy as heat, then re-emits the rest as higher-wavelength, lower-energy fluorescent light as it returns to its ground state.

Excitation and emission spectra

Each fluorochrome has a maximum excitation wavelength at which it absorbs light most efficiently, but can be excited across a specific range of wavelengths with lower efficiency. This range of wavelengths is the fluorochrome's excitation spectrum. This information is needed to select which fluorochromes

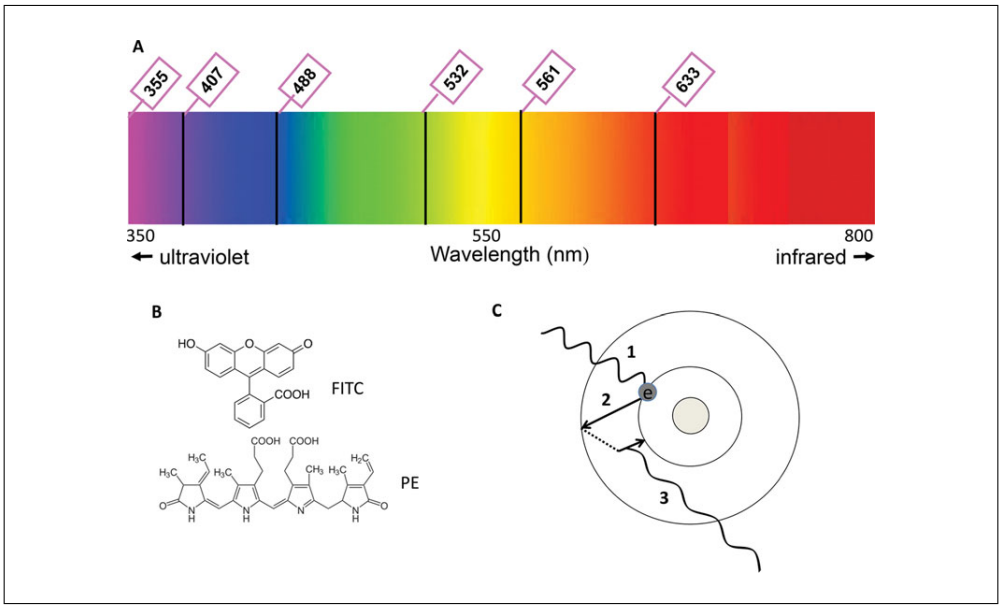

Figure 5.4.1 Light, fluorochromes and lasers. (**A**) Shows the spectrum of light wavelengths utilized in flow cytometry, as well as the lasers found on current cytometers. Most cytometers come equipped with a blue 488-nm, red 640-nm, and violet 405-nm laser, with options of yellow-green 532- or 561- and UV 355-nm lasers becoming commonly available. (**B**) Shows the structure of 2 widely used fluorochromes, FITC (fluorescein) and PE (phycoerythrin). (**C**) Shows a simple schema of the process of fluorescence. (1) An electron in resting state is excited by laser light, absorbing the energy of the laser. (2) The excited electron jumps to an unstable higher energy level, where it loses some of the energy as heat or non-radiatively, and (3) re-emits the rest of the energy as fluorescence at a higher wavelength with lower energy. This is very simplified: it is, in fact, the electron cloud of the fluorochrome molecule's conjugated double-bond system which becomes excited, expands, and then fluoresces.

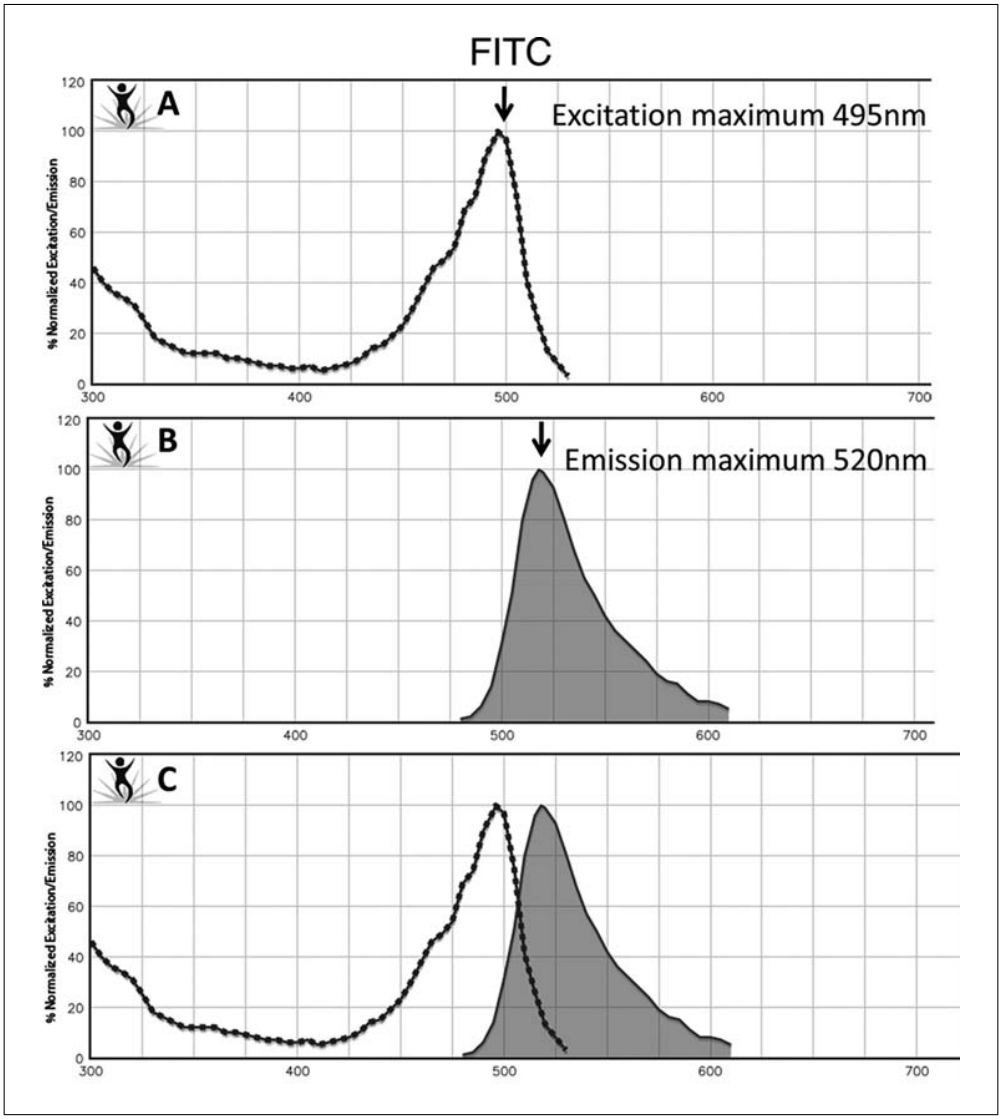

Figure 5.4.2 Fluorochrome excitation and emission spectra. (**A**) Each fluorochrome is capable of absorbing light energy over a specific range of wavelengths, but most efficiently at its excitation maximum. Here, FITC can absorb energy at all these wavelengths, but best at 495 nm. (**B**) Similarly, each fluorochrome is capable of emitting light energy over a specific range of wavelengths, but most efficiently at its emission maximum. FITC will fluoresce at all these wavelengths but highest at 520 nm. (**C**) Overlay of the excitation and emission spectra for FITC. Note the width of both these curves. All spectra prepared using the Biolegend spectral viewer; see Internet Resources.

can be excited by the lasers on the cytometers that are available to the user.

Likewise, each fluorochrome has a maximum emission wavelength, but will also emit across its entire emission spectrum. An example of the FITC excitation and emission spectra is shown in Figure 5.4.2. FITC is excited by the 488-nm laser, which is near its maximal excitation peak of 495. The emission spectrum is seen to be quite broad—FITC emits best at 520 but also emits out into the yellow to orange wavelengths, although at lower efficiency farther from the emission maximum. The emission spectra will determine which optical filter is best suited for optimal collection of the emitted light by the detector dedicated to that fluorochrome, as well as which fluorochromes can be used together.

Excitation and emission spectra of several other fluorochromes are shown in Figure 5.4.3, with maximum excitation by different lasers. PE can be excited by several of the lasers available on current cytometers at different efficiencies. APC is best excited by the 640-nm laser and to a small degree by the 561-nm laser; BV421 best excited by the 405-nm laser and to a small degree by the 355-nm laser. Note the width of the excitation and emission

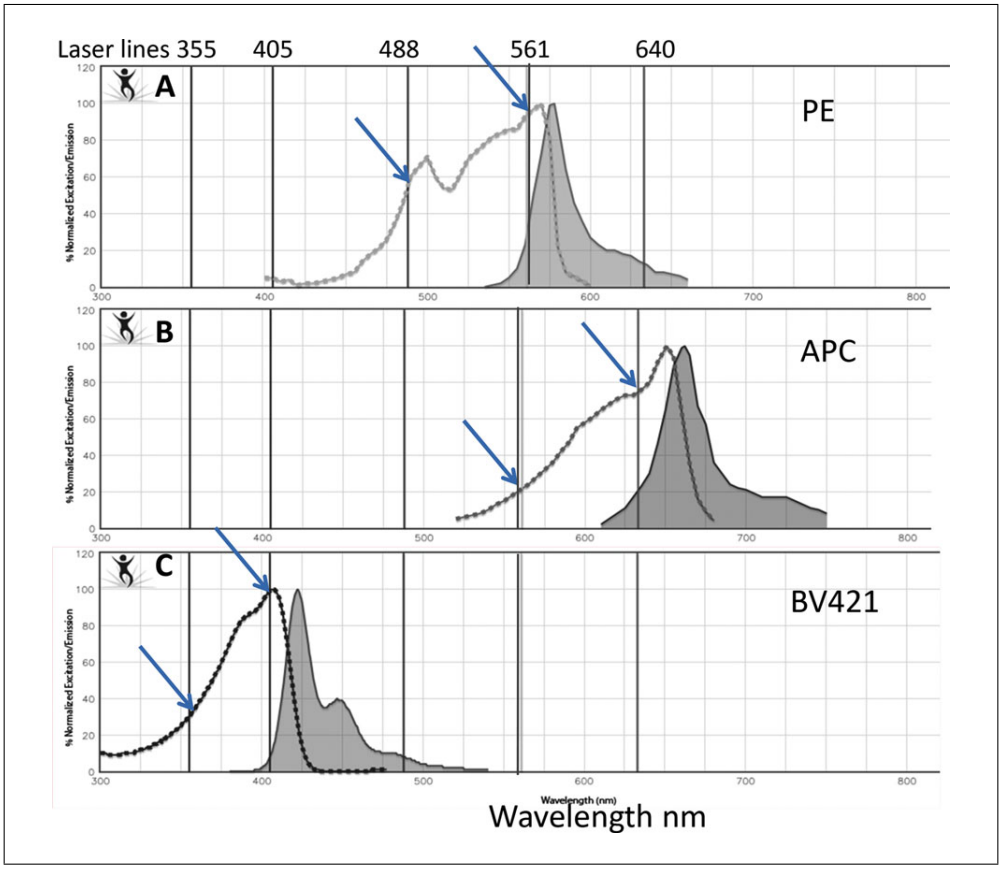

Figure 5.4.3 Fluorochromes excited by different lasers. Notice that: (**A**) PE has a very wide excitation spectrum, excited by the 488-nm laser, but more efficiently by the 561-nm. (**Panel B**) APC is excited best by the 640-nm laser, but also a little by the 561. (**C**) BV421 is excited best by the 405-nm laser but also a little by the 355-nm.

curves, which may result in excitation by more than one laser or fluorescence emission into multiple detectors.

A number of 'spectral viewer' Web sites (see Internet Resources) can be used to examine the excitation and emission spectra of each fluorochrome as well as the collection ranges of commonly used optical filters.

Tandem dyes and energy transfer

Fluorochromes are also available as tandem dyes that consist of two tightly coupled fluorochromes (Fig. 5.4.4). The laser energy absorbed by the first fluorochrome is transferred to the second fluorochrome, which then re-emits this as fluorescence. Donor fluorochrome #1 absorbs the laser light, but transfers most of the energy nonradiatively to the acceptor fluorochrome #2. Fluorochrome #2 then re-emits the transferred energy as fluorescence at its own, higher-wavelength emission spectrum. This is called energy transfer and requires the fluorochromes to be in very close proximity. The emission spectrum of fluorochrome 1 must overlap with the excitation spectrum of fluorochrome 2.

All tandems are not the same. The coupling of the dyes, and thus the efficiency of the energy transfer, varies greatly between manufacturers and between lot numbers of the same product. The dyes can uncouple if left out in the light. These factors will affect the compensation values needed to correct for leakage from the donor fluorochrome, discussed in detail below.

Fluorochrome brightness

Intrinsic fluorochrome intensity or brightness is an important characteristic of each fluorochrome and must be taken into consideration when choosing fluorochromes, even for a very basic panel. A fluorochrome's brightness is dependent on its extinction coefficient, which is its capacity to absorb light, and its quantum yield, which is the proportion of that absorbed light it can re-emit. Large variations in extinction coefficients are the major contributors to the differences seen in fluorochrome brightness. Table 5.4.3

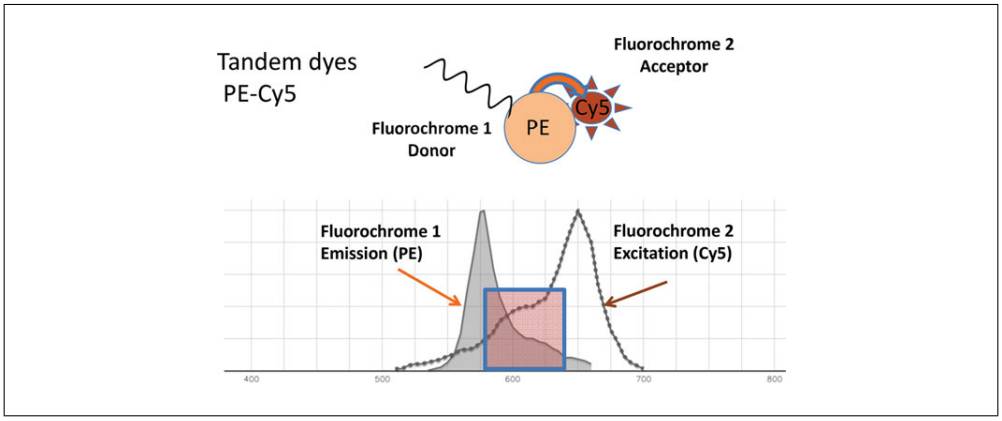

Figure 5.4.4 Tandem dyes. Tandem dyes consist of two fluorochromes tightly coupled. The donor fluorochrome 1 absorbs the laser light, and instead of emitting this as fluorescence, it transfers the energy to the acceptor fluorochrome 2, which then re-emits this energy as fluorescence at its own longer emission wavelength. For this energy transfer to occur, the fluorochrome 1 emission spectra must overlap with the fluorochrome 2 excitation spectra, and the fluorochromes must be in very close proximity.

Table 5.4.3 Fluorochrome Brightness[a]

Fluorochrome	Extinction coefficient	Quantum yield	Brightness × 10^5	Brightness relative to PE	Size (Da)
PE	1,960,000	0.84	16	100%	240,000
PeCy5	1,960,000	NA	NA	NA	241,500
APC	700,000	0.68	4.7	29%	105,000
FITC	75,000	0.5	0.4	2%	389
BV421	2,500,000	0.69	16	100%	264

[a]Fluorochrome brightness is intrinsic to the fluorochrome and is dependent on the extinction coefficient and the quantum yield (QY). Brightness = Extinction Coefficient × QY. The brightness values of commonly used fluorochromes are shown here and compared to PE, the gold standard for fluorochrome brightness.

lists the extinction coefficients and quantum yields of some commonly used fluorochromes compared to phycoerythrin (PE), which is often used as a gold standard for brightness. In addition to intrinsic fluorochrome brightness, staining intensity is affected by antigen density, antibody affinity, and cytometer characteristics, which will be discussed below.

Fluorochrome size and stability

Fluorochromes differ greatly in size: PE is 1000 times larger than FITC. Size can affect intracellular staining in that small fluorochromes can more easily penetrate the permeabilized cell membrane to reach the cytoplasmic and nuclear targets than large fluorochromes. There may be limitations getting reagents for intracellular antigens into cells, or steric hindrance of antibody-antigen binding. In practice, however, most large dyes, such as PE and its tandems, work well for the detection of intracellular antigens such as cytokines. Steric hindrance rarely occurs with the commonly used antibody-dye conjugation chemistries. The sizes of typical fluorochromes are included in Table 5.4.3.

Fluorochrome stability

The stability of certain fluorochromes can vary; exposure to light and fixatives may cause degradation, particularly with certain tandems (Hulspas, Dombkowski, et al., 2009). Care must be taken to ensure that both fluorochromes and antigens are intact after fixation or permeabilization and that buffers chosen are optimal for the staining requirements. Compensation controls, whether they be beads or cells, should be treated in the same manner as the cell samples so that they control for the effects of fixation on the fluorochrome spectral characteristics.

Resolution and sensitivity

The purpose of staining cells with fluorochrome-labeled antibodies is to distinguish the cells that are positive for a marker from those that are negative. This resolution

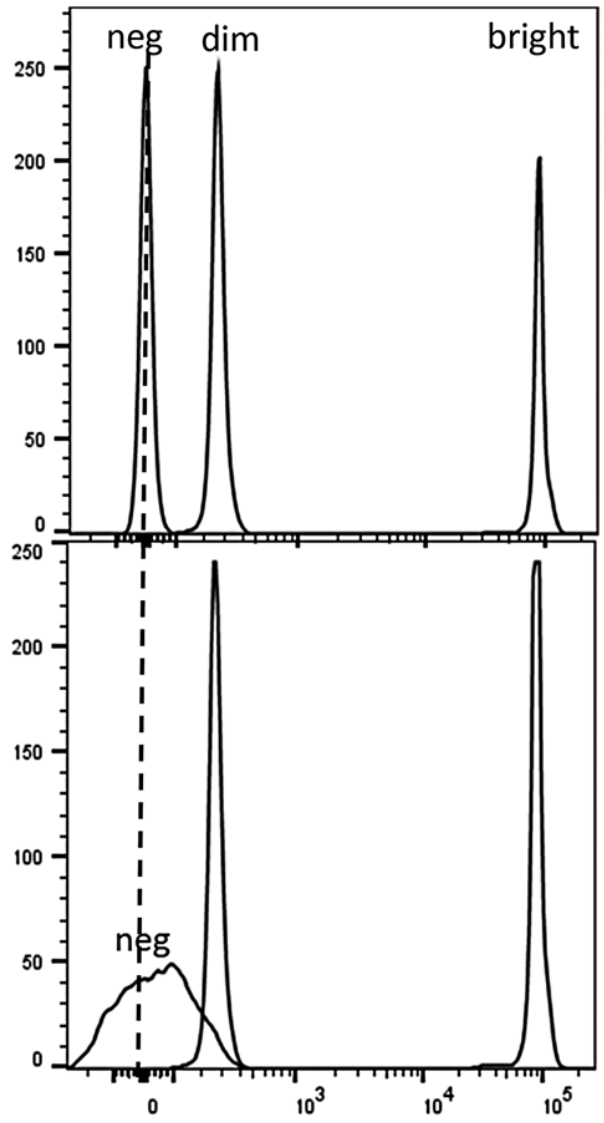

Figure 5.4.5 Resolution. The ability to distinguish positive cells from negative depends on the position and width of the positive and negative peaks. In the upper panel, both the bright and dim positive populations are easily distinguished from the negative peak. If, as in the lower panel, the position of background negative peak increases or widens to a point where it overlaps with the dim peak, discrimination of dim from negative becomes difficult. The width of the negative peak is dependent on electronic and optical properties of the cytometer, but also on reagent concentration, instrument settings and spillover from other fluorochromes.

depends on the intensity of the positively stained cells, but in great degree on the position and spread of the negative cell background peak. The schema in Figure 5.4.5 shows how increased background position or spread can diminish the separation of the peaks, problematic for dimly stained populations. The level and spread of the negative peak is due to a number of factors, primarily electronic and optical noise of the detectors and cellular autofluorescence, but can also be affected by antibody concentration and detector gain settings and, as we will see later, associated with spectral spillover from other fluorochromes and photon-counting errors.

Separation or stain index

The separation or stain index (SI) provides a quantitative measurement of how well two populations can be separated taking into account the intensity of positive staining as well as the level and spread of the background (Fig. 5.4.6; Bigos, 2007). Cells or beads stained with the fluorochrome-coupled antibody and containing both negative and

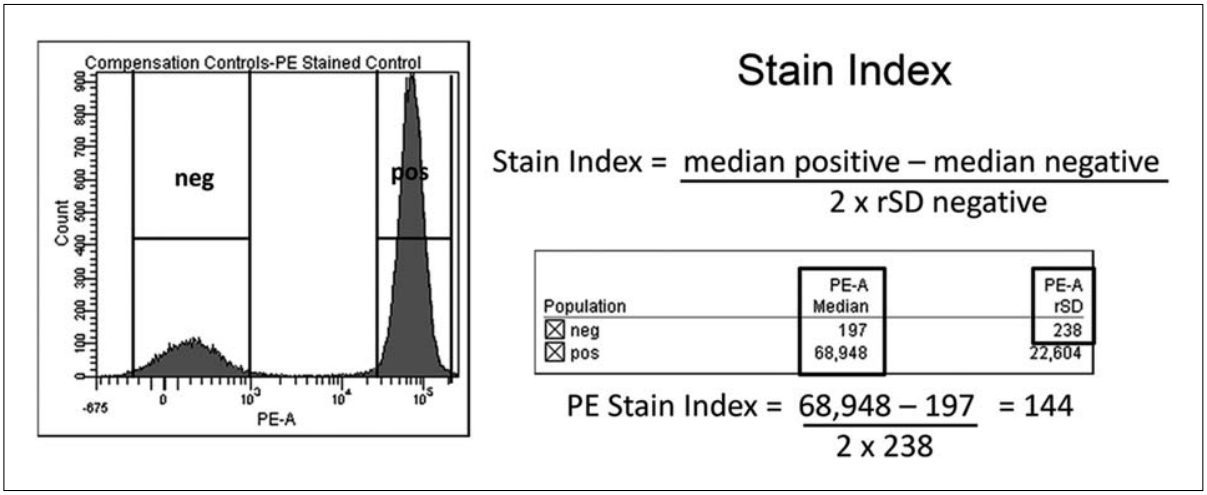

Figure 5.4.6 Stain index. Stain index measures the separation of positive and negative peaks on the cytometer, based on the distance between the medians of the positive and negative populations normalized to the robust standard deviation of the negative population. Adapted from Bigos et al. (2007).

Table 5.4.4 Fluorochrome Brightness vs. Stain Index[a]

			Stain index	
Fluorochrome	Brightness $\times 10^5$	Brightness relative to PE	Cytometer 1	Cytometer 2
PE	16	100%	**348**	262
PeCy5	NA	NA	180	131
APC	4.7	29%	238	**281**
FITC	0.4	2%	132	61
BV421	16	100%	264	145

[a]Stain index does not always correlate with intrinsic fluorochrome brightness, and all cytometers are not the same. Here, PE is brightest on Cytometer 1, compared to APC on Cytometer 2.

positive populations are analyzed on the cytometer, and the stain index is calculated using the following formula:

$$\text{Stain Index} = \frac{\text{median(positive peak)} - \text{median(negative peak)}}{2 \times \text{robust Standard Deviation(negative peak)}}$$

As can be seen in Table 5.4.4, the stain index depends not only on the intrinsic fluorochrome intensity but also on the cytometer characteristics. The brightness of the positive and the position and width of the background depend on the fluorochrome used but also on antigen density, antibody concentration, cytometer sensitivity and gain settings, reagent variables, and the biological characteristics of the markers studied. These factors will be discussed below.

Know thy cytometer: Cytometer variability

Cytometers vary greatly in their sensitivity and ability to separate populations. Laser power, wavelength and alignment, optical paths, and detector efficiency all combine to make each cytometer, even of the same brand and type, quite individual. Good daily cleaning and QC procedures as well as regular revisions by service engineers are needed to ensure that the cytometer is functioning optimally.

The comparison of stain index between two cytometers seen in Table 5.4.4 shows the effects of instrument variability on stain index and ranking of fluorochrome brightness.

Laser wavelength and power affect the efficiency of fluorochrome excitation, and thus the emission intensity. As seen earlier, PE is better excited by a 532- or 561-nm yellow-green laser than a 488-nm blue laser. The yellow-green lasers are much closer to PE's excitation maximum, resulting in substantally higher intensity. In many cases, higher laser power will also give better excitation and resolution. Optimal laser-beam alignment, usually adjusted by the service engineer, maximizes the amount of laser light focused on the cells as they pass through the flow cell.

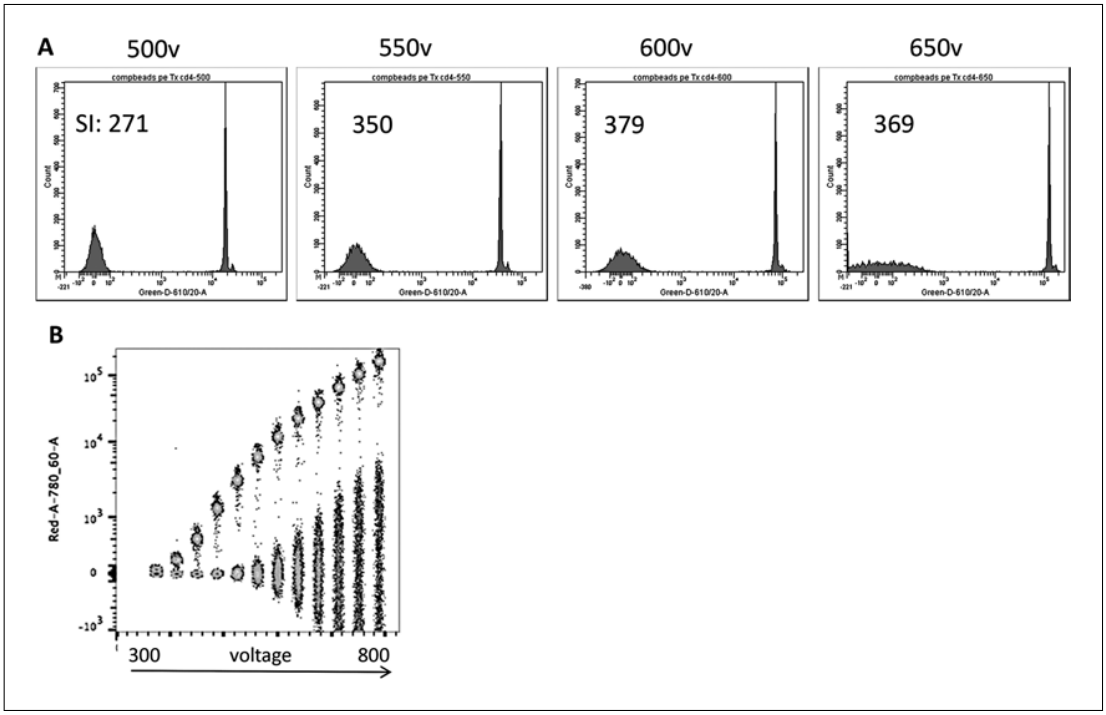

Figure 5.4.7 Optimizing detector gain settings by voltration. Fluorochrome-antibody stained compensation beads or cells are run over a range of detector gains (voltages) and the stain index (SI) at each gain calculated. The optimal gain is the lowest that gives the highest stain index. In panel **A** it can be seen that the stain index levels off at 600 V and this would be the gain of choice. In panel **B**, the effect of the background spread as the width of the negative peak increases with higher gain is evident.

Optical filters

Flow cytometers use a series of dichroic mirrors and filters to separate and direct the fluorescent light emitted by the cells so that the specific wavelengths corresponding to each individual fluorochrome reach the designated light detector. Each detector transforms the photons of this light into voltage pulses proportional to the amount of light it sees. The voltage pulses are then digitized to a channel number and all these values, cell by cell, are stored in a 'list mode' data file.

Of prime importance are the bandpass (BP) filters placed directly in front of the detector in order to let through only designated wavelengths, whose range is defined by the central wavelength of the bandpass plus a specific window on either side. Filter specifications are given using a standard nomenclature, first the central wavelength, followed by the width of the filter window. Thus, a 530/30 BP filter will let through 530 $\pm$ 15, that is, a wavelength range from 515 nm to 545 nm. The 530/30 filter corresponds well to the maximum emission spectra of FITC, GFP, or Alexa 488, which would be measured in that detector. The optical filters for each detector are targeted to the emission maximum of the fluorochrome to be measured in that channel. If need be, standard filters on the cytometers can usually be swapped out for a filter better adapted to your fluorochrome.

Another major source of cytometer variation is the light detectors (often photomultiplier tubes or PMTs). Despite the fact they are all sold and installed as the same reference and to the same specifications, detectors show substantial intrinsic differences in sensitivity (Q), background (B), and electronic noise (Hoffman & Wood, 2007; Steen, 1992; Wood, 1995, 1998), and these can change over time as the detector ages. Detectors also vary in their linearity ranges, with potential measurement and compensation errors if used outside that range. All these differences affect resolution of dim positive from negative populations, with the result that one cytometer may be more sensitive for a certain fluorochrome than its supposedly identical twin next door. Resolution of one fluorochrome may be better on cytometer A than on cytometer B, while the opposite is true of a different fluorochrome measured in another detector.

These variations reinforce the need to 'Know Thy Cytometer'. Comparative stain index data using the same antibody clone conjugated to different fluorochromes acquired on your own cytometers will give a good

idea of the strengths and weaknesses of each machine.

Detector gain settings

Optimized detector gains will improve stain index, and should be determined for each detector. There are several ways to do this (Maecker & Trotter, 2006; Perfetto et al., 2006). Figure 5.4.7 shows an example of optimizing gains using maximum stain index, but in all methods the gains must be set so that the negative cell peak is above the noise of the detector, and the positive peak is within the linearity range, and above all, not off the top of the scale.

Instrument quality control

Instrument quality control is absolutely essential to assure good-quality reproducible data. If the cytometer is not functioning properly, the data generated will be invalid and result in the irrecoverable loss of valuable time and samples. In busy labs with multiple users, even though the instruments are QC'd in the morning, clogs or bubbles occurring during the day can mean that the instrument may not be functioning properly when you arrive with your samples. Dirty fluidics or bubbles in the system will deflect the path of the cells passing through the laser beam, resulting in diminished excitation and reduced fluorescence. A simple QC protocol and template should be in place on every cytometer to quickly spot check instrument function before you start your experiment. For further information, there are references (Perfetto, Ambrozak, Nguyen, Chattopadhyay, & Roederer, 2012; Wood & Hoffman, 1998) that cover assessment of system and detector sensitivity (Q) and background (B), linearity, calibration, and cytometer optimization in depth.

Inter-instrument standardization, when setting up the same experiment across several instruments or sites, or in longitudinal studies, usually relies either on setting the best detector gains per instrument (Kalina et al., 2012) or else setting detector gains to put standard reference beads or the same compensation control beads in the same target channels for all parameters on all instruments (Maecker, McCoy, & Nussenblatt, 2012). In the second case, while the instruments are better 'standardized', gain settings may not necessarily be optimal for each instrument in terms of resolution of dim populations, so that intrinsic instrument differences can affect the outcome of the experiment. One multicenter study (Jaimes et al., 2011) includes tracking these instrument differences with an eye to detecting and resolving individual sensitivity problems.

Antibody concentration: Titrate!

Each lot of fluorochrome-coupled antibody must be titrated to determine the concentration that will maximize separation of positive and negative cells, using the same cells and same fixation and staining conditions as the experimental samples (Hulspas, 2010; Stewart & Stewart, 2001). Optimal antibody concentration can be quite different from one cell type to another. Figure 5.4.8 shows an example of differing optimal titers for the same antibody depending on whether the antigen measured is intracellular or extracellular. Too high a concentration will result in increased background and spread, particularly in fixed, permeabilized cells. The antibody concentration recommended by the manufacturer may not be valid for your cells or under your experimental conditions. Determination of the optimal antibody concentration in each experimental system can result in substantial savings on antibody costs, as acceptable population separation may well be seen using less antibody than manufacturer's recommendations.

Spectral overlap, spillover, and spread

As seen above, fluorochromes absorb and emit light over a specific but often wide range of wavelengths. Optical filters are chosen to target the maximum emission of each fluorochrome, so that the primary detector for that fluorochrome generates an accurate measurement of the target fluorochrome. If however the emission spectra of other fluorochromes partially overlap with the first, this spectral overlap will result in spillover, that is, some emission from the other fluorochromes will pass through the optical filter and contribute to the light measured in the detector for the first fluorochrome. As seen in Figure 5.4.9, FITC spillover into PE generates a population that appears to be positive for PE. There is no PE in this tube, therefore, this positivity is due only to FITC spillover. This contribution of light due to spillover must be corrected for in order to accurately quantify the light for each fluorochrome in its designated detector.

Sources of spillover

Spillover can occur in three types of situations. The first, as seen above, is when the emission spectra of the two fluorochromes have some adjacent overlap (Fig. 5.4.10A).

The second situation (Fig. 5.4.10B) occurs in the case of tandem dyes and depends on the efficiency of the dye coupling. If the dyes are poorly coupled, or have become uncoupled

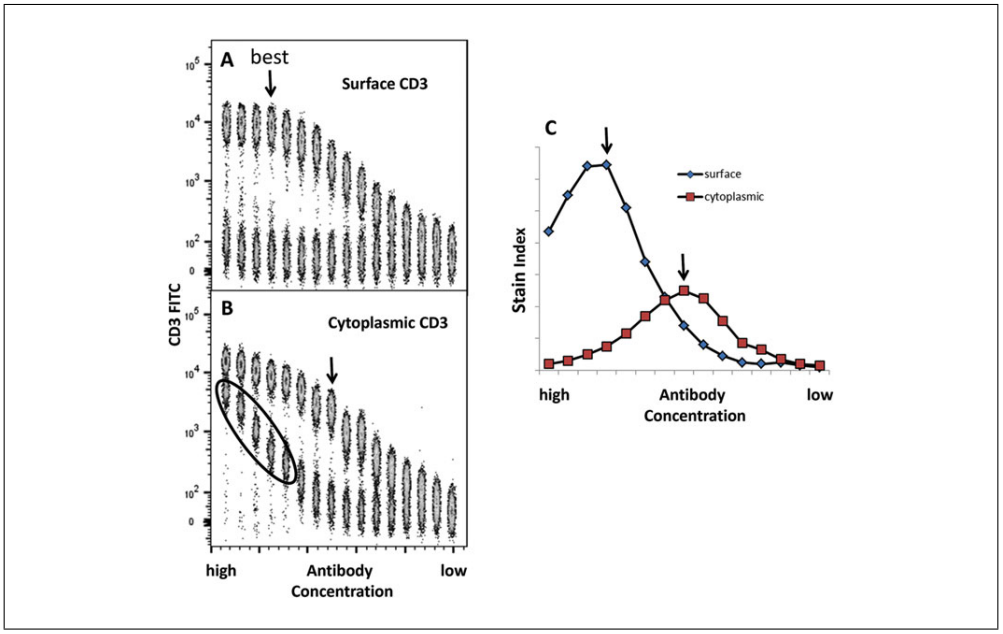

Figure 5.4.8 Antibody titration. Optimal antibody concentration will vary depending on the cell preparation and staining conditions under which the antibody is titrated. Here, titration of the same CD3 antibody gives very different results on surface stained unfixed cells (**A**) versus intracellular staining on fixed and permeabilized cells (**B**). This is due to the increased background (circled in panel B) on the negative populations induced by the fix/perm steps for cytoplasmic CD3 staining. The calculated stain index (**C**) reflects this loss of sensitivity; the best SI for the intracellular CD3 is half that of the surface staining.

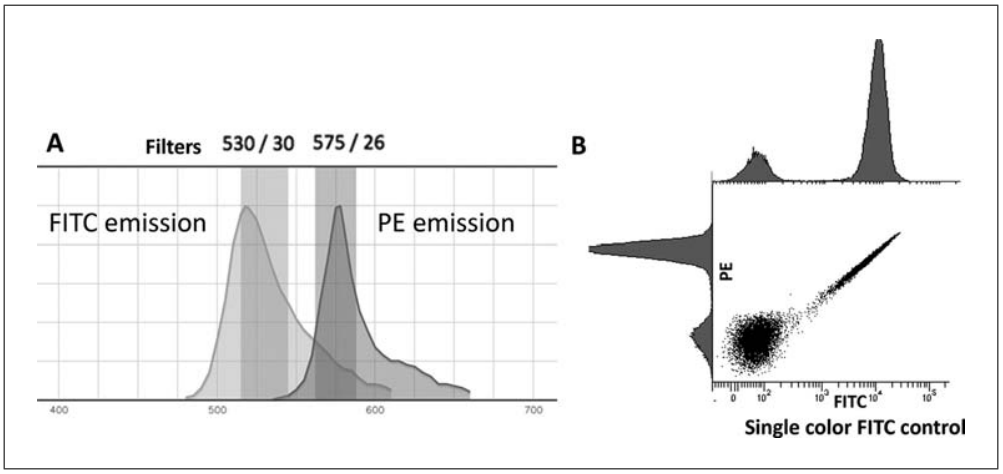

Figure 5.4.9 Spectral overlap. (**A**) Shows the overlap of the emission spectra of two fluorochromes, FITC and PE, and the optical filters through which each fluorochrome is collected. It can be seen that there is some FITC emission at the wavelengths measured through the PE filter and vice versa. (**B**) Shows the result of this spillover. This is a FITC only single color tube. There appears, however, to be a positive population in the PE channel too, but this is due to the FITC emission at the wavelengths measured in the PE detector.

due to temperature or light degradation, there will be more direct emission from the donor fluorochrome at its normal emission wavelength. In the example of PE-Cy5, there would be some spillover emission into the PE channel from the uncoupled PE. Even when the coupling is ideal, there is usually some signal from the donor dye.

The third source of spillover is in the case of cross laser excitation (Fig. 5.4.10C). Two fluorochromes with similar emission spectra, but excited by different lasers, will show spillover when one of the fluorochromes is also, but to a lesser degree, excited by the other laser. This can usually be predicted by looking at the fluorochromes' excitation

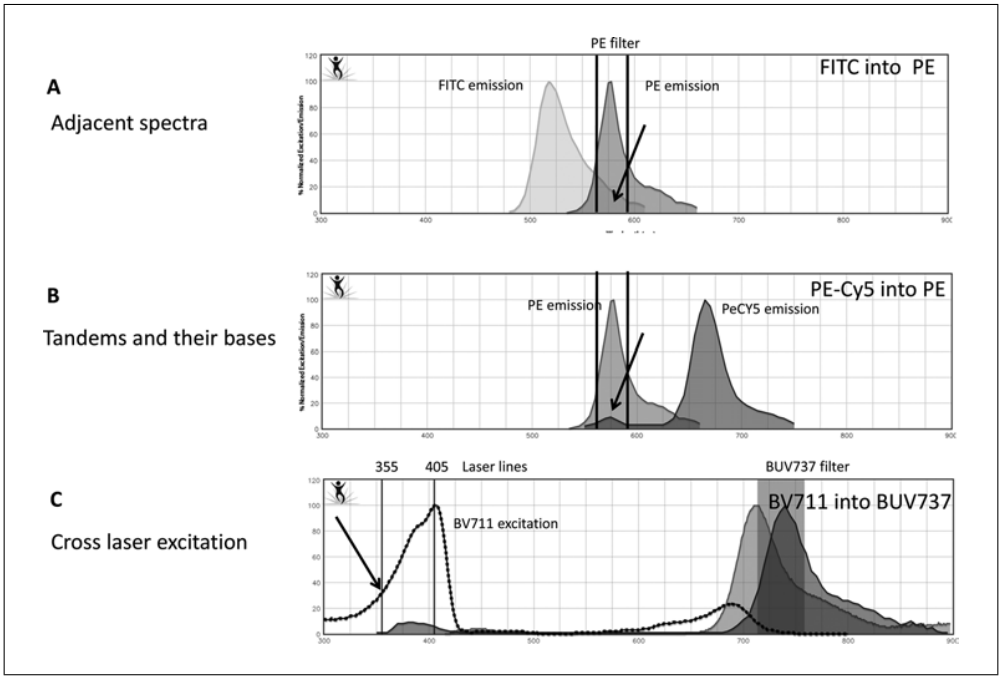

Figure 5.4.10 Sources of spillover. Three sources of spillover commonly occur: (**A**) Adjacent emission: spillover due to overlap of adjacent emission spectra. Shown here, FITC into PE. (**B**) Tandem to base: spillover from a tandem into the detector for its base (donor fluorochrome). Here, leakage from the PE moiety of PE-Cy5 will spill over into the PE channel. (**C**) Cross laser excitation: fluorochromes excited by different lasers, but with similar emission spectra, will show spillover if also excited by the other laser. Here, BV711 will spillover into the BUV780 detector, as it is also excited to a small degree by the UV laser.

spectra to see if there is excitation, even minor, by other lasers.

Figure 5.4.11 shows an example of the extent to which spillover can occur, in this case PE-Cy5 spillover into multiple other detectors.

Even when fluorochrome combinations for an experiment are chosen carefully to avoid these situations, some spillover usually occurs, so that a proportion of the light reaching the primary light detector targeted to the desired fluorochrome comes from other fluorochromes. This contribution of light signal from other fluorochromes must be measured and then removed from the signal generated by the primary detector so that the measurements from each detector will be specific for the desired fluorochrome only. This measurement and mathematical correction process is called compensation.

Compensation

In order to compensate, the amount of spillover is first measured using a series of single-color controls, one tube for each fluorochrome used. The software then uses this information to calculate a mathematical correction and generate a compensation matrix. This compensation matrix, with corrections for spillover from all colors into all detectors, is then applied to the experimental multicolor samples. Application of compensation correction to fully stained multicolor samples ensures that the signal seen in each detector is due to real signal from that fluorochrome and not to spillover from the others.

The compensation calculation is based on the slope of the line between the positive and negative medians in the spillover channels versus the primary channel (Fig. 5.4.12). In reality, the compensation calculation performed by the software on the digital data is not simple, using an inverted spillover matrix and matrix algebra, and is calculated slightly differently depending on the software. Almost all cytometry software packages have automated compensation protocols, either using the cytometer's data acquisition software or post-acquisition with third-party software.

Compensation can be calculated manually, if necessary, for simple two-color experiments on older cytometers such as the FACSCalibur, but it is preferable for both simple and complex experiments to use the automated compensation software programs available on all recent cytometers. It is possible with the older cytometers to acquire the data uncom-

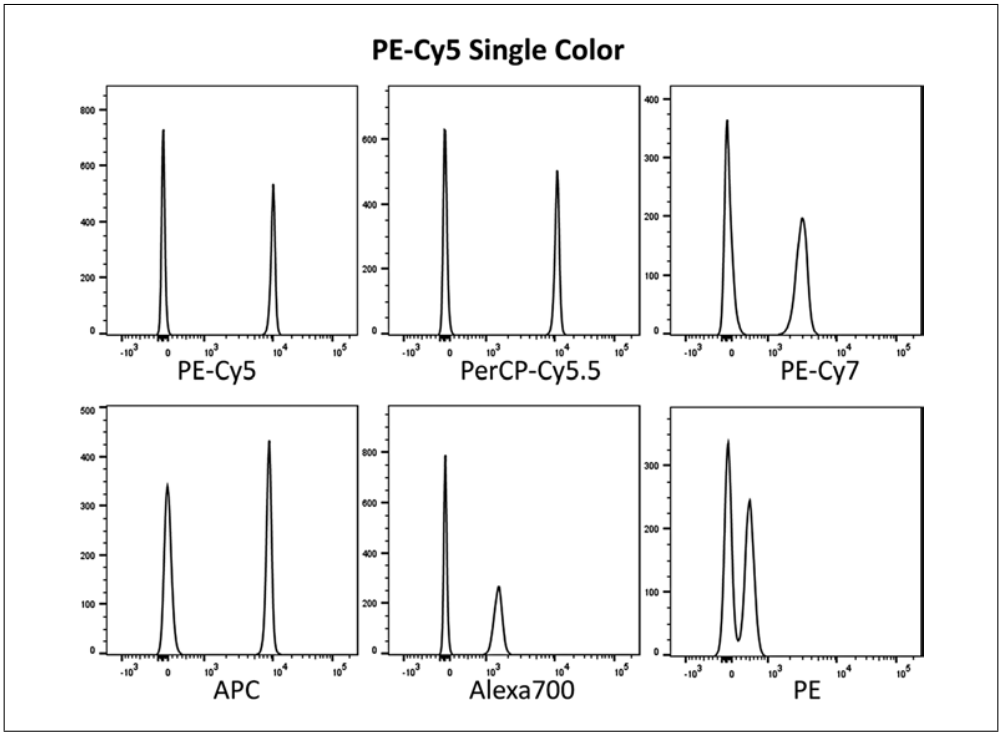

Figure 5.4.11 PE-Cy5 multiple spillover. PE-Cy5 causes spillover into other channels via all three spillover categories. This is a single color containing PE-Cy5 only. Note the substantial spillover into multiple channels: Adjacent detectors—into PerCP-Cy5.5, PE-Cy7. Cross laser excitation: into APC and Alexa 700 due to red laser excitation of the Cy5 moiety. Tandem to base: leakage from PE moiety into PE.

pensated or under-compensated, and then refine the compensation post acquisition using third-party software. The automated procedures are more accurate: they use complex matrix algebra and compensate simultaneously for all parameters, as opposed to the sequential subtraction procedure done manually. Under- or overcompensation (Fig. 5.4.13) will lead to incorrect quantification of cell populations in the fully stained samples. Accurate compensation is critical for the correct identification of sub-populations within a sample, as seen in Figure 5.4.14.

There are three myths associated with compensation values. First, there is a misconception that compensation values must be consistent for the same dye combinations: for example, that there is always a 10% overlap between FITC and PE signals, and that this will be reflected in the compensation matrix. Compensation is heavily dependent on the detector gain (PMT) settings; values will change with even slightly different voltage settings. Compensation matrices are unlikely to be similar across different instruments. The addition of new dyes into a multicolor experiment can also change compensation values, as the software simultaneously calculates all compensation values for all colors. Compensation may vary from day to day even on the same instrument, due in part to experimental variation, but also due to some imprecision in the values used for the compensation calculation. Second, users are often concerned by values over 100%. In fact, compensation removes fluorescent spillover for values of 200% just as well as it does for values of 2%; compensation values over 100% merely reflect "unbalanced" detector voltages. Unbalanced voltages result in higher signal in a secondary detector than in the primary detector (for example, FITC signal higher in the PE detector than in the FITC detector), and can introduce slight challenges when troubleshooting experiments, but these issues do not affect compensation. Finally, there is a common misconception that brighter signals require higher compensation values. Compensation values are intrinsic to the dyes used, regardless of staining intensity. In two panels that differ only by the substitution of a bright marker (CD3) for a dim marker (IL4) on the same (non-tandem) fluorochrome, the same compensation matrix can be used successfully for both, following the caveats outlined below regarding the brightness of the single color controls.

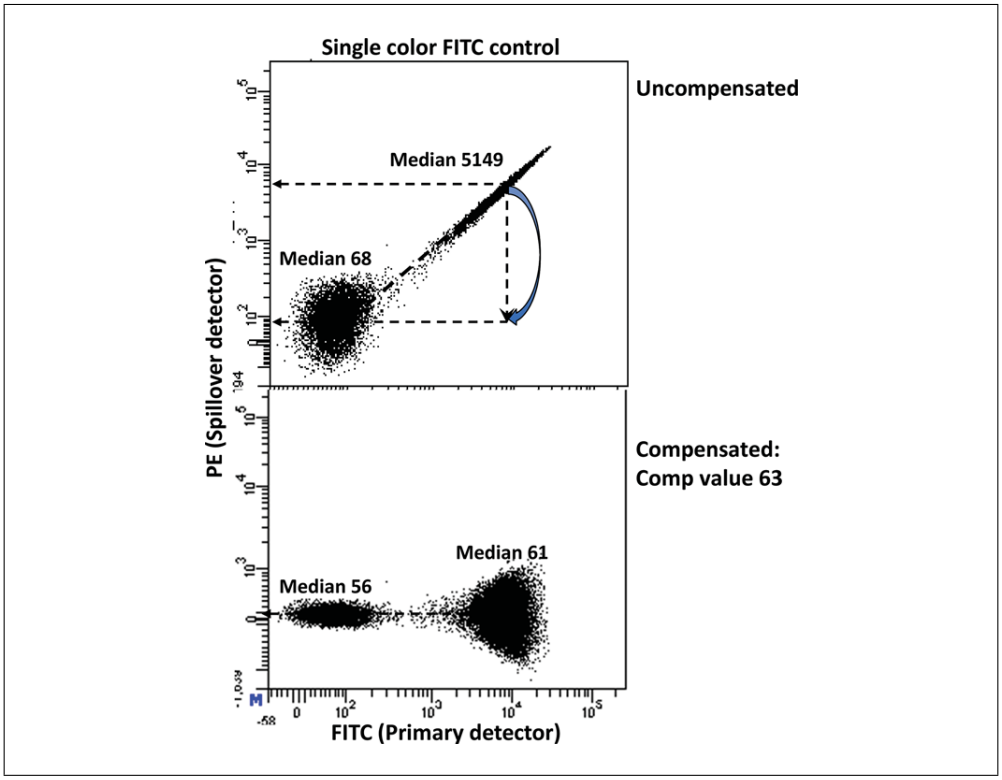

Figure 5.4.12 Compensation calculation. To calculate compensation, the median intensities of the positive and negative populations in the each channel are first measured. The compensation calculation is based on the slope of the line between the medians of the positive and negative populations. This is a FITC single-color control; thus, there should be no positivity in the PE channel—this is only due to spillover. In the upper uncompensated plot, the median of the FITC-positive cells is 5149 in the PE (spillover) channel, whereas the median of the FITC negative cells is 68. In the lower plot, which is correctly compensated using automated software, the median of the FITC positive (61) is now the same as the median of the FITC negative (56) in the PE channel.

It is important to understand the theoretical and mathematical basis for compensation (Bagwell & Adams, 1993; Roederer, 2001) and to monitor the compensation as applied to the single-color controls using $n \times n$ plots. In general, as we will see below, errors in compensation are usually due to poor-quality single-color controls.

How to compensate

As stated above, automated compensation software generates the most accurate compensation correction. A simple manual procedure, however, is given in the protocols for situations in which automated compensation is not available, e.g., on the older analog cytometers such as a FACSCalibur. The following rules apply to automated and manual compensation procedures.

To calculate a compensation matrix, a series of single-color controls for each color in the panel is needed, each with cells or beads stained with one color ONLY. A negative control, either within each single-color control tube or as a separate unstained tube, is also necessary. Data is acquired for each single color and gates are set to identify positive and negative populations. The compensation is calculated and applied so that, in the spillover channels, the medians of the positive populations are equal to the medians of the negative populations, i.e., there is no positivity seen due to other fluorochromes (Fig. 5.4.12). Compensation is dependent on detector gain/voltage settings: all single-color and multicolor samples must be run using exactly the same gains. Accurate compensation calculation relies on good quality controls with distinct, well separated positive and negative populations, and according to the following rules elaborated by Mario Roederer at NIH.

Rule #1 (Fig. 5.4.15): The fluorochrome in the single-color control must have EXACTLY the same spectral characteristics as the one in the mix. It is particularly important for tandem dyes, in which variation in lot to lot coupling efficiency affects leakage from the donor fluorochrome, to use exactly the

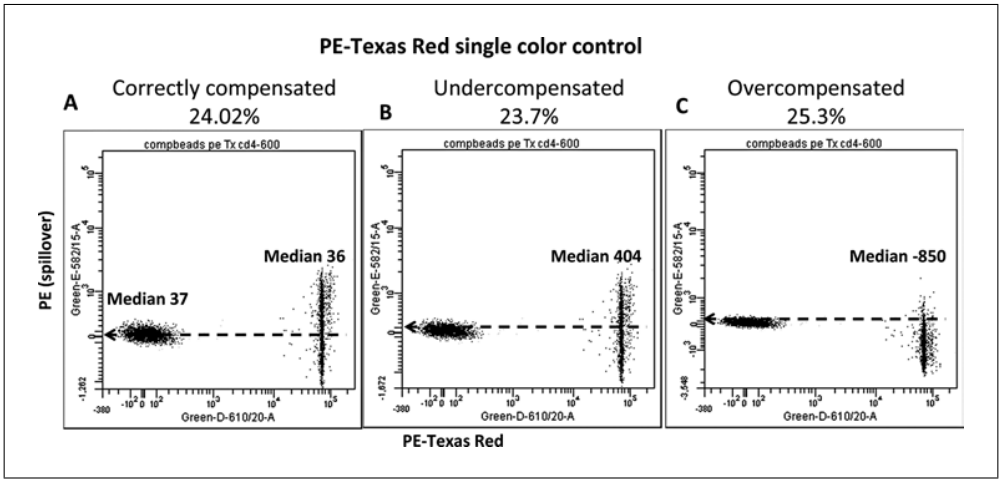

Figure 5.4.13 Incorrect compensation. In Panel **A** on the left, the data is correctly compensated. In the center, Panel **B**, the data is undercompensated; the median of the PE-Texas Red positive cells is higher than that of the negatives in the PE spillover channel. In Panel **C**, on the right it is overcompensated; the positive median is lower than the negative. Incorrect compensation will lead to incorrect quantification of double-positive cells in the fully stained samples.

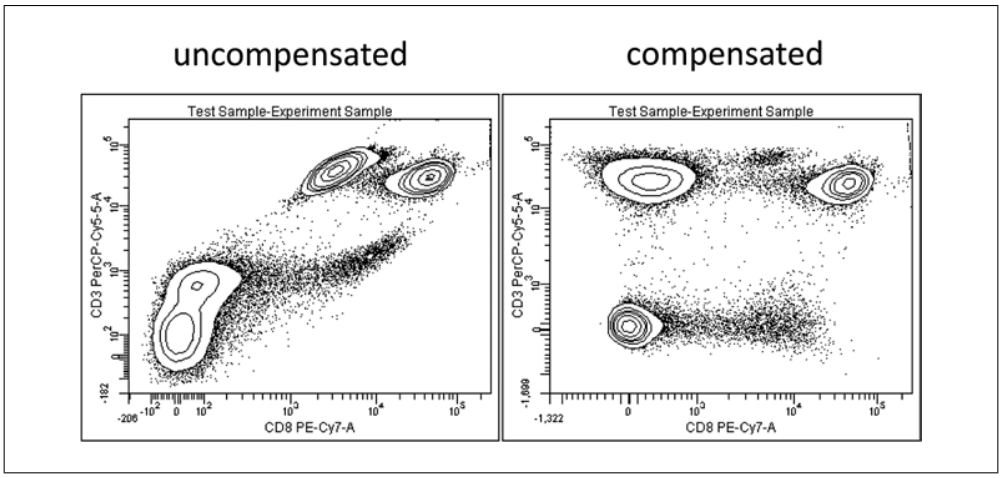

Figure 5.4.14 Correct compensation: the end result. The data from uncompensated multicolor stained cells on the left appears to show three double-positive populations, while the compensated data on the right correctly shows the true double positives.

same tandem labeled antibody as in the mix. Simple fluorochromes that appear to have the same emission spectra or are read in the same channel are not the same in the eyes of the cytometer. FITC is not the same as Alexa488 is not the same as GFP, even though they are all read in the 530/30 channel. Compensation using a FITC antibody will give an incorrect compensation value for GFP or Alexa488.

Rule #2 (Fig. 5.4.16): The positive population in the single-color control must be as bright or brighter than the positive cells in the mix. If the compensation is calculated on a dim population, brightly stained cells in the mix can easily be under- or over-compensated.

Rule #3 (Fig. 5.4.17): Within a single-color control, the negative population must have the same autofluorescence as the positive population. Compensation uses the slope of the line between the positive and negative for calculation, so they must match. Use negative beads with positive beads and negative cells with positive cells. If using cells, the negative cells must have the same autofluorescence as the positively stained cells. Use negative lymphocytes as the control for positive lymphocytes, not negative monocytes. Beads can be used for one single-color control, and cells for another, but the negative and positive population within each single color control must be autofluorescence matched.

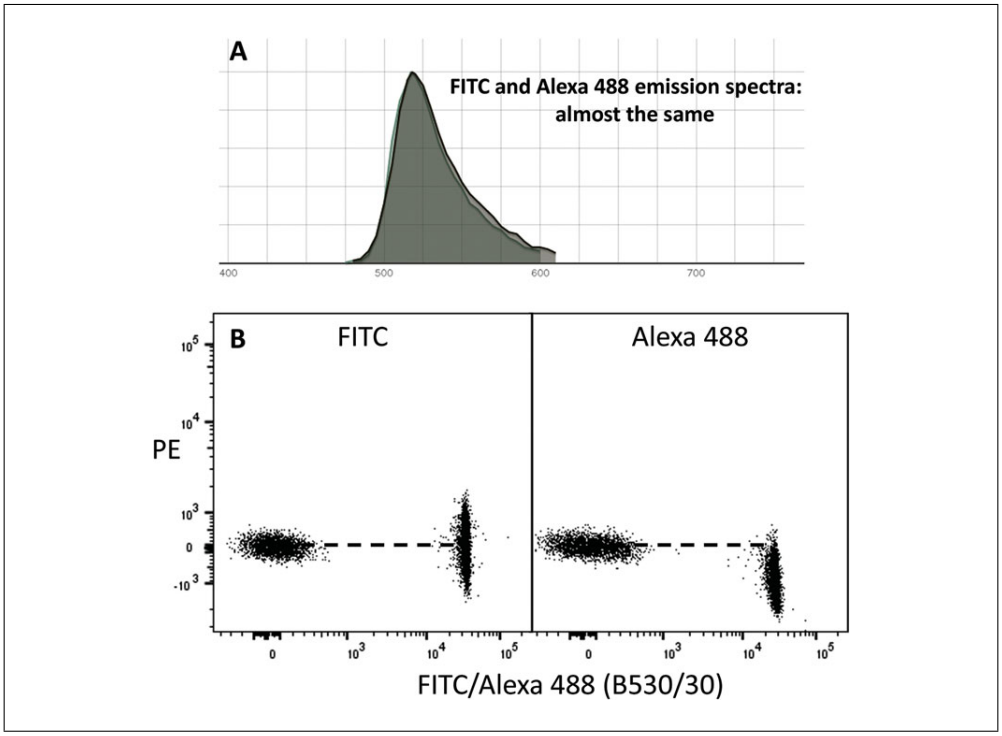

Figure 5.4.15 Compensation rule #1. The antibody in the single-color control must have exactly the same spectral characteristics as the one in the mix. This is especially important for tandem dyes, which can differ from lot to lot or due to degradation. (**A**) Even fluorochromes that appear to have the same emission spectra, such as FITC and Alexa 488, will differ in their compensation values. In panel **B** it can be seen that the compensation value against PE, calculated using a single color FITC control, results in overcompensation when applied to a single-color Alexa 488.

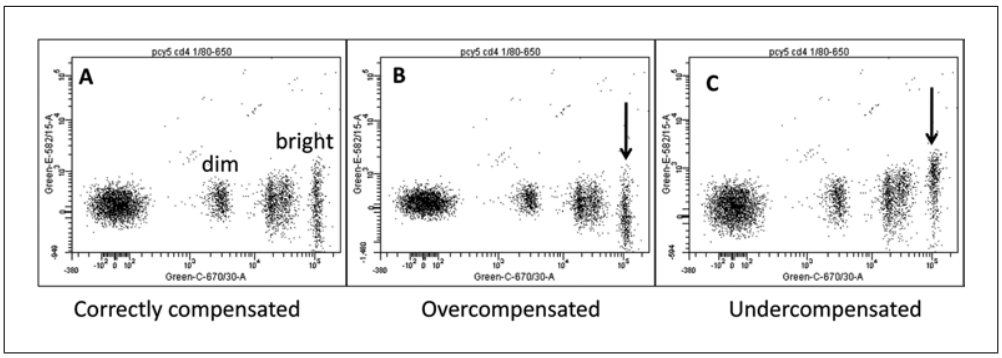

Figure 5.4.16 Compensation rule # 2. The single color must be as bright or brighter than the positive cells in the mix. In panel **A** the compensation has been calculated using the bright population as the positive and is correct for all the populations. In panels **B** and **C** it has been calculated using the dim population as the positive: here the compensation is adequate for the dim population, but the brighter populations are either over- or under- compensated (arrows).

The single color controls, whether cells or beads, should be treated in the same way, i.e., using the same fixation and permeabilization procedures and buffers as the experimental samples. Fixation, permeabilization and the buffers used in these procedures can alter the spectral characteristics of certain fluorochromes, which will affect their spillover and thus the compensation matrix.

As a new multicolor panel is being developed and tested, it is useful to run single-color beads and cells in parallel to determine which is best adapted for routine use. Single-color controls using cells are preferable unless, as is often the case, there are no, or very few, positive cells, or if the staining on the cells is very dim. In these cases, antibody capture compensation beads are preferable. These kits contain negative beads plus beads coated to

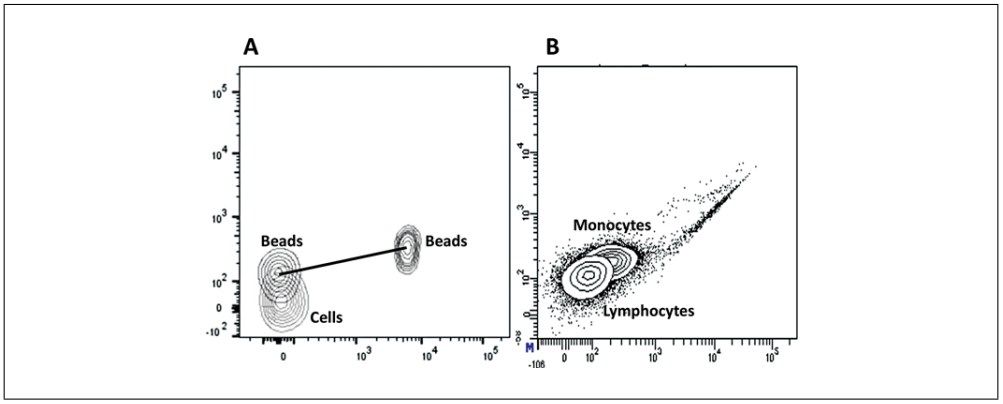

Figure 5.4.17 Compensation rule # 3. The autofluorescence of the negative must match the autofluorescence of the positive population. (**A**) It can be seen that the slope of the line used to calculate compensation would be quite different, depending on which negative autofluorescent population is used. In panel A, negative beads must be used, not negative cells. In panel **B**, negative lymphocytes must be used, not monocytes. Using a mismatched negative will generate an erroneous compensation correction.

capture antibodies. The beads are incubated with the antibody-fluorochrome (the one used in the mix, rule #1) to capture the antibody and give a tight, bright positive peak. Many kits are available on the market, some tailored to specific situations, such as high autofluorescence. In some cases, for certain fluorochromes, the beads may not give the same compensation values as cells. This is sometimes noted in the manufacturer's protocol information with the bead kit, but is the reason why the comparison with cells at the outset is recommended.

Spread

While spectral spillover is corrected by compensation to give accurate measurements of each marker, the compensated data often reveals 'spillover spread': the width or spread of the positive population peak seen in the channel receiving the spillover is due to photon-counting errors and becomes evident when the data is compensated. Figure 5.4.18 gives a visual explanation of why and how this increase in spread can reduce sensitivity. The spillover spread cannot be removed and can be problematic because it decreases the resolution of a dim double-positive population.

Spillover spread increases with higher staining intensity (Fig. 5.4.19). Thus, for the same fluorochrome, a population that is brightly stained due to high antigen density will show more spread into the spillover channel than will a dimly stained population.

It is useful and relatively easy to calculate the spillover spread matrix (SSM) in order to know which combinations engender the most spread on your cytometer. This can be done using single-color bead controls for every detector either manually, (Fig. 5.4.20A; Nguyen, Perfetto, Mahnke, Chattopadhyay, & Roederer, 2013), or automatically with the SSM calculation button in Flowjo's v9 compensation wizard. Once calculated, the spread matrix will remain valid for the cytometer as a reference for future panel development. The SSM will need to be recalculated in the case of cytometer repairs such as replacement of lasers, filters, or detectors. The spillover spread values are independent of the compensation values: a high compensation value does not necessarily mean high spread (Fig. 5.4.20B), with higher spread values usually seen in the red wavelength emissions.

Spread is additive: each channel is affected by the spillover from all of the other fluorochromes in the mix. The amount of spectral overlap, fluorochrome brightness, and the antigen density of the positive populations are major contributors to the level of spillover spread. This is in addition to electronic and optical background spread from the instrument, reagent, and cellular autofluorescence factors discussed above. An example of the impact of fluorochrome choice and spillover spread on the resolution of dim double-positive populations is shown in Figure 5.4.21.

Basics of Multicolor Panel Design

All of these fluorochrome, antigen, and cytometer variables will impact the separation and identification of the cellular subpopulations of interest. To choose the best fluorochrome-antibody combinations, even for a 2-color experiment, a number of factors

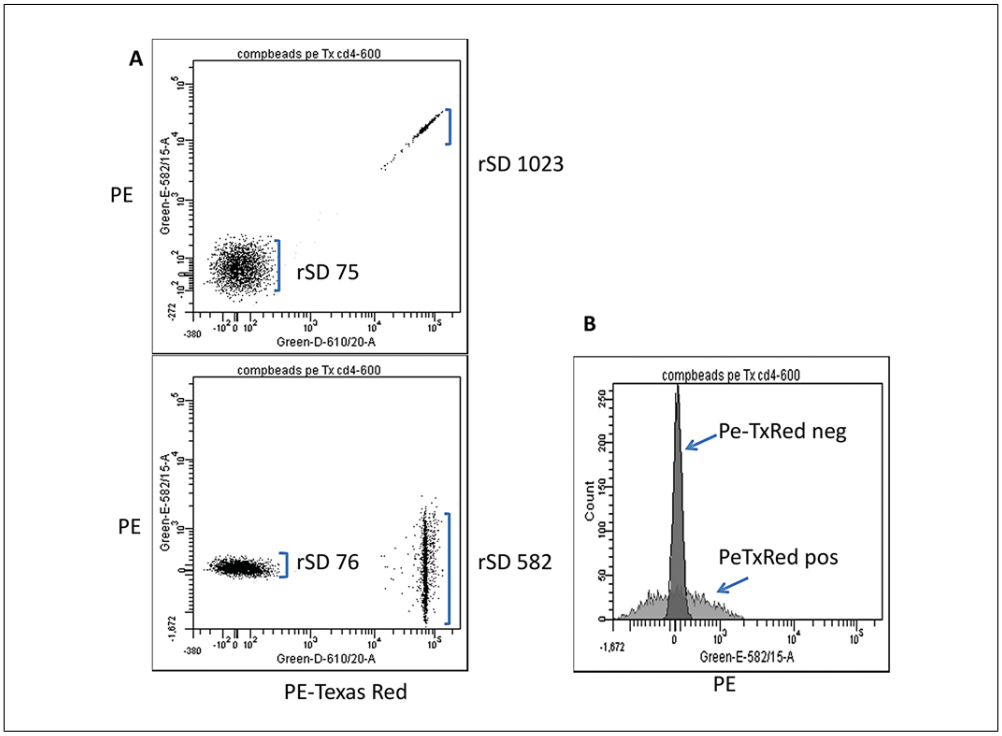

Figure 5.4.18 Spillover spread. In this example of spillover spread due to PE-Texas Red spillover into the PE channel, the width of the peaks is indicated by the rSD. In the uncompensated data, (**A**, upper), the widths of the positive and negative peaks on the PE axis look similar, but it must be kept in mind that this is a log scale. The negative peak at median 70 spans a few hundred channels with a low rSD, while the positive peak at median 17,000 spans tens of thousands, thus the high rSD. Upon compensation (**A**, lower), the positive peak is correctly compensated to have the same median as the negative, but this large channel spread remains and is now visually evident. In **B**, the PE histogram overlay illustrates the difference in spread for the PE-Texas Red positive versus negative populations. The PE-Texas Red positive population is much wider in the PE spillover channel than the PE-Texas Red negative population. A PE/PE-Texas Red double positive population would have to be quite bright for PE, well above channel 2×10^3, to be resolved.

need to be taken into consideration (Mahnke & Roederer, 2007) and are summarized below.

Fluorochrome brightness

Fluorochromes are ranked as very bright, bright, medium, or dim (Fig. 5.4.22), and overall these categories hold true across most cytometers, although there can be substantial variations between instruments. This information is easily found on the Web (see Internet Resources). Calculation of the stain index on your cytometer will give more precise information about which fluorochromes are brightest on your machine.

Antigen expression patterns and density

Antigen expression falls into three main categories, shown in Figure 5.4.23 (Mahnke & Roederer, 2007). 1° antigens are high density with on/off expression. 2° antigens also have high antigen expression but with a continuum of expression. The 3° antigens are either low density or have unknown expression levels.

You also need an idea of how many antigen molecules are on the cells you want to measure. Antigen density, or the level of expression of the target molecules in your cells, is a major consideration when choosing which fluorochrome to use for detection of those antigens (Fig. 5.4.24). For normal cells, and particularly for the human immune system, the density of many common antigens has been quantified and can be found on the Web and in the literature (Bikoue et al., 1996), sometimes in actual molecules or antibody binding sites per cell, and sometimes simply as high, intermediate, and low expression levels. These levels may vary according to disease or activation state. Often, however, the antigens you are most interested in are rare and may not be characterized, and thus are classified as 3° expressors. The titration curve of an antibody against such antigens can give a rough idea of expression level. If desired, surface

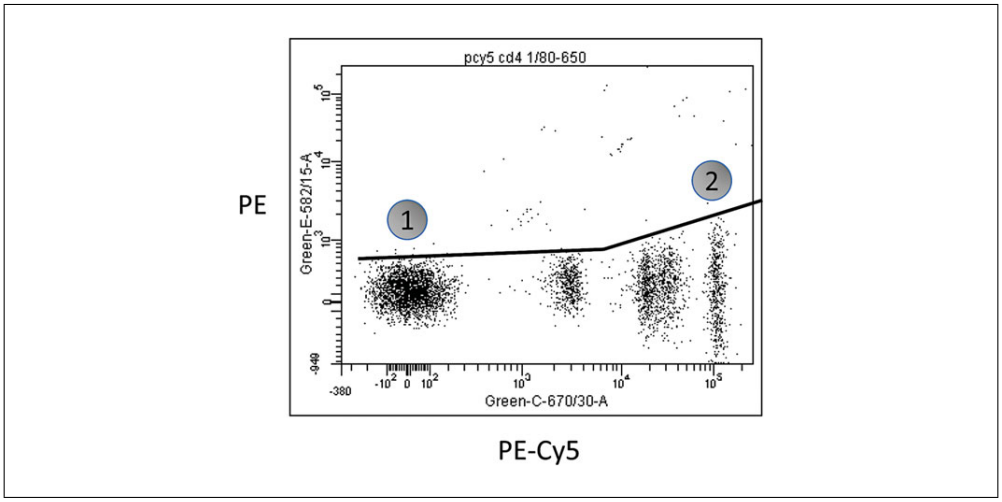

Figure 5.4.19 Spillover spread and antigen density. This graph illustrates the increase in spread due to increasing antigen density. The PE positive population can be easily resolved when it is PE-Cy5 negative (1), but will need to be much brighter as the PE-Cy5 positivity increases (2).

and intracellular biomarkers can be quantified by antibodies bound per cell using hard-dyed calibration beads and biological reference calibrators (Kantor, Moore, Meehan, & Parks, 2016; Wang et al., 2016).

Antigen co-expression and spread

As discussed above, the contribution of spread is problematic for the identification of double positive cells.

The problem of spread, however, only affects situations where cells co-express two or more antigens. The spread cannot be eliminated and must thus be avoided. Determine if, and which, antigens are expected to be co-expressed in your experimental plan (Fig. 5.4.25). Do not choose fluorochrome combinations with high spread for antigens that are co-expressed on the same cell, particularly where a high-density antigen and bright fluorochrome spread into a low-density antigen and dim fluorochrome channel. Conversely, utilize mutually exclusive expression, i.e., antigens that are not co-expressed such as CD3 and CD19, to exploit high-spread channels.

Figure 5.4.26 illustrates the improvement in resolution of dim co-expressed antigens and identification of difficult populations (Tregs) engendered by judicious fluorochrome choices.

Review: Steps for multicolor panel construction

1. Know which fluorochromes can be excited by the lasers available on your cytometer by looking at the excitation spectra of the fluorochromes you are considering.

2. Look at your cytometer configuration to know which fluorochromes are possible. Look at the fluorochrome emission spectra and compare to the optical filters on your cytometer to be sure they target the emission maxima. If necessary, better-adapted filters can usually be purchased and swapped out, depending on the cytometer.

3. Evaluate the stain index and spillover spread of the potential fluorochromes as measured on your cytometer. Look at the your SSM. Estimated values taken from the Web may not hold true for your cytometer.

4. Make a list of the fluorochrome-antibody combinations available for the prioritized antigens you need to measure. For rare antigens, you may not have much choice, so these will need to be selected first. If necessary, custom antibody-fluorochrome combinations can be specially ordered, or fabricated with kits in your lab.

5. In general, choose dim fluorochromes with low spillover spread into other colors for the high-density antigens. Save the bright fluorochromes for low expressors or uncharacterized antigens. It is wise to leave a channel open for future markers that may be added to the panel, preferably a bright fluorochrome with little spillover.

6. Look carefully at co-expression. Detection of dim double positives can be difficult when there is co-expression of two antigens on the same cell and high spread between those fluorochromes. Look at your experiment gating strategy and determine which antigens are co-expressed on which cells. Determine which of the pair is high density and which

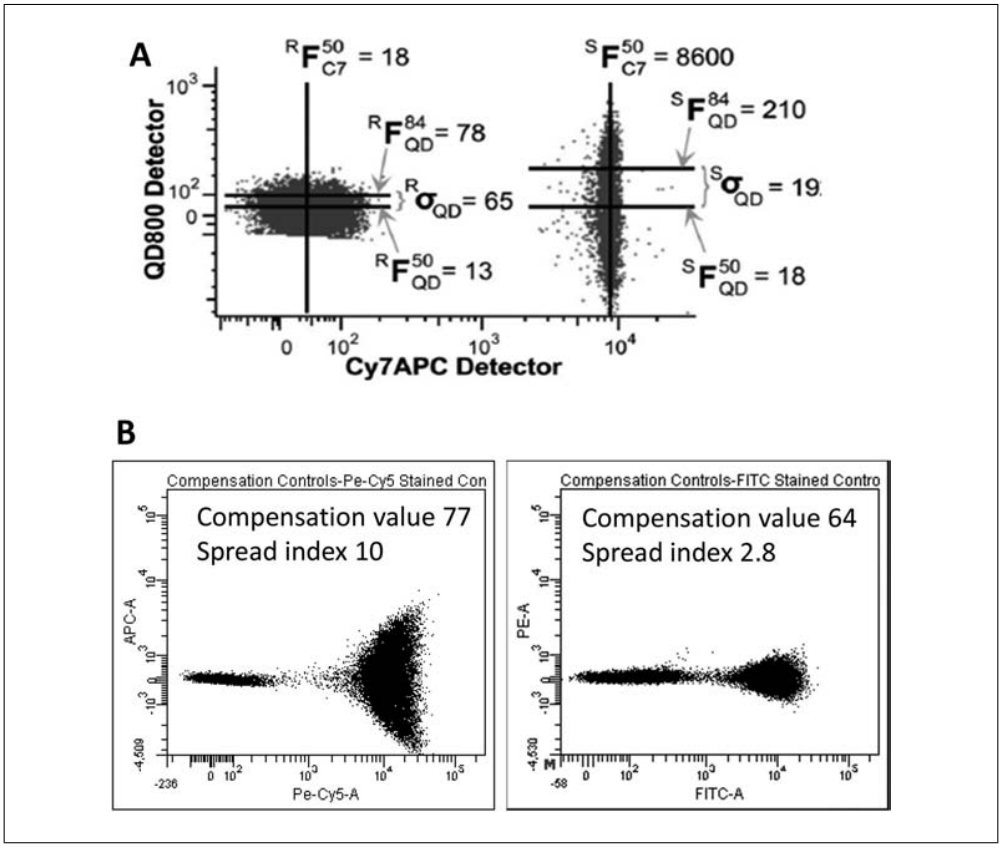

Figure 5.4.20 Calculation of spillover spread. (**A**) The spillover spreading index (Nguyen et al., 2013) uses single-color-stained beads to quantify the amount of spread between fluorochromes and detectors. The difference in spread of the APC-Cy7 positive versus the negative population in the spillover QD800 channel is normalized to the median intensity difference of the two populations in the primary APC-Cy7 channel. This spillover spread index can also be calculated automatically in Flowjo v9 to generate a spillover spread matrix (SSM), very useful for the evaluation the potential problems due to spread in the choice of fluorochromes for your own cytometer. (**B**) A high compensation value does not necessarily mean high spread. Here the compensation values are similar (and dependent on PMT voltage), while the spread index PE-Cy5 into APC is much higher (10) than FITC into PE (2.8).

is low density. Choose a bright fluorochrome for the low co-expressor and a dim fluorochrome for the high expressor, that has minimal spread into the low density channel. Multiple co-expressions will have repercussions on all the channels involved. Avoid fluorochrome combinations with high spread from a bright fluorochrome/highly expressed antigen into a dim fluorochrome/low expressor channel.

7. Even if you using only a few antibodies, spreading your fluorochrome choices across different lasers will minimize spillover and spread problems.

8. You may need to go through a few tries to find the best combination for difficult co-expression issues. Ask for test samples from the manufacturers.

There are a number of panel design aids available on the Web that help walk you through these steps (see Internet Resources), which also include search capability for available antibody/fluorochrome combinations.

Data analysis

Flow cytometry data acquired on the cytometer is saved in list mode (`.fcs`) files, the industry standard to which all cytometer manufacturers adhere. These data files list all the instrument acquisition information, additional keywords such as sample or antibody/fluorochrome information manually input by the user, and the intensity values for the fluorescence emission in each channel for all of the cells, cell by cell. These files can then be exploited to display, gate, and statistically analyze the data, either with the acquisition software or with third party analysis software.

Manual input of experiment information as keywords before data acquisition will be saved with the `.fcs` file and allow the data analy-

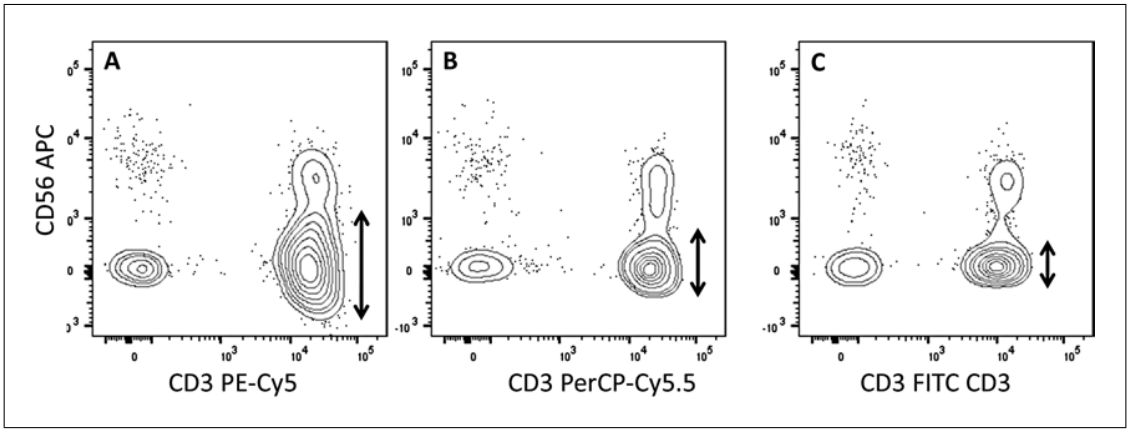

Figure 5.4.21 Effect of spread on resolution of dim populations. The effect of spread on resolution compares CD3 stained with three different fluorochromes versus CD56 APC, shown here using the same biexponential scale. Note the width of the CD3 positive, CD56 negative populations on the CD56 axis (arrows). (**A**) Poor resolution of CD56/CD3 double positive cells is due to the large spread of CD3 PE-Cy5 into the APC channel. (**B**) Better resolution using CD3 PerCP-Cy5.5, but still some spread. (**C**) Best resolution using CD3 FITC. Although FITC is a dim fluorochrome it has little spread into the APC channel.

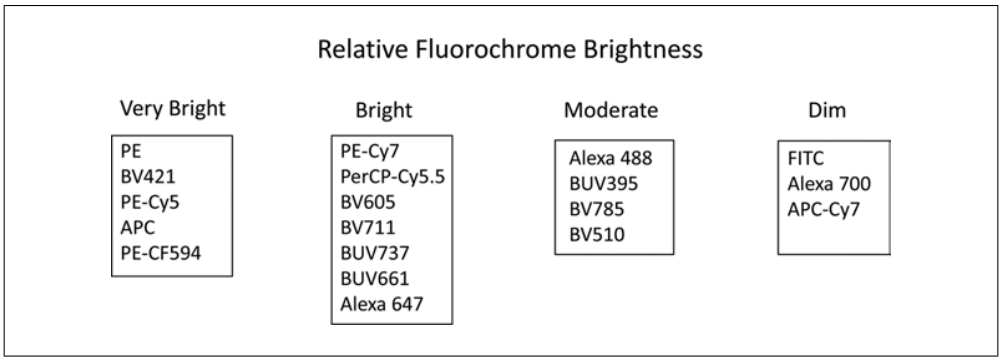

Figure 5.4.22 Relative fluorochrome brightness. Examples of relative fluorochrome brightness levels. Despite instrument differences, fluorochromes can be reliably grouped into high to low brightness levels which should be valid across most cytometers. This information can be usually be found on manufacturers Web sites (see Internet Resources). It is best of course to verify by calculating stain index on your own cytometer.

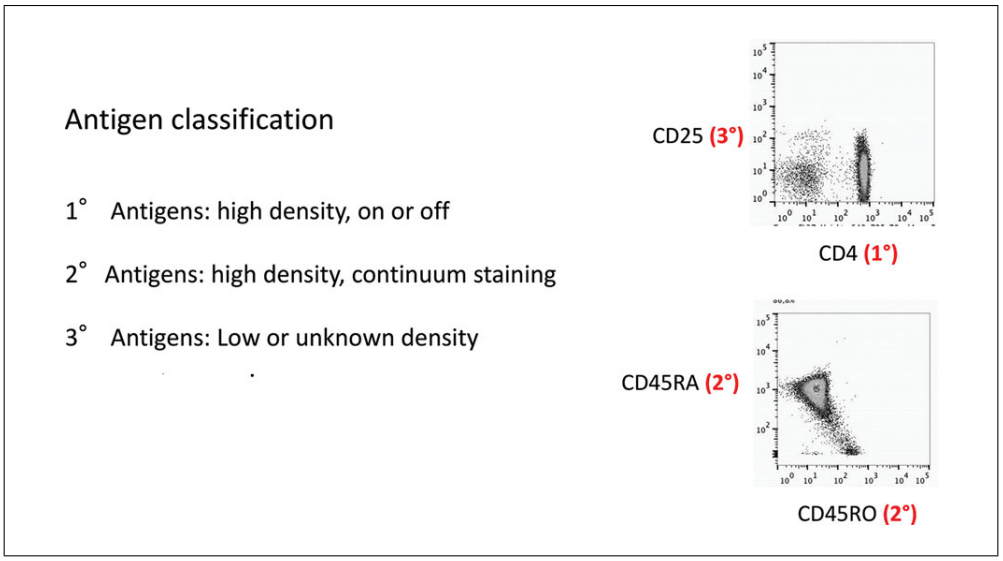

Figure 5.4.23 Antigen classification. Examples of antigen classification groups: CD4 is 1°, CD45 RA and RO are 2°, and CD25 is 3° (adapted from Mahnke et al., 2007).

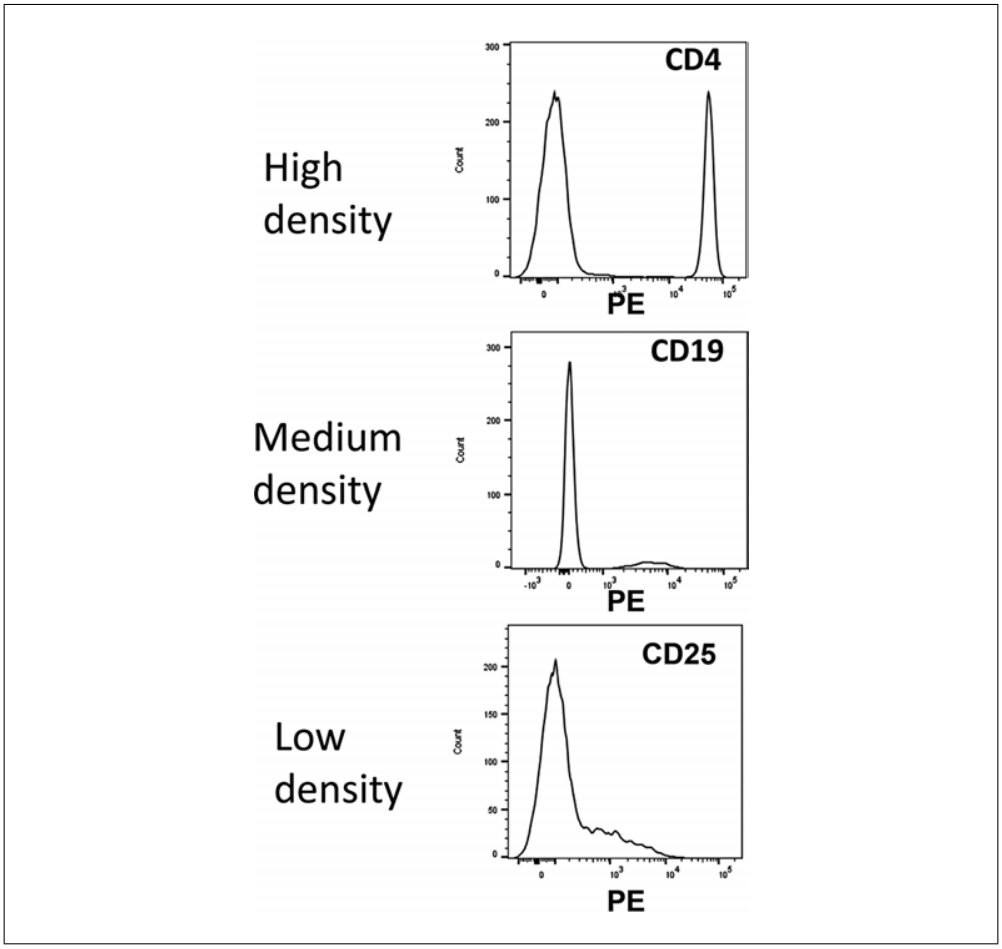

Figure 5.4.24 Antigen density. Antigen density is one of the major considerations in choosing fluorochromes in order to pair low-density antigens with bright fluorochromes and high-density antigens with dim fluorochromes. Antigen density levels can often be found with a literature or Web search. A bright fluorochrome such as PE should be reserved for low-density antigens. As seen here, it would be difficult to resolve the CD25 positive cells seen here using a dim fluorochrome.

sis software to search and group data files for different criteria, for example antibody clone, concentration, and patient information and control or treatment groups. Information on the optical configuration, laser wavelengths, and power used to acquire the data should be included. A complete nomenclature that includes all the relevant information would be: PE-CD4 data acquired using a 50-mW yellow-green 561-nm laser and a 575/15 filter would be labeled CD4-PE 561(50) 575/15, whereas the same data acquired using a 20-mW blue 488-nm laser and a 585/42 filter would be labeled CD4-PE 488(20) 585/42. As this complete nomenclature is unwieldy for plot axes, Roederer has proposed a simplified parameter naming system (Roederer, 2015) which would define the laser color by letter and the center of the bandpass, so the two parameters above, in addition to the fluorochrome and antibody information, would be G575 or B585 respectively. The complete details with laser wavelength, power, and optics would need to be included elsewhere in the publication, as defined in the MiFlowCyt standards (Spidlen, Breuer, & Brinkman, 2012). The MyFlowcyte standards for publication of flow cytometry data include recommendations for the format and inclusion of information on acquisition, analysis, and data display parameters.

Data display

Flow cytometry data is most commonly displayed using single-parameter frequency histograms or dual-parameter dot or contour plots. Data on most of the current digital cytometers is recorded in linear values, but can be displayed on linear or log scale as desired. Subtraction due to compensation, as seen above, in addition to running background subtractions

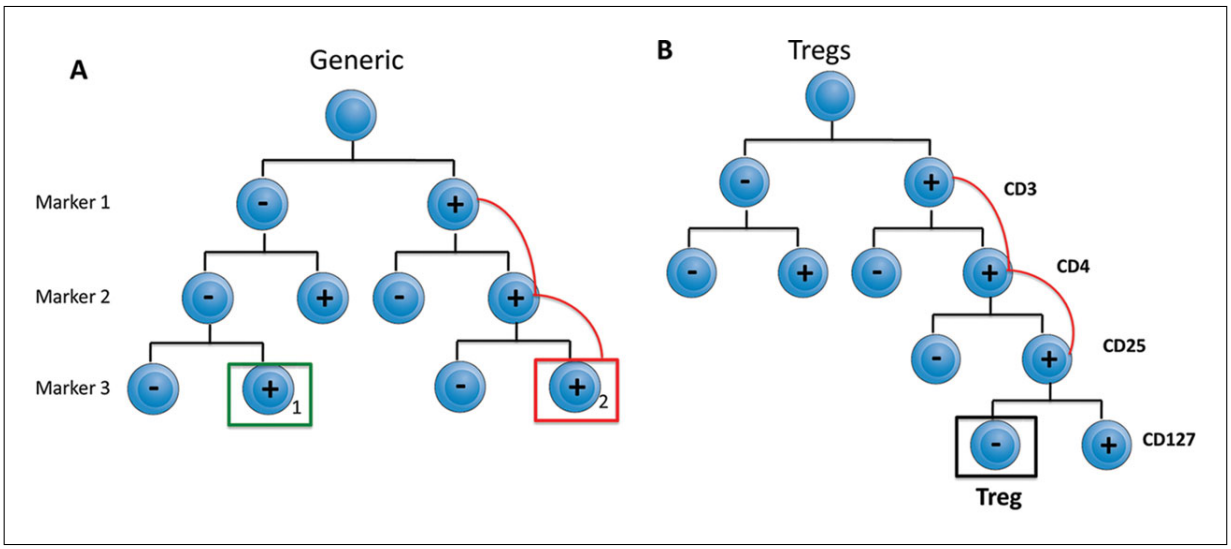

Figure 5.4.25 Determination of co-expression issues. Co-expression of two antigens on the same cell can cause difficulty in identifying dim double-positive cells in cases where there is high spread between channels. The presence of co-expression must be taken into account when choosing fluorochromes. (**A**) In this experimental schema, it can be seen that cell 1 on the left is positive only for marker 3, and thus has no co-expression issues. Cell 2 on the right, however, co-expresses all of the other markers, so care must be taken in fluorochrome choice to ensure resolution if some of the markers are dim. (**B**) The real world: the multiple co-expression pattern of cells like Tregs underlines the need for careful decisions on fluorochrome combinations.

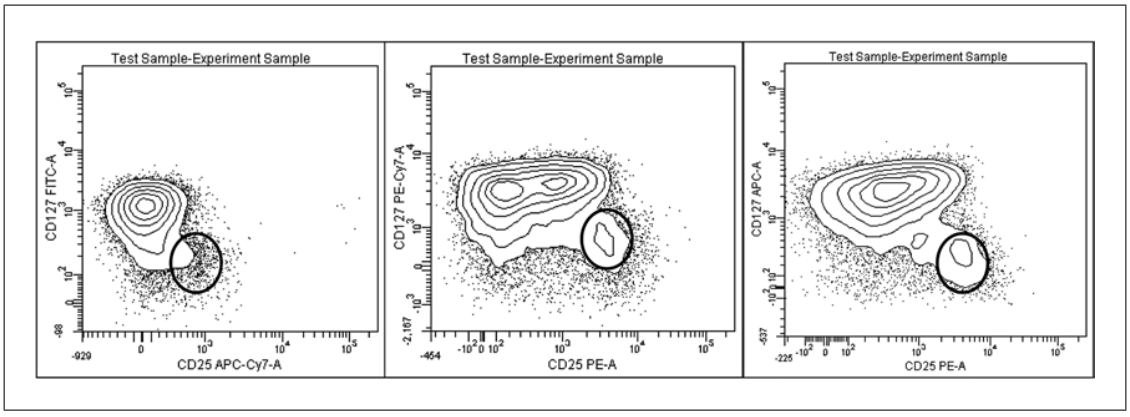

Figure 5.4.26 Fluorochrome choice affects Treg detection. Three different panels for Tregs shown here illustrate the improvement in resolution that can be gained with careful fluorochrome selection to increase intensity of staining of low density CD25 antigens and reduce background due to spillover spread.

performed by the cytometer during data acquisition, often generates sub-zero channel numbers, which cannot be correctly visualized using pure log scale. These events are either below scale and invisible or they can aberrantly appear to be two populations. To circumvent this, it is highly recommended to use one of the transformed log-scale options (Biexponential, Hyperlog, Logicle) which combine log display with linear visualization of the events around and just below zero (Herzenberg, Tung, Moore, Herzenberg, & Parks, 2006; Parks, Roederer, & Moore, 2006).

For statistical analysis, the median is the best indicator of central tendency of a peak, as it is less skewed by outliers; variance is best described by the robust standard deviation based on the median. Determination of positive vs negative cutoff points is discussed in Controls below.

Controls, controls, controls!

Good controls are essential to the acquisition and analysis of good-quality data (Hulspas, O'Gorman, et al., 2009; Maecker & Trotter, 2006). Below is a review of controls that should be run with each experiment.

1. *QC beads:* As discussed above, QC beads should be run to verify and standardize the cytometer every day, and preferably again

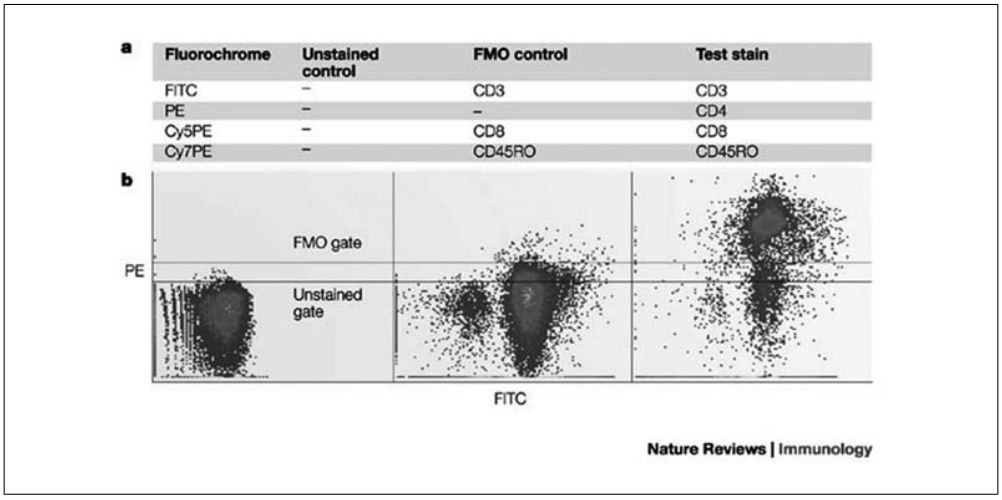

Figure 5.4.27 FMO controls. FMO (Fluorescence Minus One) controls are generally considered to be the most accurate for setting cutoffs for positivity. The FMO control shown here is for CD4-PE, which means the tube contains all the antibodies in the mix except for PE anti-CD4 antibody. The cutoff determined using the unstained control is lower and inaccurate compared to the cutoff determined using the FMO. The FMO has a higher cutoff because it controls for the contribution of spread from the other fluorochromes in the mix. (reproduced from Perfetto et al., 2004).

just before beginning acquisition of your sample data.

2. *Compensation controls:* Compensation controls should be run with each experiment. Minor day-to-day variations of the cytometer, slight changes in detector settings, and instability of tandem dyes over time can affect position of positive and negative peaks, and the compensation matrix calculated on previous runs may not be valid.

During the initial development of a new panel, it is recommended to run single color compensation controls using both cells and beads, to elucidate potential problems with one or the other. Once the best choice for each color has been determined, whether cells or beads, only that one will be run routinely.

3. *FMO controls:* FMO, or 'fluorescence minus one' controls are the current preferred norm for determining positive and negative cutoff points on parameters where the staining is dim or there are few positive cells (Perfetto, Chattopadhyay, & Roederer, 2004). These control cells are stained with all of the antibodies except for the difficult one of interest.

During the initial development of a new panel, it is useful to run an FMO for every color. These should be examined closely to elucidate unexpected problems or interactions. On a routine basis, however, only FMOs necessary to determine difficult cutoffs will be needed. Examples in Figure 5.4.27 show the difference in cutoff and results generated by using an unstained versus an FMO control (Perfetto et al., 2004).

Isotype controls

Isotype controls have generated a good deal of controversy in the flow cytometry community in the recent past (Keeney, Gratama, Chin-Yee, & Sutherland, 1998; O'Gorman & Thomas, 1999). Historically isotype controls were used to determine cutoffs for positive cells, but as seen above, FMOs furnish a more valid cutoff. If they are used, isotype controls must be matched to the experimental antibody for antibody sub-type, fluorochrome/antibody ratio, and concentration (Fig. 5.4.28A; Maecker & Trotter, 2006). Isotype controls are considered useful to evaluate the presence of undesired staining due to Fc receptors or protein-protein interactions between antibody and cellular antigens and to troubleshoot nonspecific cell staining.

Biological controls

Often, a biological control is the only option to establish the positivity cutoff between a control and experimental, normal versus abnormal, or treated versus untreated sample. As seen in Figure 5.4.28B, the cutoff for positive and negative stimulated cells can be quite different depending on whether an isotype, FMO, or fully stained unstimulated cells are used (Maecker & Trotter, 2006). In this case, the most accurate control was the unstimulated fully stained sample. In difficult cases it also

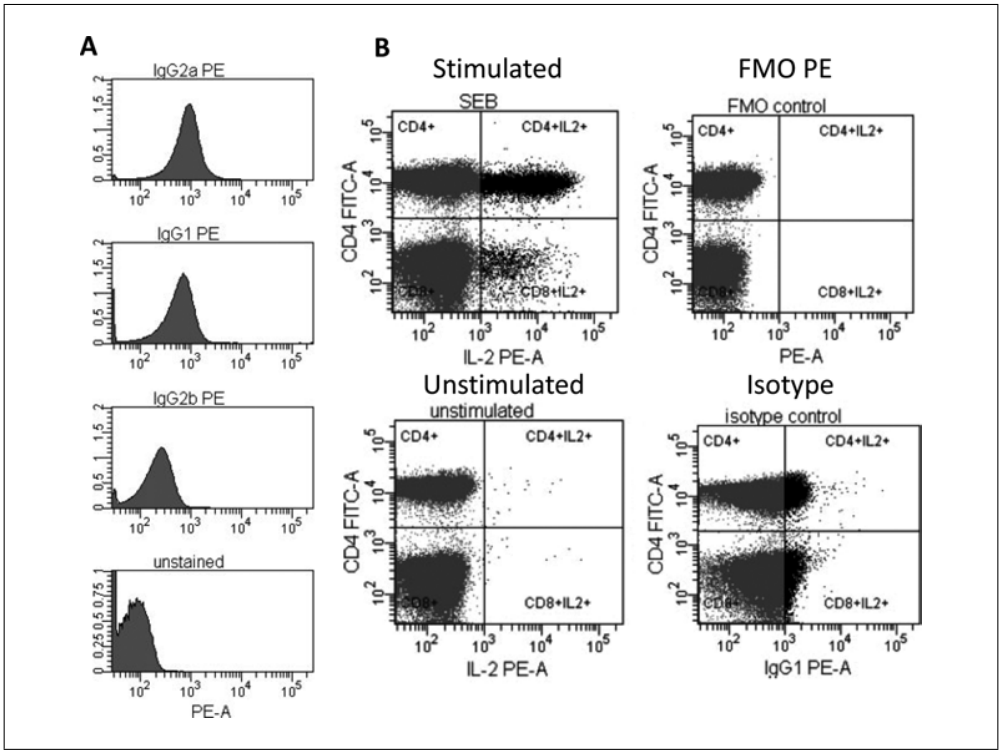

Figure 5.4.28 Isotype and biological controls. (**A**) Shows the variation seen in a series of Isotype controls of different subtypes, all used at the recommended concentration, and compared to unstained cells. Isotypes must be matched to the specific antibody for concentration, dye/protein ratio, and subtype. They should not be used to establish cutoff points for positivity. (**B**) Often, a biological control is needed to correctly determine where to establish the cutoff for positivity, in this case for IL2-PE. Here, both the stimulated and unstimulated cells are fully stained, and are compared to an FMO minus PE and an isotype control. For the IL-2 PE cutoff, the most accurate control is the fully stained unstimulated sample. The FMO cutoff would be too low, and the isotype too high. Reproduced from Maecker et al. (2006), with permission).

can be useful, if cells are available, to have an FMO of the treated or patient sample.

Troubleshooting

Eliminating artifacts

Time parameter

If fluidic instability (Fig. 5.4.29) occurs during the acquisition of a sample, either over a long acquisition time period or in a multiwell plate assay, there can be a loss of scatter and fluorescence sensitivity over the acquisition time period. If not detected, this can generate false population artifacts with diminished fluorescence or scatter. Visualization of time versus scatter and/or fluorescence as part of the data analysis schema is used to control for the stability of the data over the time period of the file acquisition. If aberrations are detected, a gate can then be set to exclude the part of the data file that is invalid. There are automated algorithms in the statistical programming language R that can do this (Fletez-Brant, Spidlen, Brinkman, Roederer, & Chattopadhyay, 2016).

Gating out false positives: Dead cells

Dead cells (Fig. 5.4.30) are sticky and can masquerade as false positives due to nonspecific sticking of antibodies. A live/dead cell marker should be included to identify and eliminate the dead cells from analysis. Dead cells are usually identified by the permeability of their cell membranes; thus, dead cell stains can diffuse freely into the cell and attach to intracellular components, often DNA; dead cells will be positive for the marker. In unfixed cells, the most commonly used stains are DAPI, PI, or 7-AAD. Fixable live dead markers are used to identify initially viable cells which then will be fixed, permeabilized, and stained for cytoplasmic or nuclear markers.

Doublet discrimination

Another source of false positivity is the presence of doublets or aggregates (Fig. 5.4.31): two cells stuck together that pass

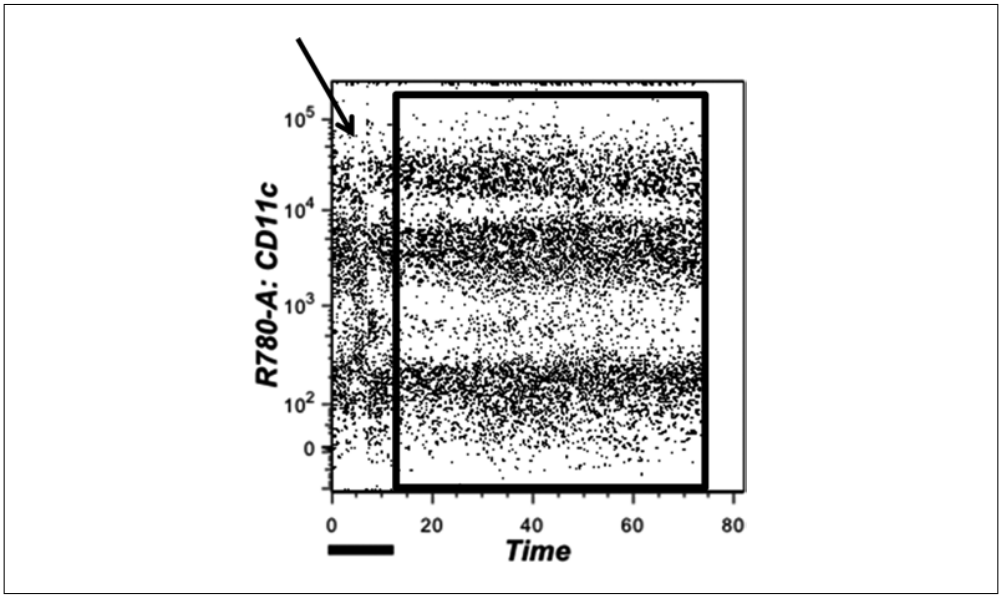

Figure 5.4.29 Time gate. Data aberrations caused by perturbations in fluidics during acquisition can be visualized by looking at the scatter or fluorescence parameters versus time. It can be seen here that data is compromised at the very beginning of the acquisition period (arrow). A gate is set to exclude these initial data points from subsequent analysis.

through the laser are seen as one cell. These cells can be two negatives or one positive and one negative, and will appear to be positive. When a doublet passes the laser, the voltage pulse generated by the detectors has a lower height and a larger width than that of a single positive cell with the same pulse area. The doublets can thus be eliminated by plotting pulse area versus pulse width or height and gating out the cells with higher width or lower height. It is never possible to eliminate all of the doublets, as only those that pass the laser in the correct orientation are distinguishable.

Fc Block

Cells with Fc receptors are able to bind the antibody/fluorochrome via the Fc portion of the antibody. These cells will appear as positive (Fig. 5.4.32). The staining is specific for the Fc receptor, but is undesirable; only the Fab′ binding specifically to the epitope of interest is desirable. The Fc-mediated binding can be blocked using a commercially available Fc block or by adding serum from the same species as the antibody source. For example, for the commonly used monoclonal antibodies raised in mice, this would be mouse serum. The serum contains antibodies that will block all of the Fc receptors on the cells, and the antibody of interest will then bind only through the Fab′ end specific for the antigen of interest. An isotype control at the panel development stage will indicate if this is a potential problem.

Other nonspecific staining

Some fluorochrome entities, the Cy dyes for example, are known to bind nonspecifically to certain cells. An isotype control can also be useful in this case.

A quick troubleshooting guide

It often happens, for beginners and sometimes advanced cytometrists, that you are in front of your cytometer, the tube is running, and there is either nothing or debris appearing on the screen. A few simple steps will help determine and resolve the problem, which stems from either (1) the cytometer, (2) the cells, or (3) the instrument settings.

1. *The cytometer:* The first step is to run quality control (QC) beads to verify that the cytometer is functioning correctly. The cytometer manufacturer's QC procedure or a manual procedure using standard QC beads can be employed. A manual procedure allows quick and frequent spot checks and can be easily set up with a template and settings to visualize QC beads in target channels on all detectors. If a problem is seen with the QC beads, it must be resolved before running the cells. Restarting both the cytometer and the computer will often solve a electronic connection problem. The most frequent problems seen are fluidics: lack of sheath pressure (someone has run the sheath dry) or a clogged sample line. Verify sheath pressure, purge air bubbles, run cleaning solution, then

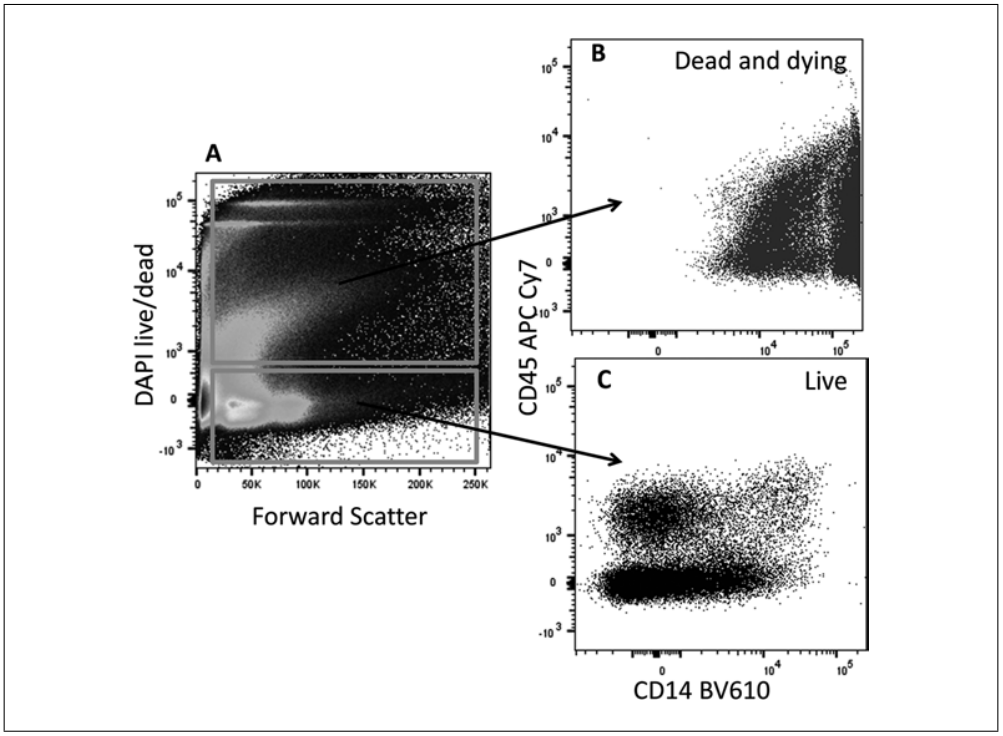

Figure 5.4.30 Dead cell elimination. Dead cells appear as false positive populations due to non-specific sticking of antibodies. In the extreme case shown here of a primary breast solid tumor biopsy (**A**), the plot on the left allows gates to be set on DAPI-negative live cells and DAPI-positive dead cells. Dead cells (**B**) have high nonspecific staining for the markers compared to live cells (**C**).

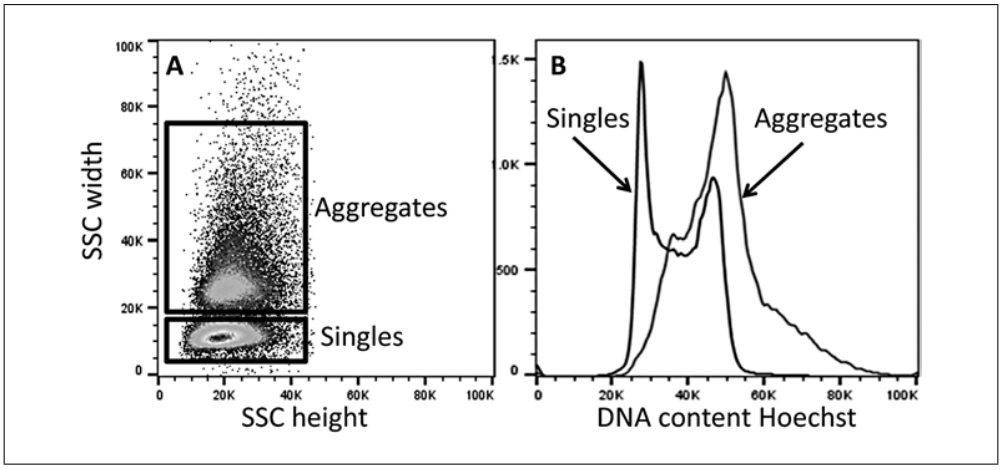

Figure 5.4.31 Doublet elimination. Aggregates of cells often appear as a false positive population, and should be excluded from analysis. Aggregates can be identified by the shape of their scatter or fluorescence pulse, with lower pulse height and higher pulse width than single cells. (**A**) Gates have been drawn on the side scatter width versus side scatter height plot to identify aggregates with high pulse width. (**B**) It can be seen that the aggregates have high fluorescence intensity and their inclusion would falsely increase the estimation of the higher DNA content population.

rerun the QC beads. If the problem doesn't resolve, a technical service call will be needed.

2. *The cells:* Look under the microscope to verify there really are cells in the tube. Addition of trypan blue will allow assessment of cell viability. Although there may have been many cells to start with, they are often lost with multiple fixation and staining steps.

3. *Cytometer settings:* After verifying that the cytometer is functioning properly, and that the cells are in the tube and intact, the problem could be the detector gain (PMT

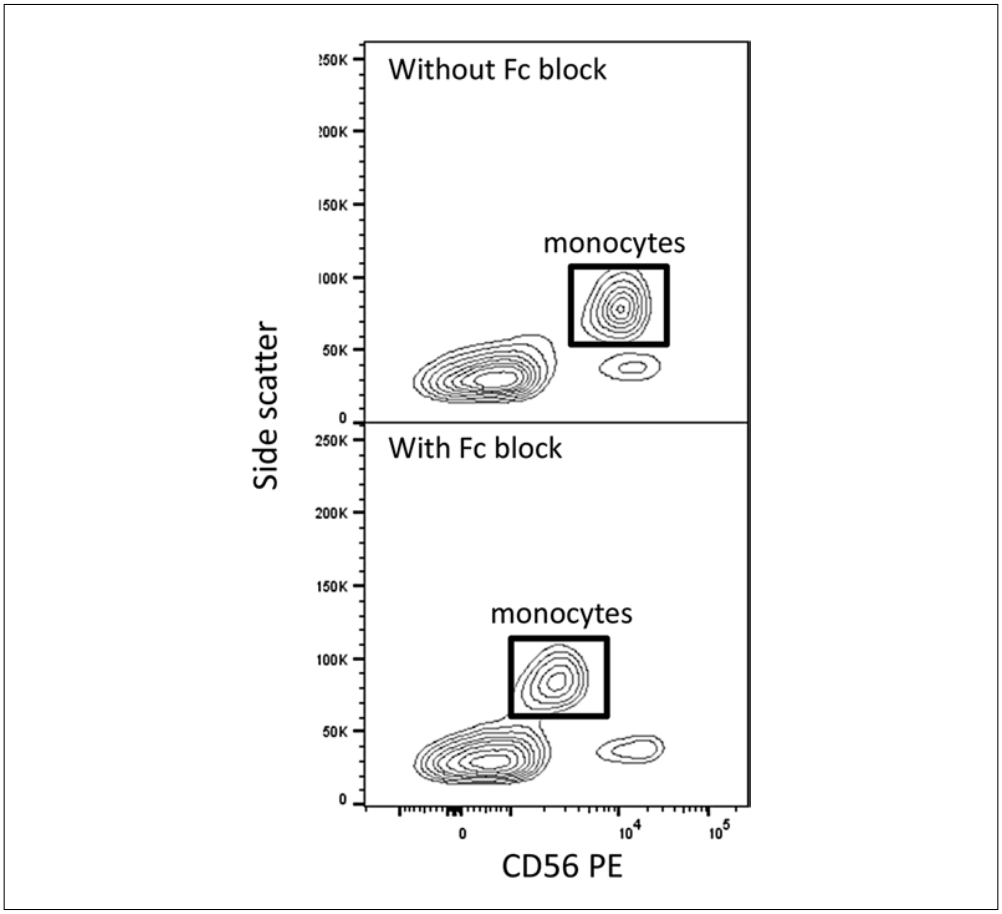

Figure 5.4.32 Blocking Fc receptors. Cells with Fc receptors can appear positive due to binding via the Fc portion of the antibody molecule. This false positivity is corrected by blocking the Fc receptor, either with Fc block or with serum Ig of the same species as the antibody. In the upper panel, the monocyte population can be seen to be brightly stained; in the lower panel, inclusion of an Fc block in the staining procedure has reduced this positivity.

voltages) and threshold settings. See the basic flow cytometry chapter in this section for an explanation of gain and threshold. The threshold setting, usually triggered on forward scatter, tells the cytometer what to consider as an 'event' or cell: anything below threshold will be not only excluded but will not appear on the screen. The cells must be above threshold or they will be invisible. Adjust the gain settings and threshold to ensure that the desired cells are above threshold. Backgating on a positive fluorescence population, CD3 for example, that identifies the cells of interest will help you set the correct gain and threshold on the trigger channel so that whole population is seen to be well above threshold on the forward scatter vs side scatter plot.

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Spectral viewers

http://www.biolegend.com/spectraanalyzer

BioLegend Spectral Viewer.

https://www.bdbiosciences.com/br/research/multicolor/spectrum_viewer/index.jsp

BD Biosciences Spectral Viewer.

https://www.thermofisher.com/fr/fr/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html#

Thermo Fisher spectral viewer.

Panel design programs

https://www.fluorish.com/

Fluorish panel design program.

https://fluorofinder.com/

Fluorofinder panel design program.

https://www.bdbiosciences.com/sg/paneldesigner/index.jsp

BD Biosciences panel design program.

Fluorochrome brightness

http://www.biolegend.com/brightness_index

Biolegend brightness index.

http://static.bdbiosciences.com/documents/multicolor_fluorochrome_laser_chart.pdf?_ga=1.193693357.1447862526.1480066966

BD Biosciences fluorochrome relative brightness.