Compensation in Flow Cytometry

The term "compensation," as it applies to flow cytometric analysis, refers to the process of correcting for fluorescence spillover, i.e., removing the signal of any given fluorochrome from all detectors except the one devoted to measuring that dye. The process of compensation is relatively simple in theory—but there are many subtle aspects that render it much more complex in practice.

Unfortunately, compensation in flow cytometry is perhaps one of the least understood processes accompanying data collection and analysis, perhaps because it is often described with the linear algebra elements needed for its computation, clouding the understanding of the fundamental process itself. Nevertheless, proper compensation is absolutely crucial for many aspects of flow cytometric analyses, particularly for the ever more popular antigen density determinations. Because compensation is often misunderstood, misapplied, and surrounded by so much incorrect mythology, many laboratories do not set compensation properly. One of the sections in this unit addresses some of the myths surrounding compensation.

Figure 1.14.1 illustrates some of the common errors in setting proper compensation. This unit is devoted to compensation in flow cytometry: why is it necessary, how is it accomplished in hardware or in software, how compensation affects the visualization of data and, finally, how best to design an experiment to achieve proper compensation. By the end of this unit, the reader should be able to set correct compensation for experiments and to recognize improperly compensated data in publications and the impact of that error on the interpretation of the results. Although a complete description of compensation requires some necessary linear algebra, understanding this math is not necessary for understanding proper compensation; the equations in the two sections can be skimmed or skipped at will.

A majority of flow cytometry users will not answer correctly all three of the questions posed in Figure 1.14.1. The correct answer to question 1 is "sample no. 2." For the second question, an answer including the use of the dashed quadrant lines is incorrect! And finally, the correct answer for the third question is: "It is impossible to determine which panel is properly compensated"—therefore, none of them can be said to be correct.

If proper compensation is so important for analysis of flow cytometric data, yet so few people know how to set proper compensation, why has flow cytometry produced such good data for so long? The simple answer to this question is that most types of data analysis do not absolutely require proper compensation. Another answer is that the problems associated with incorrect compensation are not as profound in two- or three-color analysis. However, with five-color (or higher) analysis quickly becoming available to many laboratories, these problems, if allowed to persist, can doom experiments.

For example, consider the graphs in Figure 1.14.2, which demonstrate that it is possible to compute subset frequencies properly even if the compensation settings are incorrect. However, these frequencies must be computed by comparison to a singly stained sample, not to a sample stained with isotype controls in every color! The correct gate can be set only on the sample stained with FITC-CD3 and PE-isotype control.

Figure 1.14.2 also illustrates how proper compensation is absolutely necessary for correct antigen density measurements. Here, any uncorrected spillover will contribute artifactually to the measurement. In addition, proper compensation is necessary when it is important to distinguish dim populations from negative populations; undercompensation will result in overestimating the frequency of the dim cells while overcompensation will result in underestimating their frequency.

Note that the ability to correctly calculate subset frequencies in improperly compensated data sets does not necessarily extend to three-color (or higher) experiments. Later in this unit, an example of this failure is shown. In addition, the appropriate staining controls that aid in the analysis of data (even when compensation is not completely correct) are discussed. These controls, termed "fluorescence minus one" or FMO controls, can be critical to the success of a multi-color experiment.

BASIC COMPENSATION

Compensation is the process by which fluorescence "spillover" between detectors is

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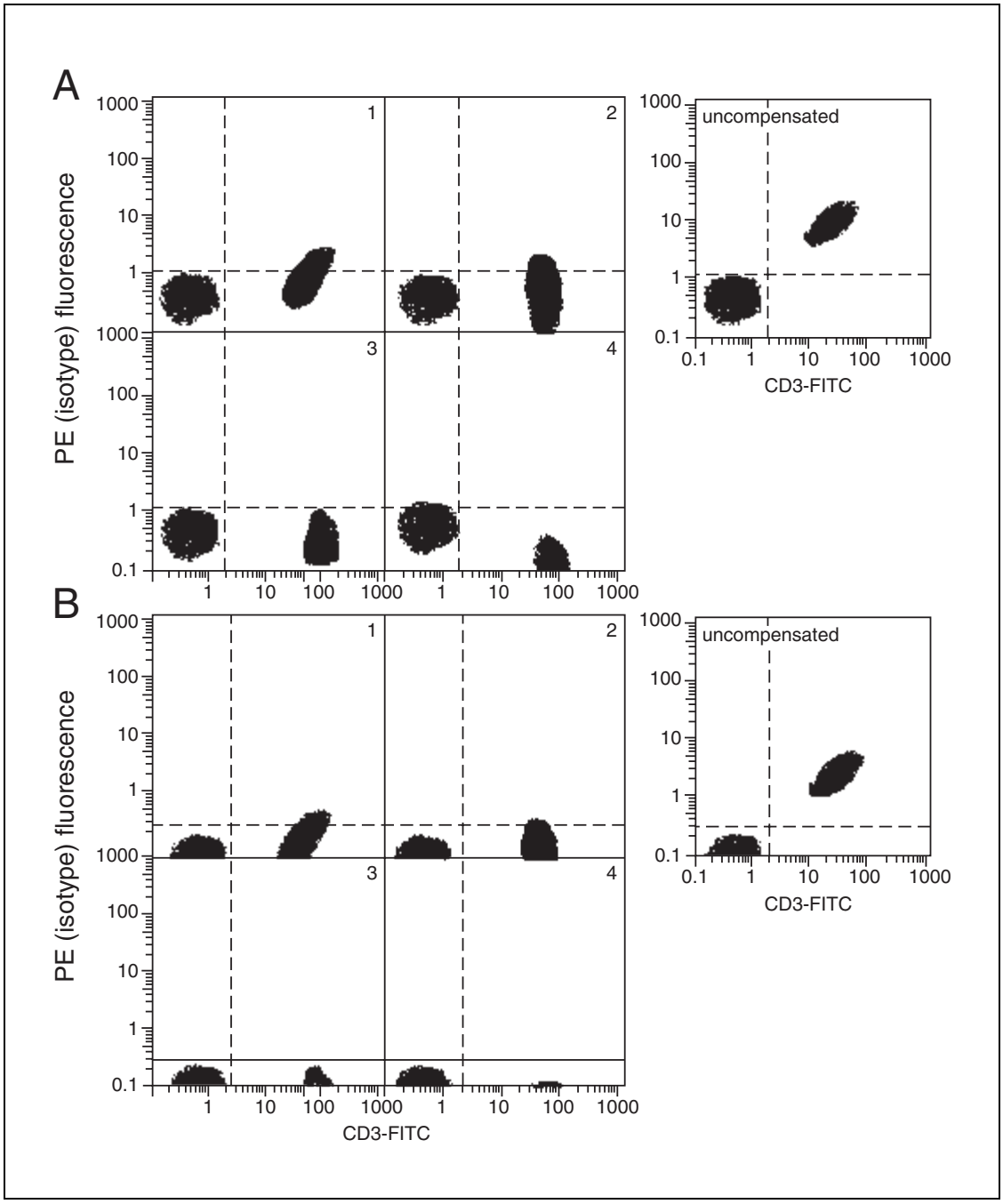

Figure 1.14.1 Can you determine which sample is appropriately compensated? In this hypothetical experiment, PBMC were stained with FITC-conjugated anti-CD3 and PE-conjugated isotype control. In both (**A**) and (**B**), the top graph shows the uncompensated data. Each of the numbered sections of the lower graphs represents the data as the compensation setting (between the FITC and PE detectors) is increased. In panel B, the data are shown for the same sample, with a much lower PMT voltage for the PE detector used during the collection. Answer three questions: (1) Which sample in panel A (no. 1, 2, 3, or 4) is properly compensated? (2) On what basis did you make this decision? (3) Which sample in panel B is properly compensated? See the introductory paragraphs of the text for the answers.

mathematically corrected. This procedure became necessary with the advent of two-color, one-laser analysis (Loken et al., 1977). Every fluorescent molecule emits light with a spectrum characteristic of that molecule. These emission spectra overlap, in some cases very significantly. For example, see the spectra shown in Figure 1.14.3 for fluorescein (FITC) and phycoerythrin (PE).

A two-detector system can be designed to discriminate FITC fluorescence from PE fluorescence. The emission light is split according to wavelength and distributed to the detectors, each of which has a different filter that eliminates the light within all but a narrow region of the spectrum. Thus, the FITC fluorescence is predominant in the detector with a 530-nm filter; the PE fluorescence is predominant in the detector with a 575-nm filter. However, some FITC fluorescence appears in the PE detector because of the emission overlap of these two fluors. This signal is termed spillover, because it spills over from the FITC detector to the PE detector. Note that it is impossible to design filter sets that will detect emissions from only FITC or PE; spillover will always occur when this dye combination is used.

Thus, whenever FITC is present, one will get a signal in the 530-nm band, and also some signal in the 575-nm band. Any PE present will also contribute to the 575-nm band. How then do we calculate how much of the 575-nm signal is from PE, and how much is from FITC? This is the process termed "compensation": i.e., correcting the PE detector signal for the amount of FITC fluorescence in the PE band.

One-Way Compensation

If a fluorescent dye emission is collected through two different light-collecting detectors that have different bandpass filters (i.e., detect light within different areas of the spectrum), one can always estimate how much emission is in one of these detectors based on how much is in the other. This is because the two signals will always vary proportionately (see Fig. 1.14.4). The constant proportionality means that we can exactly determine the area under the curve in the orange fluorescence detector (i.e., orange fluorescence signal) based on the area under the curve in the green fluorescence detector (green fluorescence signal).

The ratio of these two values is computed based on a sample that has no fluorescent molecules *except* FITC. This sample is termed a "compensation sample" or a compensation control, since it is key for determining proper compensation. For a typical flow cytometer, the FITC emission in the orange fluorescence detector is ~15% that in the green fluorescence detector. Therefore, if we subtract 15% of the green fluorescence signal from the orange fluorescence signal, then no matter how much FITC is present, the "corrected" orange fluorescence signal will always be zero.

That means that PE can now be added to the system, and the amount of signal in the orange fluorescence detector after subtracting 15% of the signal in the green fluorescence detector will represent the "true" PE fluorescence–irrespective of the presence or absence of FITC. This is "one-way" compensation: correction for the emission of a fluorophore in a second detector.

Two-Way (Pairwise) Compensation

From the spectra in Figure 1.14.3, it is apparent that PE also has an emission signal in the FITC (green fluorescence) detector. By collecting a sample of cells stained only with PE, one determines this ratio, which is typically ~2%. This is the compensation control for PE.

But if some of the PE emission shows up in the green fluorescence detector, and one uses the green fluorescence signal to estimate the amount of FITC emission in in the orange signal, won't this make it impossible to correct for the spillovers and compute the true fluorescences? An incomplete answer is to recognize that there are, in an n-color experiment, n unknowns (the amount of each dye present on a cell) with n measurements; this system can be solved exactly. Indeed, a bit of math shows that it is possible to do the compensation exactly (the reader may skip to the next section to avoid the equations).

${}^{x}F_n$ is defined as the amount of signal in detector n that originates from fluorophore x. Therefore, ${}^{f}F_1$ is the amount of fluorescein signal in the green FITC detector; ${}^{p}F_2$ is the amount of PE signal in the orange fluorescence detector. D_n is the measured signal in detector n. For the time being, assume that there is no background autofluorescence; autofluorescence will be dealt with later. Therefore:

$$D_1 = {}^{f}F_1 + {}^{p}F_1$$

Equation 1.14.1

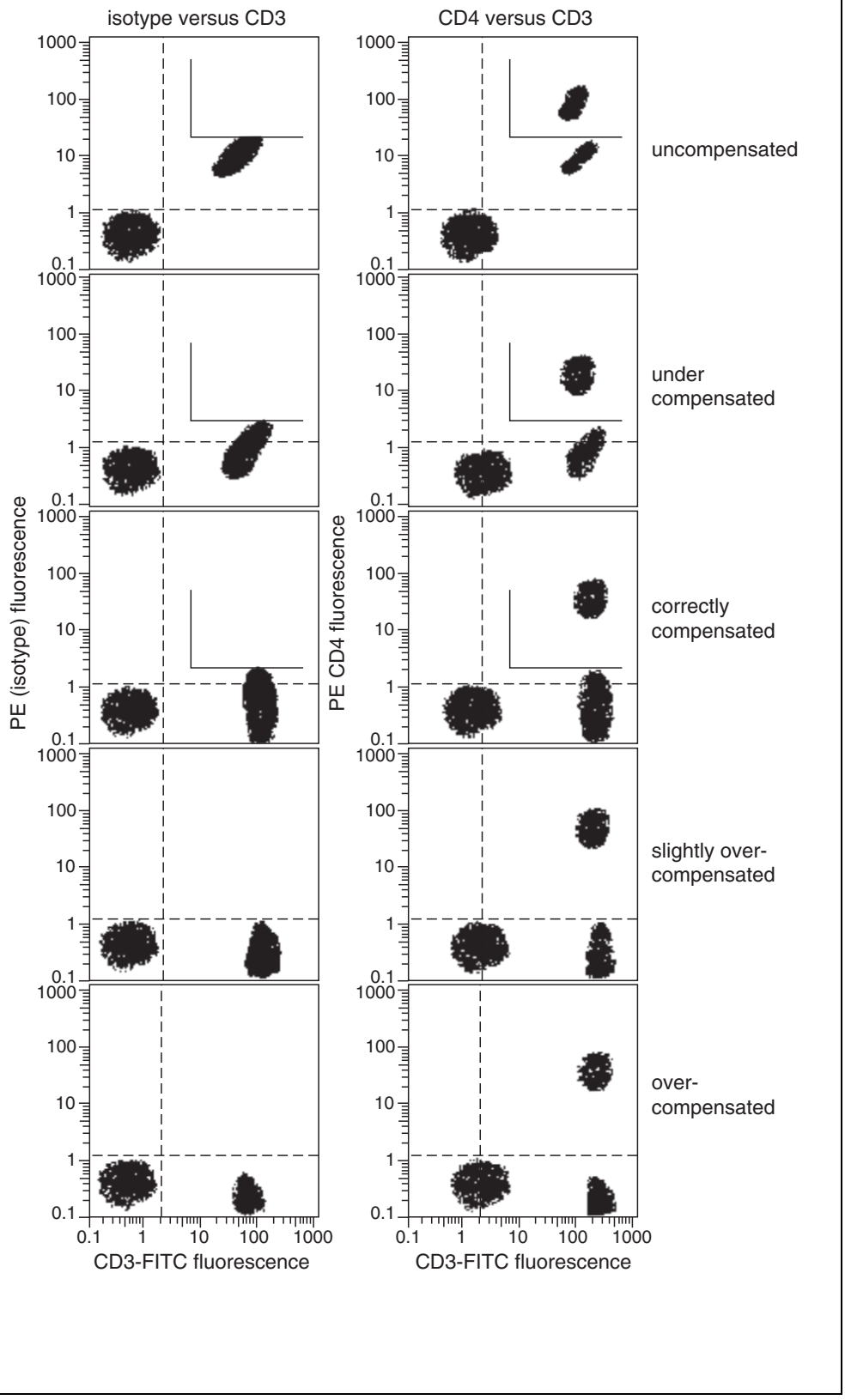

Figure 1.14.2 *(legend at right)*

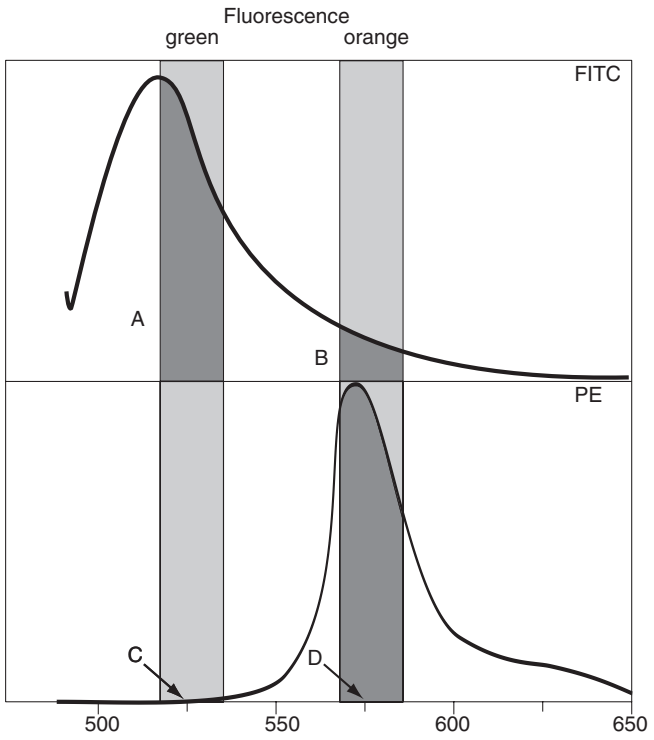

Figure 1.14.3 Fluorescence emission spectra for FITC and PE. The emission spectrum (the wavelengths of light generated by excitation of these molecules) is shown for an excitation at 488 nm (the same as the argon-ion laser line). FITC emission is maximal at ~515 nm; typically, a filter centered on 530 nm is used to collect the emitted light (shaded region). The emission of PE is farther red, with a maximum at ~575 nm; typically, a filter centered on this emission maximum is used to collect PE. Note that FITC has some emission in the wavelength bands used to collect PE fluorescence (**B**); typically, the amount of light in the 575-nm band is ~15% of that in the 530-nm band (**A**). The PE has very little emission in the 530-nm band (**C**), usually less than 2% of the emission in the 575-nm band (**D**).

Figure 1.14.2 (*left*) Incorrectly compensated data can often still be analyzed. These graphs represent data collections of PBMC stained with CD3-FITC and either CD4-PE or a PE-isotype control. The top graphs are uncompensated; the FITC into PE compensation setting is increased for each successive panel. The quadrant lines (dashed lines) represent what would be set based on an unstained sample (or a sample stained with isotype controls for both colors). The solid line represents a gate set based on the singly-stained isotype control from the left panels. Note that the computation of the percentage of CD3 cells that express CD4 ($CD3^+CD4^+$) can be correctly performed on any of the panels irrespective of the compensation setting—but *only* if the gate is set based on the singly-stained sample (left). The quadrant setting based on a complete isotype control stain (dashed lines) would result in incorrect frequencies. Only the correctly compensated sample (middle panels) shows the correct amount of CD4 fluorescence on the $CD3^+CD4^-$ cells (i.e., no different than autofluorescence); the undercompensated samples have apparent CD4 fluorescence and the overcompensated samples have apparent negative fluorescence. Thus, antigen density measurements are particularly sensitive to improper compensation settings.

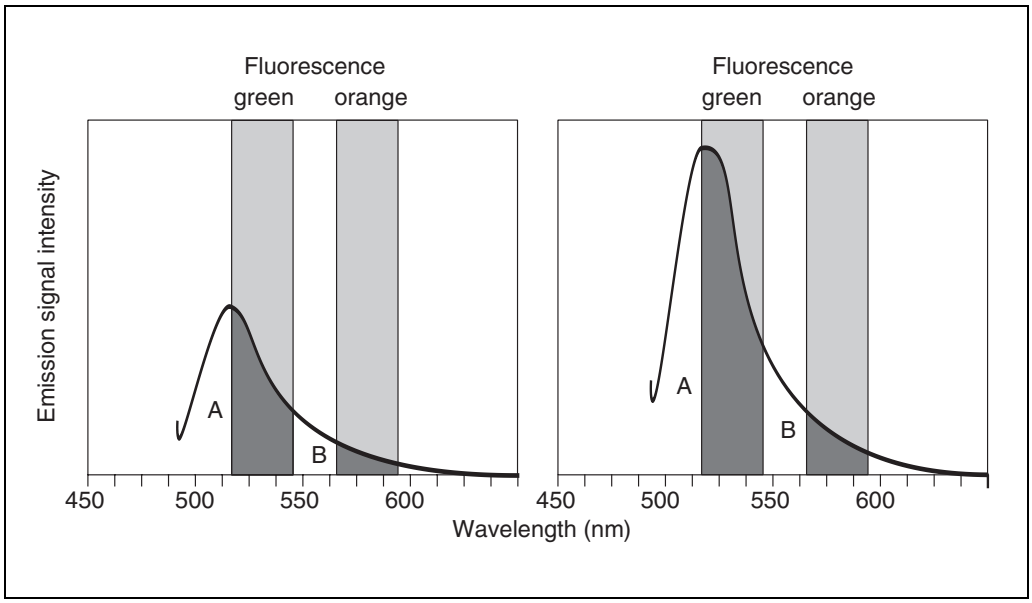

Figure 1.14.4 Emission spectra as a function of concentration (or amount) of dye. The FITC emission spectrum is shown for two samples; the right panel has twice the concentration of FITC as the left. The ratio of emissions in the two detector bands, $r = B/A$, is the same irrespective of the amount of dye. After this ratio is determined for one sample, the signal B in a different sample can be determined simply by measuring A and multiplying by r.

$$D_2 = {}^{f}F_2 + {}^{p}F_2$$

Equation 1.14.2

The amount of fluorophore x is proportional to ${}^{x}F_n$ (where n is the primary detector used to collect that fluorophore's emission); thus, to know how much FITC or PE there is in the sample, one needs to calculate ${}^{f}F_1$ and ${}^{p}F_2$.

It is known (from Fig. 1.14.4) that the ratio of ${}^{f}F_1$ to ${}^{f}F_2$ is a constant, as is the ratio of ${}^{p}F_2$ to ${}^{p}F_1$, and these constants of proportionality are defined as follows:

$${}^{f}S_2 = \frac{{}^{f}F_2}{{}^{f}F_1}$$

Equation 1.14.3

$${}^{p}S_1 = \frac{{}^{p}F_1}{{}^{p}F_2}$$

Equation 1.14.4

Therefore, ${}^{x}S_n$ is the spillover coefficient for fluorophore x between the primary detector for the fluorophore and detector n. This value is determined from the compensation controls, where the sample fluorescence comes only from a single fluorophore. For example, for the FITC compensation control:

$$D_1 = {}^{f}F_1 + {}^{p}F_1 = {}^{f}F_1 + 0 = {}^{f}F_1$$

Equation 1.14.5

$$D_2 = {}^{f}F_2 + {}^{p}F_2 = {}^{f}F_2 + 0 = {}^{f}F_2$$

Equation 1.14.6

$${}^{f}S_2 = \frac{{}^{f}F_2}{{}^{f}F_1} = \frac{D_2}{D_1}$$

Equation 1.14.7

Thus, the FITC spillover coefficient is the ratio of the signals measured in each detector for the FITC-stained sample. Similarly, the PE compensation control can be used to calculate:

$${}^{p}S_1 = \frac{{}^{p}F_1}{{}^{p}F_2} = \frac{D_1}{D_2}$$

Equation 1.14.8

Note that the values for D_1 and D_2 in Equation 1.14.8 will be different from those used in Equation 1.14.7, since they represent the signals measured for the PE compensation control.

Once the spillover coefficients are known, they can be used on the costained samples to calculate the "true" fluorescences ${}^{x}F_n$. Rewriting equations 1.14.5 and 1.14.6 above:

$$D_1 = {}^{f}F_1 + {}^{p}F_1 = {}^{f}F_1 + \left({}^{p}F_2 \times {}^{p}S_1 \right)$$

Equation 1.14.9

$$D_2 = {}^{f}F_2 + {}^{p}F_2 = \left({}^{f}F_1 \times {}^{f}S_2 \right) + {}^{p}F_2$$

Equation 1.14.10

These two equations have two unknowns (${}^{f}F_1$ and ${}^{p}F_2$), which can be solved exactly:

$${}^{f}F_1 = \frac{D_1 - \left({}^{p}S_1 \times D_2 \right)}{1 - \left({}^{p}S_1 \times {}^{f}S_2 \right)}$$

Equation 1.14.11

$${}^{p}F_2 = \frac{D_2 - \left({}^{f}S_2 \times D_1 \right)}{1 - \left({}^{p}S_1 \times {}^{f}S_2 \right)}$$

Equation 1.14.12

PAIRWISE COMPENSATION ON FLOW CYTOMETERS: THREE-COLOR COMPENSATION

When performing compensation on an instrument, compensation controls are typically used to set the appropriate compensation coefficients. In the case of pairwise compensations (i.e., compensating for spillover between two detectors), the compensation coefficients are essentially the same as the spillover coefficients defined above. Thus, when compensating between FITC and PE, for example, one would use a FITC-stained sample to set the green fluorescence into orange fluorescence compensation to correct for FITC spillover into the PE detector, and then use a PE-stained sample to set the orange fluorescence into green fluorescence compensation.

Most laboratories now perform three-color experiments, using a combination of fluorochromes such as FITC, PE, and PE-Cy5 or PerCP as the third color. There is a significant spillover from PE into the red fluorescence detector, and minor yet detectable spillover from the red fluorescence into the orange. Thus, most instruments provide for two pairwise compensations: between green fluorescence and orange fluorescence and between orange fluorescence and red fluorescence. This compensation is performed as a simple extension of the two-color compensation noted above; i.e., with two sets of two-color compensation settings.

For example, after performing the FITC-PE compensations, one would turn to PE-PE-Cy5. The PE compensation sample is used to set the orange fluorescence into red fluorescence compensation setting, and the PE-Cy5 compensation sample is used to set the red fluorescence into orange compensation setting. This process results in four compensation coefficients, two for each pairwise compensation.

Note that this process does not directly correct for the amount of FITC fluorescence in the red fluorescence detector (nor vice versa), and is therefore an incomplete compensation. Is this a problem? Often, the answer is no, but one must be careful because it can become a significant problem. To determine if this is a problem, display the FITC versus PE-Cy5 detectors after setting compensation, for the FITC and the PE-Cy5 compensation controls. Spillover between the green and red fluorescence detectors means uncorrected compensation that can significantly affect the data. The only way that one will be able to correct for this will be to use software compensation after data collection.

COMPLETE COMPENSATION

This section extends the equations given above to provide the complete solution to compensating any number of detectors. To avoid the mathematics, the reader may skip to the next section (see Autofluorescence).

The general solution to compensating n detectors is an extension of the equations above. The procedure is much the same: for each fluorophore, the spillover coefficient between the primary detector for that fluorophore and every other detector being measured is determined based on the compensation control for that fluorophore. Extension of equation 1.14.1 to n fluorophores (again, ignoring the contribution of autofluorescence):

$$\begin{aligned} D_1 &= {}^{1}F_1 + {}^{2}F_1 + {}^{3}F_1 + \ldots + {}^{n}F_1 \\ D_2 &= {}^{1}F_2 + {}^{2}F_2 + {}^{3}F_2 + \ldots + {}^{n}F_2 \\ &\vdots \\ D_n &= {}^{1}F_n + {}^{2}F_n + {}^{3}F_n + \ldots + {}^{n}F_n \end{aligned}$$

Equation 1.14.13

Or, more simply:

$$D_m = \sum_{i=1}^{n} {}^{i}F_m$$

Equation 1.14.14

Likewise, we know that the contribution of fluorophore *i* to detector *m* will be equal to the contribution of fluorophore *i* in detector *i* multiplied by the spillover coefficient ${}^{i}S_m$. Note that, by definition, ${}^{i}S_i = 1$. We define the "true" fluorescence of fluorophore *i* as T_i:

$${}^{m}F_i = T_i \times {}^{m}S_i$$

Equation 1.14.15

Therefore, we can rewrite equation 1.14.14 in terms of the spillover coefficients and the "true" fluorescences:

$$D_m = \sum_{i=1}^{n} (T_i \times {}^{m}S_i)$$

Equation 1.14.16

In linear algebra terms, *D* is the vector of measured fluorescences, *T* is the vector of true fluorescences, and *S* is the matrix of spillover coefficients whose diagonal elements are all 1:

$$D = T \times S$$

Equation 1.14.17

To determine the values in *T*, equation 1.14.17 can be solved by premultiplying by the inverted matrix of *S*:

$$S^{-1} \times D = S^{-1} \times T \times S$$

Equation 1.14.18

$$S^{-1} \times D = T$$

Equation 1.14.19

Complete compensation, therefore, requires an inversion of the spillover coefficient matrix followed by multiplication by the measurement vector. The inverse of the spillover matrix is also termed the compensation matrix; the elements of the compensation matrix are the compensation coefficients. Note that the compensation coefficients are therefore different from the spillover coefficients!

The spillover coefficients are closely related to the spectrum of a fluorophore: they convey the amount of a fluorophore's emission in each of the detectors. The compensation matrix tells how much of each detector's value must be subtracted in order to determine the final calculated true fluorescence. Table 1.14.1 and Table 1.14.2 illustrate the difference between these two for a simple three-color matrix, for an experiment utilizing the fluorophores FITC, PE, and PE-Cy5.

The incomplete compensation that is typically done on instruments, using pairwise compensations between green/orange and orange/red, would be identical to this matrix with the exception that the corner (nondiagonal) elements would have values of 0.0.

Note that while the spillover from FITC to PE-Cy5 is 4% (Table 1.14.1), the actual compensation value needed is only 0.17% (Table 1.14.2), because of the similarity between FITC and PE in terms of the ratio of orange to red signal (i.e., from Table 1.14.1, for FITC it is 0.04/0.18 = 0.22 and for PE it is 0.21). Thus, the compensation of orange into red for PE removes the FITC component from the red fluorescence. (Incidently, this this fortuitous relationship explains why most experiments do not require an explicit compensation between FITC and PE-Cy5—most of this compensation is taken care of by the combination of FITC to PE and PE to PE-Cy5.)

AUTOFLUORESCENCE

Autofluorescence throws a small kink into compensation, but, as it turns out, does not change one's ability to deconvolute spillovers. Cellular autofluorescence is present in all detectors to varying extents, and provides a background that varies from cell to cell. There are three ways to deal with autofluorescence.

One way is to devote a single detector to autofluorescence measurement. Because the autofluorescence spectrum of similar cells is generally identical, autofluorescence can be treated as just one more type of fluorescent molecule. Then, by compensation, one can actually correct for the contribution of autofluorescence to all detectors. This process can significantly enhance sensitivity for detection of low-density antigens. Autofluorescence compensation is more fully described in published references by Roederer and Murphy (1986) and Alberti et al. (1987).

This process works best for cell types that have a lot of autofluorescence (like cultured cell lines, fibroblasts, or large highly cytoplasmic

Table 1.14.1 Typical Spillover Matrix for a Three-Color Compensation[a]

Fluorophore	Detector		
	Green	Orange	Red
FITC	1.000	0.180	0.040
PE	0.009	1.000	0.213
PE-Cy5	0.005	0.029	1.000

[a]Note: The diagonal elements are 1, since the contribution of each fluorophore to its cognate detector is defined to be 100%. In this table, the FITC into PE spillover is 18%; the PE into FITC spillover is 0.9%.

Table 1.14.2 Typical Compensation Coefficient Matrix for Three-Color Compensation[a]

Fluorophore	Detector		
	Green	Orange	Red
FITC	1.0000	–0.1802	–0.0017
PE	–0.0079	1.0000	–0.2126
PE-Cy5	–0.0047	–0.0281	1.0000

[a]Note: The matrix in Table 1.14.1 has been inverted. These values correspond to what would be set on an instrument for performing pairwise compensation (after negation; i.e., the green to orange compensation is 18.02%, and to red it is 0.17%).

cells). It does not work very well for lymphocytes, nor does it work well for channels where the signal level is very low (like the red fluorescences). This is because the error in the ability to measure the autofluorescence exceeds the actual signal level; hence, the correction process does not help at all.

The second way is an exact mathematical treatment of autofluorescence. This requires collection of one more control sample: an unstained sample. The mathematics required to do this calculation is no longer linear, but affine. This topic is dealt with in detail by Bagwell and Adams (1993). Note that the limitations of autofluorescence correction noted above (i.e., trying to work with cell types that have very low autofluorescence, like lymphocytes) apply to this method equally.

The third way to deal with autofluorescence is to simply ignore it: applying the matrix algebra described above still works to make the detector measurements independent, such that only autofluorescence and the specific fluorescent molecule of interest contribute to each detector. The only effect that this has on the measurement is that the absolute amount of autofluorescence is no longer comparable to uncompensated (and unstained) cells.

However, this is virtually never a problem; the absolute amount of autofluorescence is in most cases irrelevant. In fact, the "true" fluorescence for a given cell population is then simply determined by subtracting the mean (or median) fluorescence intensity (MFI) for unstained cells from the MFI for stained cells, when both are collected with the same compensation settings; in fact, this "true" fluorescence is correct, independent of the presence or absence of autofluorescence.

Standard compensation in the presence of autofluorescence, therefore, works just fine: the resulting values are independent of the presence of other reagents, and are proportional to the amounts of the fluorescent molecules present.

Note, however, that if the autofluorescence of the stained cells in the compensation sample is different than that of the unstained cells in the compensation sample, then the computed compensation will be incorrect! Thus, one could not use CD14-FITC, which stains highly autofluorescent monocytes, to compensate

against unstained lymphocytes, which have low autofluorescence.

RESONANCE ENERGY TRANSFER (TANDEM) DYES

In the late 1980s, PE-Cy5 became the first resonance energy transfer (RET) dye to be regularly used in flow cytometric analysis. RET dyes are comprised of two covalently linked fluorophores having the property that the emission spectrum of one (the donor) overlaps with the excitation spectrum of the other (the acceptor). Under the appropriate conditions, the tandem dye has spectral properties that consist of a fusion of the properties of the individual dyes: an excitation spectrum that is the sum of the donor and acceptor but an emission spectrum that is principally that of the acceptor (*UNIT 1.12).*

In the particular case of PE-Cy5, the tandem is excited by the 488-nm argon laser line, exciting the PE molecule, but emits light at ~680 nm. The energy of excitation of the PE is transferred to the Cy5 before the PE itself emits light with its own characteristic spectrum, ~575 nm. Cy5 itself is not excited by the 488-nm line directly.

Were the efficiency of energy transfer between PE and Cy5 perfect, then the pair could be considered to be simply a well-characterized single fluor with a defined emission spectrum. However, the efficiency of transfer is never 100%, and, in fact, can vary significantly between different lots of Cy5PE. This variation has significant implications for compensation.

Inefficient transfer from donor to acceptor means that the residual energy is emitted directly by the donor. In the case of the Cy5PE tandem, this appears as normal PE emission, ~575 nm. The less efficient the tandem, the more 575-nm emission occurs. This is manifested by a greater spillover between the 680-nm detector and the 575-nm detector, requiring greater compensation.

Because of lot-to-lot variation of Cy5PE conjugates, this means that different compensation values can be necessary for each lot of a tandem. While the compensation may be correct for one tandem, another may be undercompensated or overcompensated.

To date, manufacturers have achieved a quality control in the manufacture of tandem dyes such that most conjugates from a single manufacturer are very similar and require essentially the same compensation value. However, this is not the case for tandems from different manufacturers.

In general, when several different tandem conjugates in a single experiment are used, compensation controls should be generated for each in order to determine whether or not a single compensation value can be used for all tandems. If the tandems are sufficiently different that this is not the case, then the compensation must be adjusted individually for each different conjugated tandem.

This problem can be a minor nuisance when only a single tandem type is used. However, in the last few years a number of different tandems have been introduced for use in flow cytometry. Almost certainly, it will be impossible to guarantee identical spectral properties for all different conjugates of these many tandems (e.g., PE-Texas Red, ECD, PE-Cy7, APC-Cy7). Therefore, it will be necessary to be able to apply compensation matrices specifically calculated for each lot of tandem used in any given panel. This can be achieved only with significant software support, either by selective modification of the hardware compensation values depending on the stains used for each tube, or by applying the appropriate coefficients during analysis (software compensation).

In any case, it is important to remember that the use of tandem dyes in flow cytometry introduces special problems for compensation. Each different tandem used in an experiment must be carefully characterized to determine whether a common compensation value can be used or whether compensation values must be tailored to each conjugate.

COMPENSATION: EFFECT ON VISUALIZATION OF DATA

Compensation has a profound impact on the visualization of data. After all, the goal of compensation is to remove the covariation in two measurements to provide a display for which the measurements are independent (and hopefully show "rectilinear" distributions—i.e., distributions are found vertically or horizontally displaced from unstained cells in the absence of co-expression of markers). However, this is often not the case, particularly as the far-red dyes (e.g., Cy7 tandems, APC tandems) are used more often. Indeed, compensation leads to visualizations of the data that appear wrong to most users (Fig. 1.14.5 and 1.14.6). A full description of this artifact is found in Roederer (2001); a summary is provided here.

As illustrated in Figure 1.14.5, the process of compensation cannot change the "width" of the uncompensated distribution. Ideally, the uncompensated events would lie on a very tight

line extending at a 45° angle from the unstained population. In reality, the events fall about this line. This distribution away from the line is driven by errors in the measurement process. While there are small errors in the electronics that can contribute to this spread, the most significant source of error is photon-counting statistics (Roederer, 2001).

Any fluorescence measurement is performed by integrating the number of photons collected from the cell as it passes through the laser. In the deeper-red channels, the number of events actually counted can be relatively small—a few dozen or hundred. A fundamental aspect of counting events is that the minimum error (standard deviation) associated with any count is equal to the square root of the number of counts. Therefore, a cell with 100 photon events at the detector has an associated error in this measurement of ±10%; i.e., running many cells with exactly the same number of fluorescent molecules through the system will give a distribution that has a width of at least 20% of the signal level. It is this counting error that leads to the spreading of the distribution in the uncompensated cells.

Unfortunately, one aspect of the log-log display commonly used to view fluorescence data is that an apparently small distribution at high-signal levels becomes enormous when shifted down (by the linear compensation process) to low-signal levels. This is illustrated in Figure 1.14.5.

It is important to be able to distinguish uncompensated (or under-compensated) data from properly compensated data. Note that the relationship between two detectors for uncompensated data (and under-compensated data) is linear, because of the proportional relationship of spillover (Fig. 1.14.4). In a log-log display, a linear relationship is a straight line with exactly a 45° slope. After compensation, the spreading in the distribution is related to the square root of the signal (because counting error is proportional to the square root of the signal). A square-root relationship in a log-log plot is a straight line with a 22.5° slope. Note that the slope of the right graphic in Figure 1.14.5 has a shallow slope (1:2 decade/decade), compared to the left graphic (1:1). This can aid in understanding whether data may be properly compensated.

Finally, it is crucial to realize that the spread in the properly compensated distribution cannot be "corrected." This spread arises from fundamental counting errors that contribute to the *minimum* possible measurement error. Furthermore, this spread is a nonlinear relationship (square-root); compensation is a linear process. Thus, simply turning up the compensation value will not straighten out this distribution and will only lead to problems with data analysis. Again, for a full discussion of why overcompensating the data cannot help, see Roederer (2001).

GATING AND ANALYSIS CONTROLS: FMO CONTROLS

Given that properly compensated data can show a spreading distribution into other detectors, it becomes apparent that discriminating positive from negative events is no longer a simple process of selecting a single threshold for positivity. The distribution shown in the previous discussion is reproduced in Figure 1.14.6A, for the purpose of asking the question: How do we identify cells that would be positive for an APC-Cy5.5 (*y*-axis) stain? For this purpose, the author introduces the concept of fluorescence-minus-one (FMO) gates.

In trying to determine the optimal gate position for a given color, it should be apparent that the best control on which to base this position is one in which the same cells have been stained with everything *except* the one reagent of interest. After all, this is the most rigorous control as only one variable has changed between the two samples; therefore, any differences between the two distributions can be ascribed solely to the addition of the reagent of interest. This control sample, which has every fluorescence except one, is termed an FMO control; any gate based on this sample is an FMO gate. A one-dimensional (1-D) FMO gate is defined as the gate that best discriminates positive from negative events in the channel of interest when viewing a (1-D) histogram of that channel (i.e., at the high end of the fluorescence distribution of the FMO sample). A 2-D FMO gate would be that devised on a two-dimensional graphic of the parameter of interest versus another parameter for the FMO sample. Figure 1.14.6 contains several examples of FMO samples and gates to illustrate this process.

In Figure 1.14.6A, the question was how to best identify positive events. Note that events that are below the putative isotype gate (a gate defined on a fully unstained sample) are easily identified as negative (open circles). However, at least some events that are stained only with CD57-APC would be considered positive with this gate—they rise over the gate because of the spreading in the distribution (see Fig 1.14.5).

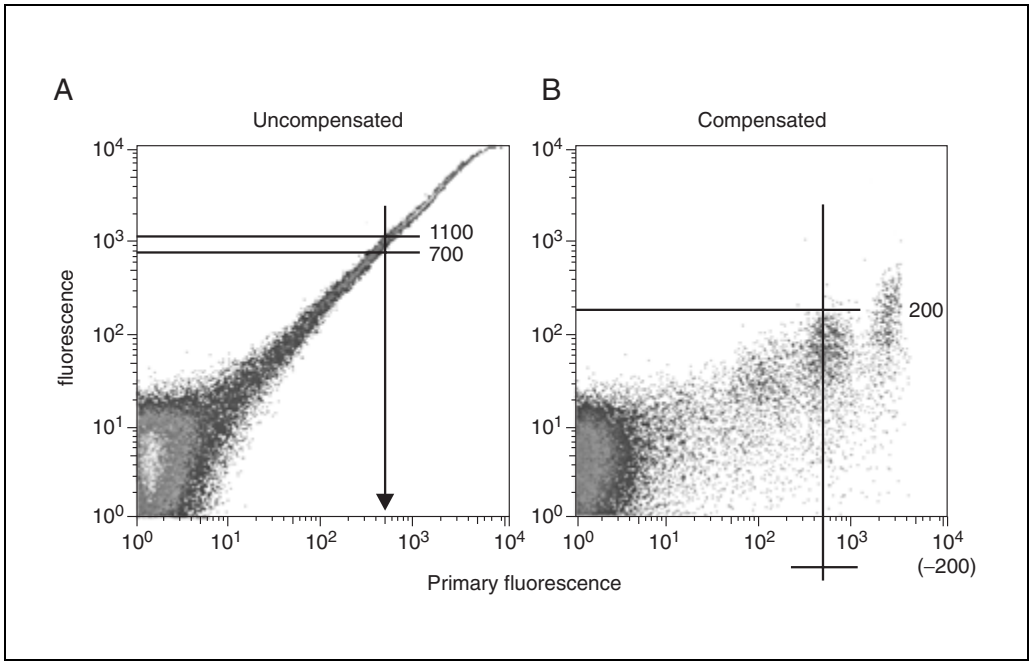

Figure 1.14.5 Measurement errors lead to spreading of properly compensated distributions. This example shows data collected for PBMC stained with APC-conjugated anti-CD57. This antigen shows a "smear" of events from negative to very bright, leading to an excellent visualization of compensation. (**A**). Uncompensated distribution of the APC signal versus the APC-Cy5.5 signal. There is considerable spectral overlap between these two channels; in addition, as they are in the far red, the number of actual photons counted is relatively small. The goal of compensation is to remove the contribution of the primary fluorochrome from the spillover channel (arrow). Note that compensation is a linear process, in which the amount to subtract from any given cell is based on the primary fluorescence value. Thus, every cell on the line of the arrow will have the same amount of fluorescence removed from it, an amount that is proportional to the primary fluorescence value (i.e., proportional to ~500). Since the same amount has to be subtracted from every cell on the line, the vertical width of the uncompensated distribution cannot change in absolute amounts. The bottom of this distribution is at ~700 fluorescence units, the top at ~1100. Therefore, after compensation, the vertical width of this distribution must still be 400 units. (**B**) The same data, after correct compensation. Note that the vertical width of the distribution at the line is still 400 units, centered at ~5 ± 200. All events below a value of 1 are forced onto the axis; hence the accumulation of a large number of events at the very bottom. The distribution must extend all the way up to 200. The width of this distribution is determined principally by photon-counting statistics (see text).

Therefore, a sample that is stained with everything except APC-Cy5.5 is used to define the limit of the negative population (FMO control). Note that the 1-D FMO gate (drawn on a histogram of APC-Cy5.5 for the FMO sample, shown as the upper dotted line in Fig. 1.14.6) would be accurate in that all events above this gate would truly be positive for APC-Cy5.5. However, this gate misses many events that are in fact positive—for example, the left-most event in the middle pair (shaded circle). The 2-D FMO gate, which curves upward with the spread of the negative events, can accurately discriminate the positive events from those events that are in the negative distribution and are likely negative.

Note that the APC-Cy5.5 fluorescence of the two central events is the same, yet one is clearly positive and the other is in the negative distribution. Thus, within the APC-Cy5.5 distribution, there are areas in which both positive and negative events can overlap. The multivariate approach to FMO gating will always be superior for identifying a greater fraction of positive events.

Figure 1.14.6B illustrates the use of the FMO gating to most accurately enumerate CCR5-positive T cells. This example shows clearly that the threshold for discriminating CCR5 positivity will depend on the amount of CD8-APC-Cy7 fluorescence, the threshold for positive cells is higher for the $CD8^+$ T cells than for the $CD8^-$ T cells. This example shows why

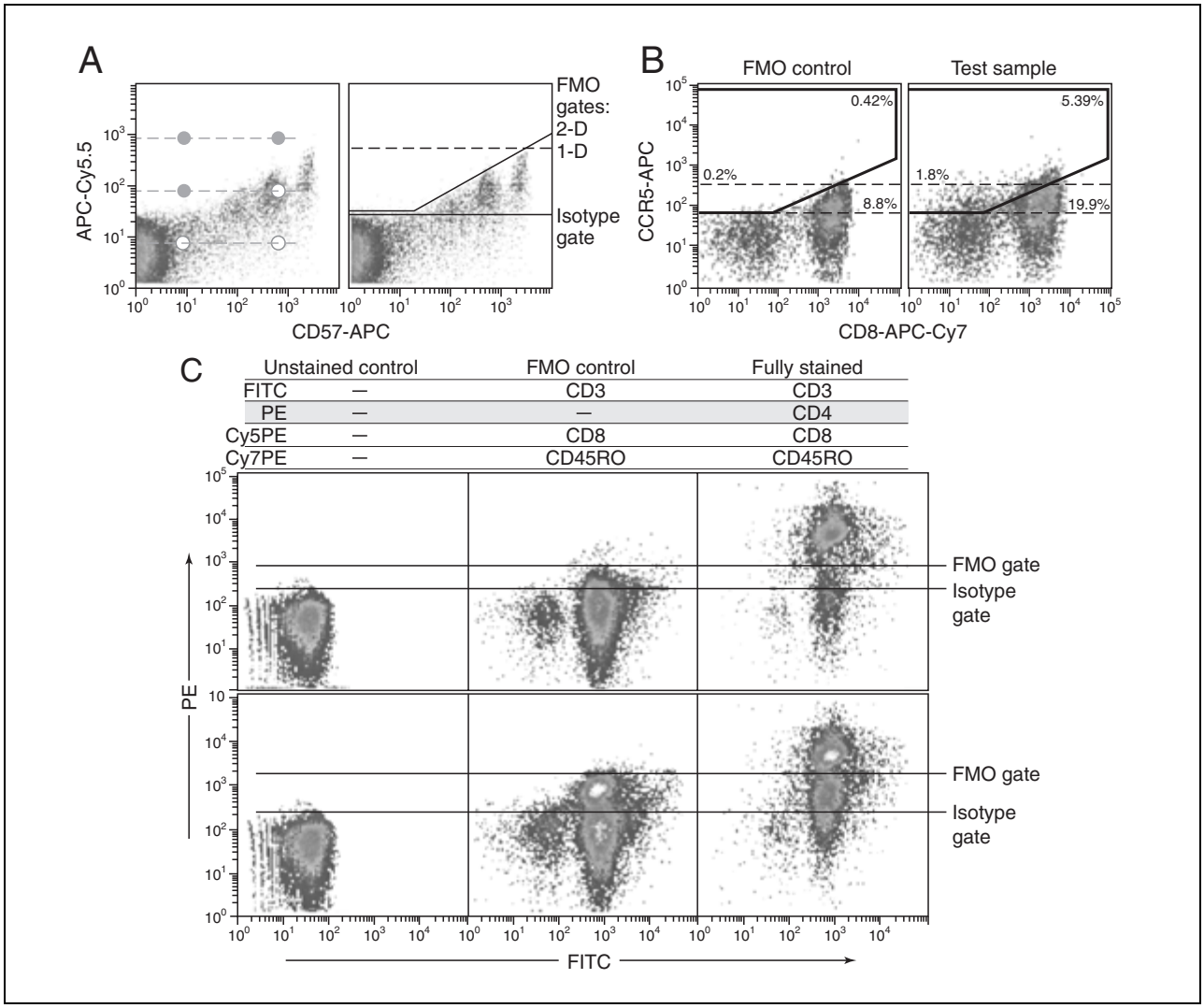

Figure 1.14.6 Fluorescence Minus One (FMO) gates are an accurate way to identify positive versus negative events. See text for full discussion. (**A**) The distribution as shown in Figure 1.14.5 is reproduced here. In these two graphs, cells were stained only with CD57-APC. The isotype gate is that defined by an unstained (or fully isotype-stained) sample. The 1-D FMO gate is defined by the CD57-APC-stained sample, examining only the distribution of APC-Cy5.5 and setting the threshold above all events. The 2-D FMO gate is defined by the limit of APC-Cy5.5 distribution when viewing this two-dimensional graph. (**B**) Human PBMC were stained and gated for CD3 (not shown), CD8-APC-Cy7, with (right) and without (left) CCR5-APC. The expression of CCR5 is dim, and accurate discrimination of positive and negative events is necessary. Percentages show the fraction of CD8 T cells within each "positive" gate: the isotype gate (lower dotted line), the 1-D FMO gate (upper dotted line), and the 2-D FMO gate (solid polygon). (**C**) A four-color (single-laser-excited) staining combination is used to illustrate the utility of FMO gates. The goal in this illustration is only to identify the CD4 T cell population accurately. The stains used for each sample are listed in the table above the graphs. In the upper 3 graphs, the data are properly compensated. The lower 3 graphs represent exactly the same data, except that the PE-Cy7 into PE-Cy5 compensation setting is off by 20%. Note that the fact that this compensation was incorrect is not evident, as neither of these channels is viewed. The effect on the PE channel of this incorrect compensation is due to the interaction of compensation settings across channels (were compensation set by spillover values instead of compensation values, this would not occur; hence, the recent trend by manufacturers to provide control over compensation via the spillover domain). In this sample, the CD8 T cells (PE-Cy5$^+$) have uncorrected fluorescence in the PE channel and show up as a separate, dull population. The "isotype" gate is that defined by the fully isotype-stained sample (far left); the FMO gate is that defined by cells stained with everything *except* CD4-PE. Without the FMO gate, it would be nearly impossible to know where to set the discriminating gate.

full isotype-stained cells are inadequate and in the sample with no CCR5-APC, the isotype-based gate shows 8.8% positive events! The difference between a 1-D FMO gate (1.8%) and a 2-D FMO gate (5.4%) further shows that the multivariate FMO gate will always reveal more of the truly positive events.

Finally, Figure 1.14.6C illustrates that FMO gates can even overcome incorrect compensation. In this simplistic example, the goal was to define an appropriate gate to distinguish CD4 T cells. As shown in the properly compensated samples, and like previous examples, only the FMO gate (in this case, a 1-D FMO gate) gives what is a proper division between the CD4 T cells and other cells. Importantly, the FMO gate (which is then in a different position) works for the data when there is incorrect compensation.

It might be appreciated that there are as many FMO controls as there are colors in the experiment. However, this is typically not necessary. FMO controls are necessary only for those channels where accurate identification of the positive and negative populations is crucial, and where the separation between these populations is not great (i.e., dimly-expressed antigens or those that have continuous distributions from negative to bright). A typical staining panel might require only one or two FMO controls. Nonetheless, it is apparent that an unstained cell sample (or one stained with all isotype-control antibodies) is useless compared to an FMO control.

COMPENSATION CONTROLS: CELLS

No matter how compensation is to be set, the most critical factor is the selection of good compensation controls. To properly calculate the spillover coefficients necessary for the computation process, one must have samples that are stained to different extents for each fluorophore of interest (Fig. 1.14.7).

A good strategy to adopt is to choose the brightest reagent among the ones being compensated to use as the compensation control. For regular immunophenotyping studies, one might alternatively select a very highly expressed antigen, such as CD8 or CD45, to stain for the compensation control.

Figure 1.14.7 shows why it is necessary to have a positive and a negative population to set compensation properly: it is the ratio of the *differences* in the fluorescences between these two populations that is critical for defining the compensation. Strictly speaking, a true "negative" population is not necessary; it is only necessary that two different levels of staining be present. Thus, in Figure 1.14.7, proper compensation could be set by using only the dim and the bright populations. Mathematically, the ratios of the differences between orange fluorescence and green fluorescence for each of the three pairs of populations in Figure 1.14.7 are identical. Thus, there is nothing special about a negative population with respect to the calculation of proper compensation.

From Figure 1.14.7, one can also ascertain another crucial criterion for setting proper compensation: that the two populations used for setting compensation have the same autofluorescence and other background fluorescence values. Otherwise, the values for Δgreen and Δorange will be due not only to the fluorescent stain, but also to differences in background fluorescences between the two cell types. This would result in incorrect compensation.

Ideally, the best compensation control is one that uniformly labels all cells in a population very brightly. These stained cells can then be mixed with unstained cells in equal numbers to generate two different peaks. For immunophenotyping of hematopoietic cells, CD45 represents an excellent choice. For each color in an experiment, label cells with CD45 conjugated to that fluorochrome. After washing the cells, mix them with equal numbers of unlabeled cells; this will be the compensation control.

Although simple, this process is already more cumbersome than many laboratories are willing to perform. The next best compensation control is to use a bright stain that resolves two populations of cells within the same sample. CD8, which is highly expressed on CD8 T cells, is an ideal choice for immunophenotyping of blood cells.

Note that if one chooses a reagent that specifically binds only to lymphocytes, one must first select a scatter gate for lymphocytes before setting compensation. For example, consider the use of conjugated CD8 as a compensation control for staining peripheral blood mononuclear cells (PBMC). CD8 T cells, which are lymphocytes, will be brightly stained. The unstained cells will include both lymphocytes and monocytes; thus, as a whole, their autofluorescence is significantly higher than that for lymphocytes alone. If one were to set compensation based on this ungated population, the result would be undercompensation.

One approach taken by some laboratories for generating compensation controls is to stain a single sample of cells with antibodies that bind to exclusive populations within the sam-

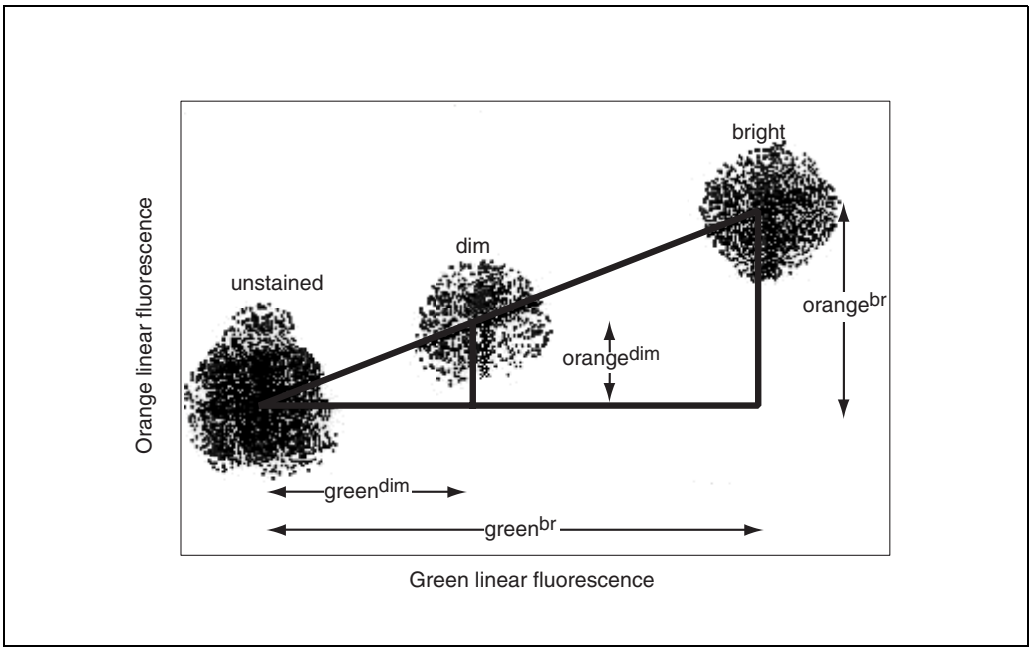

Figure 1.14.7 The most accurate compensation is achieved with the brightest compensation controls. This graph shows three populations of cells stained only with FITC: negatives, a dim population, and a bright population. Linear fluorescences illustrate the principle; the same principles hold true for logarithmic amplification. The correct spillover coefficient is the ratio of the Δorange signal to the Δgreen signal (for either the dim or the bright cells). In an ideal world, this ratio is the same for dim or for bright cells. However, the Δorange and Δgreen values will have an inherently greater proportionate error for the dim cells than for the bright cells; therefore, the spillover coefficient will be less accurately determined on the basis of the dim population than on the bright population. For logarithmic scaling, this problem is exacerbated because dim populations can have orders of magnitude less fluorescence than bright populations (remember, the error of the measurement varies with the square root of the absolute value of the measurement).

ple. For example, for compensating a three-color analysis of PBMC, a single sample could be stained with reagents for CD19, CD8, and CD4. Since these reagents, for the most part, stain unique subsets of cells that have roughly the same autofluorescence, with $CD8^-$ NK cells providing the negative population, the single sample provides all the necessary populations.

However, this forced economy of sample will inevitably lead to poor compensation at some point, for several potential reasons. First, there may not be enough of any given subset ($CD19^+$, $CD8^+$, $CD4^+$, or triple negative) to provide a statistically accurate determination of the fluorescence distribution. Second, the autofluorescence of all these populations may not necessarily be the same for every cell sample. Third, CD19 and CD4 are relatively dim reagents; it is likely that other reagents in the panels are brighter than this (see discussion above regarding Fig. 1.14.7).

Finally, this approach becomes untenable for experiments with more than three colors, or with cell samples for which it is impossible to find enough distinct populations that can be uniquely labeled with reagents.

There are too many potential problems with this method (simultaneous compensation stains on the same cell sample) to warrant its use; it should be discontinued.

In summary, the ideal compensation control has a good representation of two or more populations of cells with as great a difference in fluorescence between these populations as possible. In addition, these populations must have the same autofluorescence (and other background fluorescence values). Finally, the ideal compensation control is labeled with only one of the colors of the experiment; there should be one compensation control for each color. Remember that with the use of tandem (RET) fluorophores like PE-Cy5, one compensation control may be needed for each different conjugate of the tandem in the experiment (see discussion of Resonance Energy Transfer Dyes).

COMPENSATION CONTROLS: BEADS

Recently, manufacturers have provided "compensation beads" that can be used to set up compensation. The only beads that work well for this purpose are those that bind to the actual reagents that are used in the experiment. Do not rely on the manufacturer to provide the actual fluorochrome; it may not spectrally match the one being used. Beads that are coated with antibodies, which bind to the reagents of use, provide an excellent compensation control, as the signals most closely reflect those obtained with cells.

There are several advantages to using antibody-capture beads as compensation controls. (1) They will work equally well with any reagent being used (assuming that the bead captures the reagent). Since the beads capture the antibody, 100% of the capture beads will be fluorescent. If using an antibody that is expressed by a very small fraction of cells, it would be nearly impossible to use cells to compensate that reagent. (2) The beads are highly uniform. The precision with which the spillover can be calculated with beads is therefore very high. This is most important not in the primary detector channel, but the spillover channels. For example, a FITC compensation-control cell sample has a small amount of fluorescence in the PE-Cy5 detector—but this amount is not much above autofluorescence. To accurately compensate FITC out of the detector, however, the amount of spillover must be precisely determined—a difficult chore given the large variation in autofluorescence from cell to cell. Because the beads have essentially no autofluorescence and have a very uniform binding capacity, the spillover can be determined with great precision. (3) The beads, after labeling, are very stable and can be re-used for several experiments. (Note: tandems of PE, such as PE-Cy5 and PE-Cy7, exhibit changes in emission spectra—and therefore compensation requirements—with exposure to light; therefore, it is not recommended that compensation controls be used across experiments unless they are carefully stored in the dark and the cells are stained in the dark as well!)

Antibody-capture beads have a couple of disadvantages. (1) They may not be as bright as some very bright reagents on cells. While the precision of the beads is very high, they probably should not be used to compensate samples that are more than ten-fold brighter than the bead itself. (2) They cannot be used for non-antibody reagents (such as live/dead discriminating markers) or for reagents that do not bind the capture beads. For reagents that fall under these categories, regular cell-stained compensation must be performed. Note that it is perfectly acceptable to use beads to compensate some colors and cells to compensate other colors in the same experiment.

FACTORS AFFECTING COMPENSATION VALUES

In a perfect world, one could determine the emission spectrum for a given dye and use that to calculate the correct compensation value to set on the flow cytometer—never having to run compensation controls in the first place. Unfortunately, this isn't the case.

As shown previously, the spillover coefficients are determined by the ratio of the measured signals in two detectors for a compensation control (a singly stained sample). Therefore, anything that affects the signal level will directly affect the compensation values necessary to correct for the spillover. These include any number of factors, from instrument sensitivity—e.g., how well the optics are aligned and what filters and optical elements are in use—to photomultiplier sensitivity and electronic gains during signal processing.

Until instrumentation advances to the point where these factors are internally calibrated and set each time during setup, they must be assumed to have varied from the previous experiment. Thus, compensation controls must be collected in order to determine the correct spillover coefficients for that experiment.

A direct corollary of this is that should any of these factors change during an experiment, the compensation will no longer be correct. Therefore, once the compensation values are set, one cannot change PMT voltages, filters, or the optical focusing without risking that subsequent samples will no longer be properly compensated. Further, should one encounter a nozzle clog or other incident that requires any adjustments or change in apparent sensitivity, then one should reanalyze the compensation controls to make sure that the compensation values are set properly.

As noted above, the tandems of PE exhibit time-dependent changes in emission spectra with exposure to room light. Therefore, it is critical that the compensation samples and the stained samples be treated identically with regards to light exposure. In general, it is advisable to keep stained cells under cover to block light. This aspect becomes crucial when the compensation samples are generated at a dif-

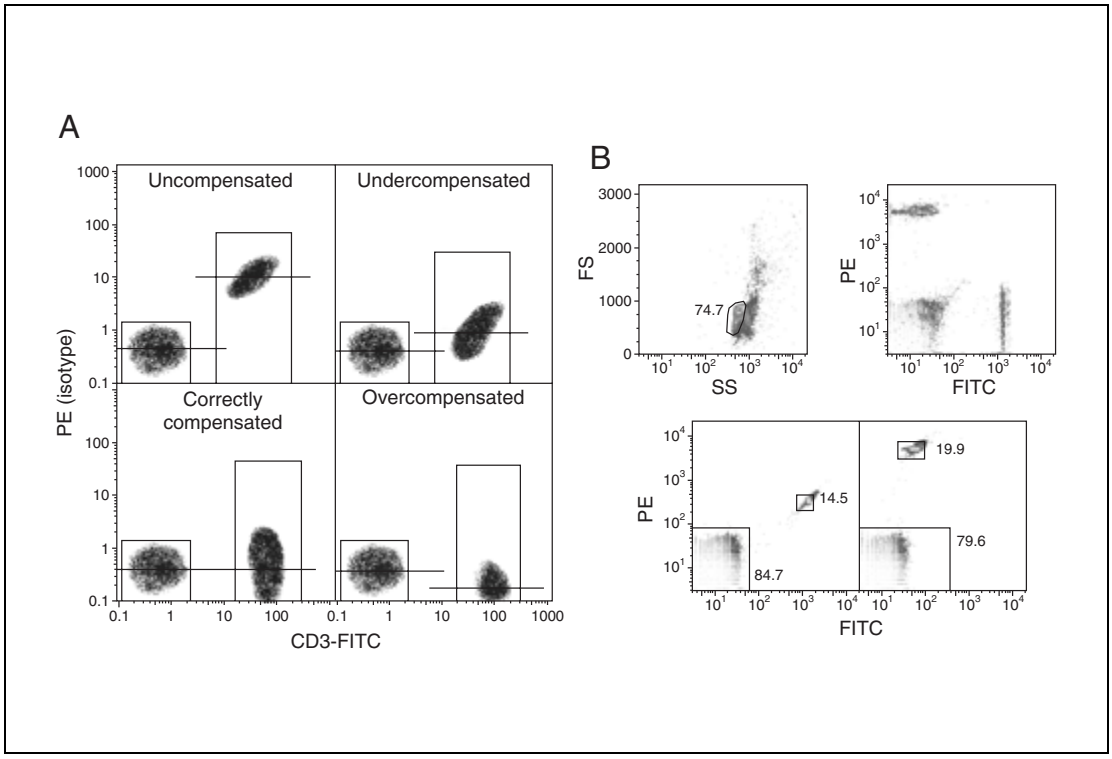

Figure 1.14.8 *Proper compensation is set when the centers of positive and negative populations align. (***A**) In a hypothetical experiment, cells stained with CD3-FITC and PE isotype control were collected at different compensation settings. The horizontal line is drawn through the median of the population. The boxes indicate the analysis gates used when the median fluorescences were computed. Proper compensation is achieved when these centers align; note that the properly compensated positive population extends *above* the top of the negative population (i.e., above where an isotype gate would be set based on the negative population). (**B**) An example of using antibody capture beads to set compensation. On the top left panel, a forward- and side-scatter gate is drawn tightly around the main population to select only singlet events. The bottom panels show the distributions in the FITC and PE channels for beads labeled with FITC and PE antibodies, respectively; the beads represent a mixture of capture beads with identical but noncapturing beads as the blank. Gates are drawn around the positive and negative bead populations. Software can be used to automatically align the populations, or manually gate settings to align the medians. The top right panel shows a mixture of unlabeled, FITC-, and PE-captured beads. Note that the distribution of the beads is very small, allowing for precise determination of the compensation required. Also note that the distribution in the compensated channel is visually much larger (and similar to that of the blank beads). As shown in Figure 1.14.5, this apparent widening of the distribution is simply a visual artifact of moving the distribution from the bright to the dim area of a logarithmically scaled graph. In this case, because there are many photons being measured for each bead, photon-count- ing statistics has not widened the distribution beyond that of the background distribution. Numbers indicate the percentage of events displayed within each gate.

ferent time than the stained samples. Compensation samples stained on a different day must be carefully stored and judiciously used. Particular care must be taken to make sure that they are appropriate for the experiment. The effect of light exposure is to increase the compensation required between the primary (e.g., PE-Cy5) detector and the PE detector. Note that the compensation required between PE-Cy5 and APC will not change. The light sensitivity has not been observed for APC tandems.

COMPENSATION MYTHS

Because of the subtleties that accompany the compensation process, and affect the visualization of compensated data in non-intuitive ways, it is not surprising that a number of myths have become prevalent regarding compensation.

Following are a few of the myths, with a short discussion of each one.

1. *Compensation is dependent on brightness*. In other words, the amount of compensation required depends on how bright a reagent is. This is incorrect. As illustrated in this unit, the degree of compensation is an intrinsic variable that relates to the spectrum of a fluorochrome. In fact, if compensation were brightness related, one would never be able to compensate properly.

The source of this myth is that brighter compensation samples appear to require greater compensation settings. However, this is only because of the inability to properly judge proper compensation with the dimmer samples. They appear to be properly compensated when the compensation setting is still too low. This myth can be easily dispelled simply by showing that a properly compensated bright sample results in proper compensation for dimmer samples as well. This also illustrates why it is important to choose the brightest stain possible for the compensation control.

2. *Compensation introduces error*. This, too, is incorrect. It is based on the observation that properly compensated data appear to spread out (as shown in Fig. 1.14.5). However, it should be noted that the error is already present in the data; compensation simply makes it more evident in the visualization process. In fact, compensation does not introduce any error, it only makes extant errors more evident.

3. *One can not compensate properly with beads, or one can only compensate properly with the same cells that are used in the experiment*. Remember that compensation is a property of the fluorochrome, not the cells. There-

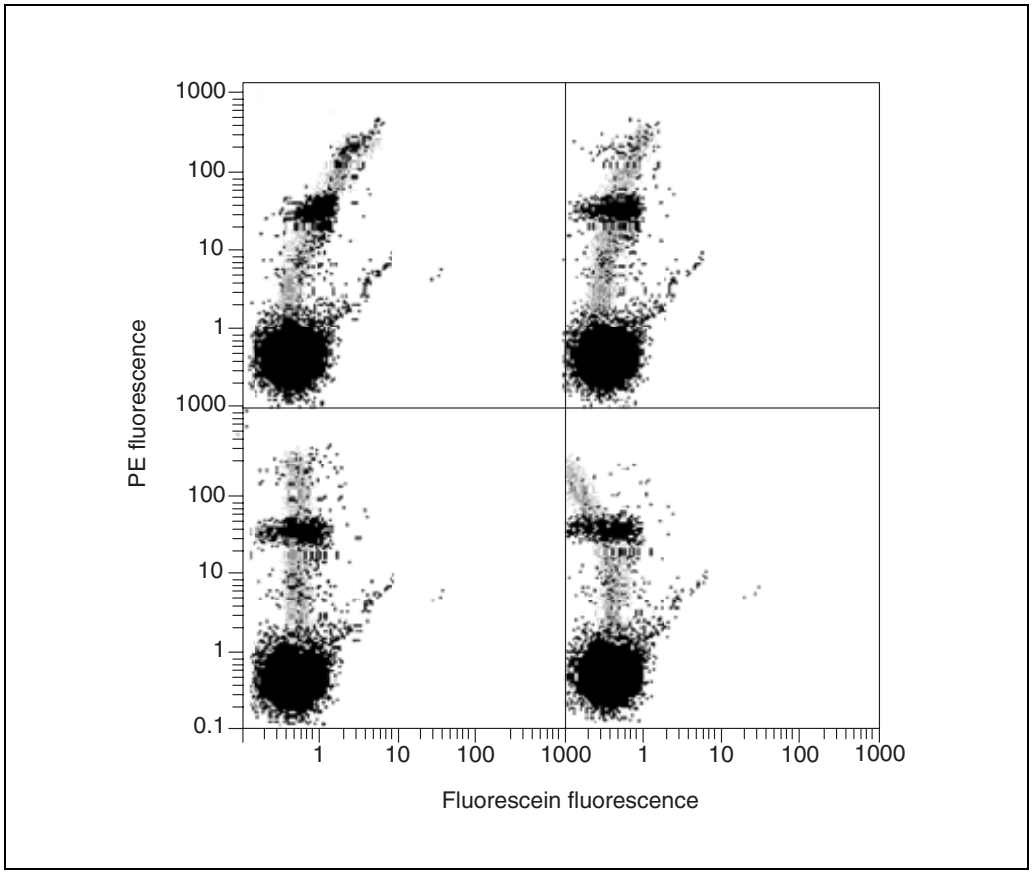

Figure 1.14.9 Setting appropriate compensation. This is a peripheral blood mononuclear cell (PBMC) sample stained with a PE-conjugated reagent and collected at several different compensation values (into FITC). The top left panel is uncompensated, the top right is undercompensated, the bottom right is overcompensated, and the bottom left is properly compensated. The bold gray lines have been added for emphasis only; no quantitative relationship is implied by the shapes. In this example, the cells have been gated for lymphocytes, so the highly autofluorescent monocytes do not interfere with the setting. Note in particular that while the main PE^+ population appears reasonably well compensated in all graphs where some compensation is set, the brightest cells clearly indicate the incorrect compensation level. This is a clear example of why only the brightest stain should be used to estimate correct compensation!

fore, proper compensation requires only that one can measure in each of the detectors the amount of fluorescence coming from a given fluorochrome. It is entirely appropriate to use monocytes for one compensation control, lymphocytes for another, and beads for a third. However, for each control sample, the positive and negative (or dim) gates must be applied such that the two gated populations would have the same autofluorescence if the sample were unstained.

4. *The compensation value is too high/low [in absolute value], or one can not have compensation settings over 100%*. In fact, there is no real meaning to the absolute value of the compensation setting. Remember that changing the PMT voltage will force a change in the compensation value, without changing the actual quality of the measurements in any way. Do not assign importance to the fact that one instrument has a given compensation setting of 30%, whereas another has the same setting as 15%; these absolute values are not comparable. For a given instrument that has been carefully calibrated, compensation values *should* be roughly comparable from day to day, but that is the extent to which the absolute values can be used. Likewise, there is no magical significance to a value of 100%; compensations of 200% can be used just as well as 2%.

5. *One can use the same compensation settings every day*. This time-saving approximation is no more than that: an approximation. For experiments where identification of relatively low-expression stains is important, or where more than three or four colors are involved, compensation controls should be generated concurrently with cell staining, and should be used to precisely set proper compensation.

THE PROTOCOL: HOW TO SET COMPENSATION

This section is devoted to setting the compensation on the instrument manually. The use of software compensation obviates all these steps by automating the process and ensures that the compensation values are correct. Even when using software compensation, one should pay special attention to the discussion above regarding compensation (see Factors Affecting Compensation Values and Compensation Controls: Cells). To perform software compensation, follow the instructions given by the manufacturer.

Proper compensation is set when, *on average for a population of cells*, there is no contribution of any given fluorophore into each of the other detectors collecting fluorescence. For example, a population of cells stained with only FITC should have the same median PE fluorescence as would another aliquot of the same cells left unstained.

Figure 1.14.8 illustrates this process. Starting with no compensation set, and viewing the dot display for the two parameters being compensated, the appropriate compensation control is slowly increased until the centers of the two populations are equal. Note that this is not necessarily when the tops of the two populations line up! The reader who does not understand this should refer to the section above (see Compensation: Effect on Visualization of Data). It can be difficult to determine when the centers of the populations are equal; once one has achieved approximately the correct setting, one should collect some events, and, using a software analysis package, calculate the medians of the two populations (high and low) to ensure that they are indeed equal. Note that it is preferable to use the median rather than the mean, since the median is a better estimator of the central tendency of a population, especially if any of the events are off-scale on either side.

Figure 1.14.8 also illustrates another important facet. Before beginning to adjust any compensation values, one must ensure that the negative population is on-scale sufficiently so that its median is above the axis. Otherwise, it will be impossible to determine when one has set compensation properly. Note that if the positive cells are so bright that to keep them on-scale the voltage must be turned down to the point where negative cells are off-scale at the low end, then one must design a compensation sample having the positive and a dim population, both of which are on-scale. This can be accomplished, for example, by diluting the reagent used in the positive stain by 100-fold or more before staining the cells.

Figure 1.14.9 is an illustration to help the reader learn how to view fluorescence plots to decide if compensation is proper or not. By mentally drawing a line through the centers of all the populations, even rare ones, one can often determine if samples are over- or undercompensated. Often in the literature one finds examples like the bottom-right panel in Figure 1.14.9, where the main population appears reasonably well compensated, but in fact the system is significantly overcompensated. Always use the brightest possible cells in determining appropriate compensation.

Four steps must be taken to ensure proper compensation. These steps should be taken with every experiment.

1. Make compensation controls that consist of cells with positive and negative (or dim) populations. The positive and negative populations must have the same autofluorescence as the negative populations. Use as bright a reagent as possible for each control.

2. Set the PMT voltages high enough that an entirely unstained population is completely off the lower axis for every parameter being measured.

3. Set an analysis gate such that only cells with the identical autofluorescence characteristics are being viewed (e.g., a lymphocyte gate).

4. Increase the compensation setting until the centers of the positive and negative populations (*not* the upper limits) are equal.

Even after taking these steps, caution is still needed. Since most commercial instruments can perform only pairwise compensations, one should make sure that there are no significant uncorrected spillovers between other detector pairs (especially green fluorescence and red fluorescence). Do not change any instrument settings, or else the compensation settings will have to be reset by reanalyzing the compensation controls under the new settings. Finally, remember that different tandem dye lots, such as PE-Cy5 conjugates, can have significantly different compensation requirements; make sure that the compensation setting is appropriate for all the reagents used in an experiment.

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