

Three Rules for Compensation Controls

First and foremost, there must be a single-stained control for every parameter in the experiment!

In addition, there are three *rules* for “good” compensation controls:

- 1) Controls need to be at least as bright or brighter than any sample the compensation will be applied to**
- 2) Background fluorescence should be the same for the positive and negative control**
- 3) Compensation controls MUST match the exact experimental fluorochrome**

1) Controls need to be at least as bright or brighter than any sample the compensation will be applied to

An important consideration is to select the sample with the brightest fluorescence of the experiment. “Dimness” is relatively irrelevant. Only brightest matters, and that is so that low spillovers can be accurately estimated. For example, if a spillover is so low that a MFI of 10,000 doesn't cause enough spillover to be above autofluorescence, then the system assumes no compensation is necessary. At a MFI of 100,000, the spillover becomes apparent and then compensation value can be accurately assessed. Compensation is only about estimating the slope. The bottom line is that because the compensation coefficients are computed based on the RATIO of the DIFFERENCE in MFI's (of the spillover channel and the primary channel), so small absolute errors in the position of the negative control become irrelevant as the positive controls become brighter. The error in the compensation coefficient is the sum of the absolute errors in the MFI's of both the negative and the positive control; the latter has an inherently much larger absolute error than the former.

2) Background fluorescence should be the same for the positive and negative control

Any carrier for binding fluorochromes can be used for single stain compensation controls, such as cells or particles. However, the positive and negative carrier of a parameter must have the same autofluorescence. This is because compensation is a subtraction algorithm. It is imperative to NOT include autofluorescence in the compensation calculation, so if the positive and negative have the same autofluorescence, then the autofluorescence contribution to the compensation spillover calculation will be zero. If this is met, one can apply the compensation matrix to any population. For example, one can compensate on particles and apply that to cells.

3) Compensation control fluorochromes MUST match the exact experimental fluorochrome

Each fluorochrome has a unique emission profile. Therefore, the amount of spillover will be different, even for fluorochromes that emit light at about the same wavelength (e.g. FITC and Alexa Fluor 488). This rule is even more restrictive when applied to tandem dyes. Each lot of tandem dye (PE-TR, PE-Cy5, PerCP-Cy5.5, APC-Cy7, etc.) should be considered unique and require its own single stain control. If a user is using two different lots of PE-Cy7 in an experiment, then they need to have two PE-Cy7 compensation single stain controls, one from each lot. Different lots will have different conjugation ratios, i.e. more Cy7 conjugates to PE or less.

One final note

Finally, compensation controls must be treated in the same manner as experimental samples. This is because exposure to light and treatments like fixation/permeabilization may alter the fluorochrome, particularly the tandem conjugation ratio, i.e. lose some Cy7 on each PE molecule.

Compensation particles versus cells for single stain controls

There is no difference in the accuracy of the two approaches for compensation. However, compensation particles do have numerous benefits over using cells. First and foremost, precious sample does not need to be wasted on single stain controls. All the cells can then be used for the experimental samples. In addition, compensation particles typically provide the brightest signal possible for any given parameter. Compensation particles are also more precise. The reason that particles are more precise for compensation is fairly straightforward. Cells have a large variance in background fluorescence (a high CV), higher than particles. This means that the spillover computation has significant error for compensation coefficients where the measurement of spillover fluorescence on cells is dominated by the error in the autofluorescence measurement. On the other hand, the particles have a much smaller error in the distribution of background fluorescence, meaning that the spillover computation is far more precise.

Of course, particles have some limitations.

1) Compensation particles cannot be used for dyes like PI, DAPI, or EMA -- but they can be used them with amine-reactive viability dyes. (Also, some manufacturers are now providing specific dyes preloaded into particles to use as single stain compensation controls).

2) Particles do not bind all antibody reagents and in some cases they simply are not bright enough.

3) For some experimental conditions using tandems (e.g. permeabilization/fixation), one must ensure that the fluorescence spectrum of the experiment does not alter the emission spectrum of the tandems attached to particles in a different manner than it would the tandems attached to cells.

So, in fact, in many experiments, a user may have one or two cell-based compensation controls for some parameters used together with bead based compensation controls for the other parameters.

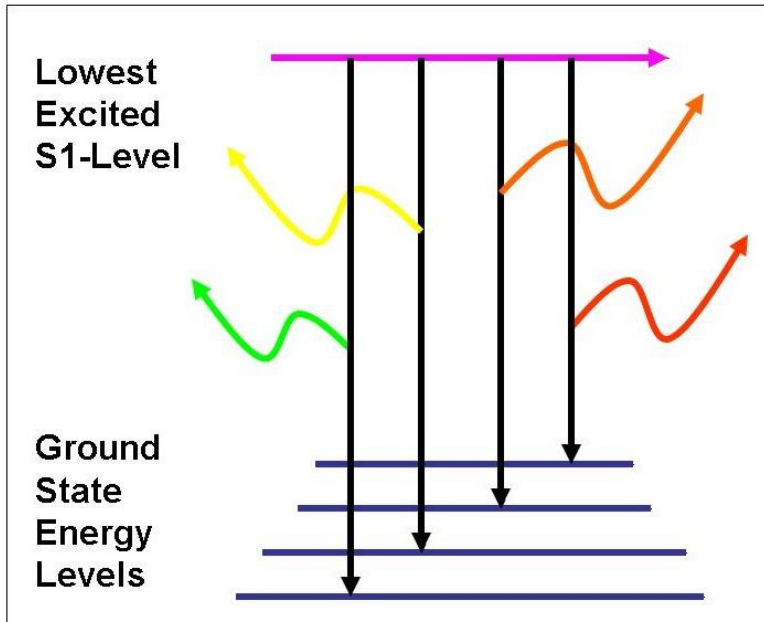
More on Compensation:

What is compensation?

Compensation is the process which corrects the detected "spillover" of the emission of one fluorochrome into the detector designed to collect the emission from another fluorochrome. The primary purpose is to allow the measurement of the true fluorescence in the fluorescence channel contaminated by the spillover.

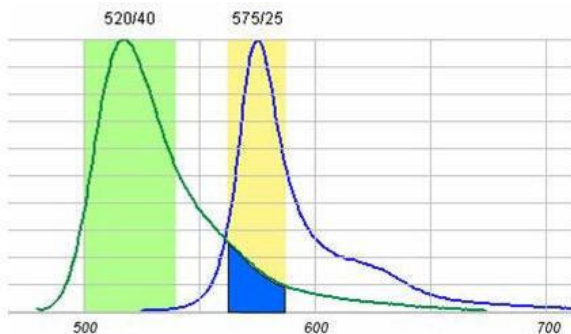
Why do we need compensation?

The reason for this "spillover" derives from the nature of fluorescence. Compensation is necessary because the fluorescence emission of a fluorochrome is not monochromatic or necessarily even close. Indeed, some fluorochromes emit light over a very broad range and/or are excited by multiple wavelengths of light. Fluorescent molecules are ones which are able to absorb photon energy from a high intensity light source. The absorbed energy raises the excitation level of the fluorochrome. The molecule, like all matter, does not like to exist in this excited state, preferring its ground energy state. Once the excitation source energy is removed the molecule reverts to its ground energy state by releasing energy in some form. The forms of this released energy include heat, vibrational energy, or light. Fluorescent molecules are those which release at least some of the absorbed energy as light. Fluorochromes releasing larger amounts of light (higher quantum



efficiency) are of course more desirable for use in flow cytometry or microscopy. The release of energy can occur from the relaxation of electrons from various excitation levels - highly simplified in the figure to the left. These of course release energy of higher or lower energy corresponding to the distance between the excited energy state and the ground state and thus, if the released energy is light, shorter (green in figure to left) or longer (red) wavelengths, respectively.

Thus, the emission of the fluorochrome occurs over a range of wavelengths. The detectors (PMTs) for the various fluorochromes are (or can be) identical so the color of light detected by each PMT is determined by the optical filters placed in front of each detector. The spillover is also a function of the optical filters' ability to separate these emissions. Thus, there is "real" light from one fluorochrome (e.g. fluorescein) that can get into the detector for another fluorochrome (e.g. FITC into R-PE - see blue area of figure below). If this



"spillover" is not corrected our ability to separate the emissions from multiple fluorochromes is compromised as is our ability to determine the true level of fluorescence in the contaminated channel. Please note that this does not mean this cannot be done. Rather, the emission from cells bearing single and multiple fluorochromes e.g. FITC only and FITC+PE is resolvable but the two populations can be very close together (sometimes seemingly not resolvable). They do however occupy discrete positions in a flow cytometric histogram. Note that the emission from a particular fluorochrome may not be detected in every other fluorochrome channel depending on the emission spectrum of that fluorochrome. See Invitrogen spectra viewer or BD Biosciences spectra viewer for common fluorochrome excitation and emission spectra. Note that proper compensation is essential for resolving dim populations from negative populations and for estimating antigen densities but it is not as critical for resolving bright populations from negatives.

Additional Resources for Understanding Compensation:

- Mario Roederer's [web page](#)
- Bagwell CB, Adams EG. Fluorescence spectral overlap compensation for any number of flow cytometry parameters. 1993. Ann NY Acad Sci. 677:167–184.
- Roederer, Mario. Spectral Compensation for flow cytometry: Visualization artifacts, limitations, and caveats. 2001. Cytometry 45:194-205.

Logicle Transform:

- David R. Parks, Mario Roederer, and Wayne A. Moore. A New “Logicle” Display Method Avoids Deceptive Effects of Logarithmic Scaling for Low Signals and Compensated Data. 2006. Cytometry 69A:541–551.
- James W. Tung, David R. Parks, Wayne A. Moore, Leonard A. Herzenberg, and Leonore A. Herzenberg. New approaches to fluorescence compensation and visualization of FACS data. Clinical Immunology 110 (2004) 277– 283.
- Leonore A Herzenberg, James Tung, Wayne A Moore, Leonard A Herzenberg & David R Parks. Interpreting flow cytometry data: a guide for the perplexed. 2006. Nature Immunology 7:681-68

Hyperlog Transform:

- Bagwell, C. Bruce. HyperLog—A Flexible Log-like Transform for Negative, Zero, and Positive Valued Data. 2005. Cytometry 64A:34–42.
- Bagwell, C. Bruce. The HyperLog Transformation for Compensated Data. slides from 2005 Los Alamos lecture

How do we perform compensation?

Looking at the emission curves of two fluorochromes (e.g. FITC and R-PE - see above), we can estimate the percent area of the fluorescein emission curve that gets into each of the detectors. What we can also appreciate is that if the amount of fluorescein emission increases (due to the presence of more molecules of fluorescein) that not only will the amount of light getting into the 520/40 filtered detector increase but the amount of light emitted from fluorescein that gets into the 575/25 filtered detector (i.e. R-PE) will also increase. However, the percent/ratio will be the same. In a typical flow cytometer setup the percent of fluorescein emission in the PE channel is around 25% of the fluorescein detected in the "FITC" channel. It should also be apparent that if one modifies the filters in one or both detectors that this would also alter the ratio. Thus, to determine the amount of correction we measure the "FITC" emission in the "FITC" channel and subtract (in digital cytometers actually correct) a percent of this value (i.e. ~25%) from the measured fluorescence in the PE channel. As one can see PE gets into the FITC channel a small amount (usually less than 1-2%) and thus we have to correct in both directions (i.e. two way compensation). As we add more colors this can get more complicated with multiple fluorochromes each emitting into a number of another detectors. Of course, in some cases the emission from a fluorochrome may not get into the detector for another (e.g Pacific Blue does not get into R-PE). To deal with this increased complexity (although it works with the simple cases as well) we use matrix algebra to solve for all the correction values. For a more complete practical discussion see www.drnr.com/compensation/.

To perform compensation we must first determine the amount of the spillover from each fluorochrome into each of the other fluorochrome channels we wish to measure simultaneously in a particular experiment. We do this by performing so-called single color compensation controls. In these controls we stain cells (or other particles e.g. B-D CompBeads or Spherotech Compensation Particles) with each fluorochrome reagent individually. Thus, for example if our experiment will simultaneously stain with FITC-anti-CD4 and PE-anti-

CD8 the two single color control tubes will be FITC-anti-CD4 and PE-anti-CD8. However, single controls have some specific requirements. First, they optimally will contain both cells negative and positive for the reagent/fluorochrome. Secondly, the positives in a given single control must also be **BRIGHTER** than any other cells will stain with that **FLUOROCHROME** in the experiment to which the compensation values will be applied. Thus, if you wish to use a set of compensation values in an experiment which will, e.g. have several different FITC labeled reagents, you must use the brightest reagent for the FITC single color control. If using different cell types (see below for caution about autofluorescence) use the cell that stains the brightest with the brightest reagent. Note it may not be necessary to prepare a single color control for every reagent - see below. Also it is important to be sure that you use or gate on cells that have the same autofluorescence for both the negative control and positive control. For instance, you would not want to use a gate that includes monocytes and lymphocytes if your single control stain is e.g. FITC-CD14. CD14 is positive on monocytes but negative on lymphocytes. However, monocytes and lymphocytes have different levels of autofluorescence and, thus, the compensation calculations will be incorrect. More about autofluorescence below.

Fluorochromes currently may be divided into two groups - **mono-molecular fluorochromes** and **bi-molecular fluorochromes**.

Mono-molecular fluorochromes are those which are composed of a single fluorescent molecule - e.g. fluorescein (FITC), R-PE, APC, AlexaFluor dyes (e.g. AlexaFluor 488). Bi-molecular fluorochromes are those composed of two fluorescent molecules - in all cases currently one is a fluorescent protein and the other is a small molecular weight fluorochrome e.g. R-PE-Cy7, R-PE-AlexaFluor 610, APC-Cy7, PerCP-Cy5.5. When preparing single color controls for mono-molecular fluorochromes it is only necessary that the fluorochromes be the same. With mono-molecular fluorochromes usually only one single color control tube is required for each fluorochrome if the cautions noted above are heeded. If multiple antibodies labeled with a particular fluorochrome are to be used in the experiment it is not necessary to do a single color control for each reagent only each different fluorochrome. Thus, it is not necessary that the reagent sticking the fluorochrome to the cell/bead is the same (assuming it is not also fluorescent). Thus, if the reagent (e.g. monoclonal antibody) to be used in the experiment does not produce a suitable single color control (not enough positives or negatives or the positives are dim) one can simply substitute another reagent (e.g. monoclonal antibody) labeled with the same fluorochrome.

Bi-molecular fluorochromes are more difficult. These reagents are produced by covalently linking a small molecular weight fluorochrome to a fluorescent protein then labeling the reagent (e.g. monoclonal antibody) with the bimolecular fluorochrome using homo- or hetero-bifunctional reagents to link the protein part of the fluorochrome (e.g. R-PE) to the protein reagent (e.g. monoclonal antibody). The problem is that there can be substantial variation in the resulting fluorochrome-reagent from lot to lot. This will result in fluorochromes with - potentially - very different spectral properties. Compensating with one lot of fluorochrome (e.g. R-PE-Cy5) while using a different lot of the fluorochrome on the reagent in the experiment will create substantial errors in the compensation. If purchasing reagents the vendors do not tell you which lot of fluorochrome was used to label the reagents, thus, you must always assume that the spectral properties are different. Practically this means that you must use exactly the reagent for the single color control that you use in the experiment. Furthermore, if you will use in the experiment several different reagents labeled with the same bimolecular fluorochrome you must prepare a single control for each reagent/bi-molecular fluorochrome combination. Then each reagent/fluorochrome combination must use the single color control that matches to set the compensation - i.e. you will need to use a separate compensation matrix for each different lot within an experiment. What if your reagent/bimolecular single

color control does not meet the requirements of a good single color control? Then you must find another cell/particle (e.g. BD CompBeads or Spherotech Compensation Particles if using monoclonal antibodies) to stain for your single color control that will produce a suitable control. However, the cell or particle you choose must reasonably have the same autofluorescence as the cells in the experiment. Also you must use as the negative population the same type of cells as those in the positive population as noted above. In addition, set the detector voltages using either the negative control cells from the experiment or the single color control cells depending on which has the lower autofluorescence. However, use the negatives that go with the positive cells/beads to set the compensation -- see autofluorescence below).

Remember once the detector voltages have been set you cannot alter them or you will have to completely redo all the single color controls and the compensation adjustment.

Process Overview:

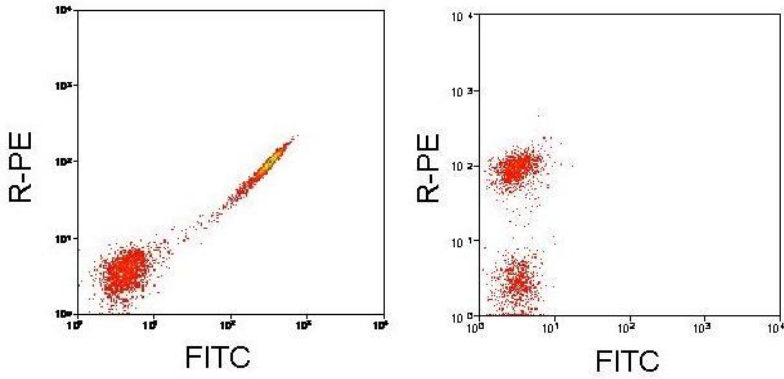
- 1) use negative control cells to set the detector voltages
- 2) gate on a single population of cells from FSC vs SSC plot
- 3) run the single color controls and collect data (on "digital" machines - CyAn, LSRII, MoFlo XDP. Reflection)
- 4) adjust the compensation either manually or by computer.

Autofluorescence and compensation.

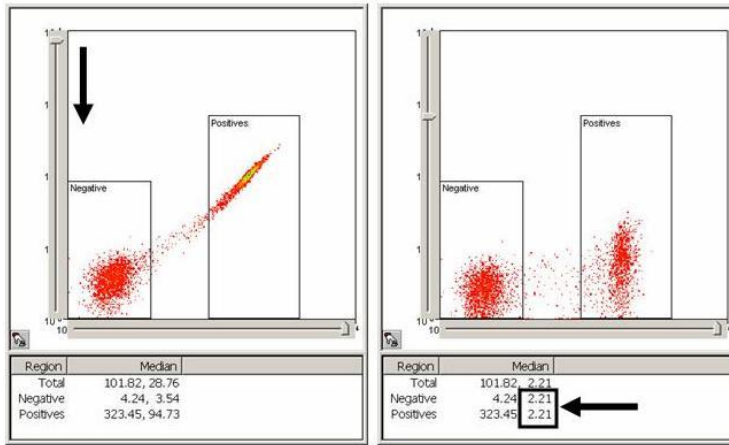
Autofluorescence does not affect compensation but it does affect how we set up compensation. For example, if we set up compensation using a cell/particle with low autofluorescence and then use cells with higher autofluorescence in the experiment the compensation will work just fine assuming no voltage changes. What is important as stated above is that the negative cell and the positive cell in a given single color control **MUST** have the same autofluorescence. If the autofluorescence of the sample cells, however, is substantially more than the cell/particle used for the single color controls then the positive cells of the sample cells may fall off-scale on the high side of the data. Thus, the voltage must be reduced to get the positive cells back on scale but that then causes the negatives in the single color control to be off-scale low and, thus, preventing those cells to be used as a compensation control. If this happens then the user must find another cell/particle that has autofluorescence that more closely matches the sample. B-D CompBeads are made in only a single level of autofluorescence. However, Spherotech is rumored to be considering making their comp particles in a variety of autofluorescence levels.

Practical aspects to performing compensation.

Perhaps one of the most difficult tasks for new users of flow cytometry is to understand how to adjust compensation and when it is correctly set. Having it set correctly is very important particularly in certain situations as stated above. Fortunately, advances in flow cytometry hardware and software has largely eliminated this difficulty but it is useful for flow cytometry users to understand the concepts. We will demonstrate this in a simple example first and then describe how to perform more complex compensation. Consider the example of simultaneous FITC and PE staining. In the figures below are shown the displays of the uncompensated single controls for FITC and PE (left and right respectively). Notice that the FITC signal spills over into the PE channel (left) much more than

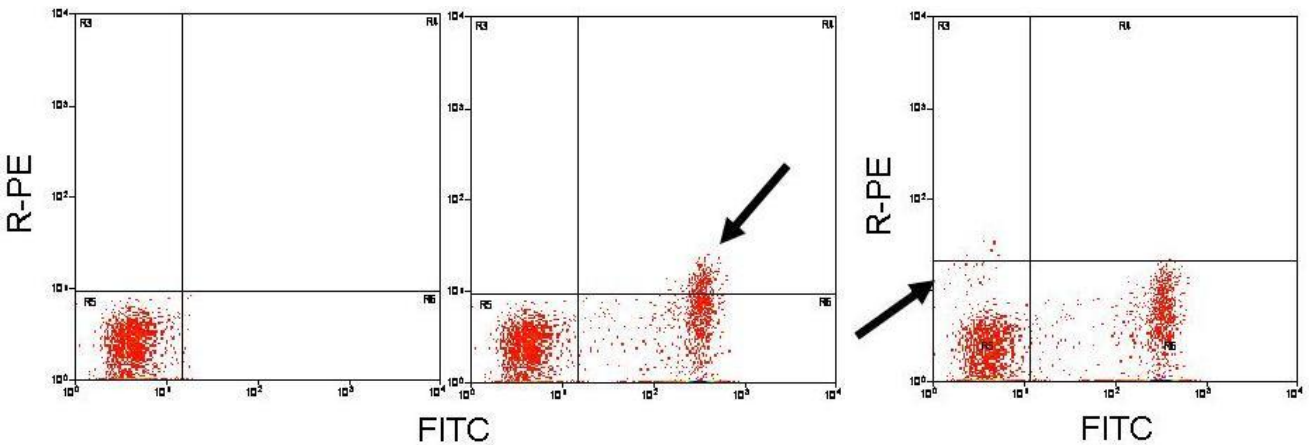


the PE spills over into the FITC channel (right). This can also be seen in the spectral curves for FITC and PE presented above. In the figures below right you can see the way manual compensation appears in Summit. Activating compensation creates compensated parameters and presents adjustment sliders on each axis. Shown is the adjustment of compensating the PE channel for the spillover of FITC. Adjust the slider on the

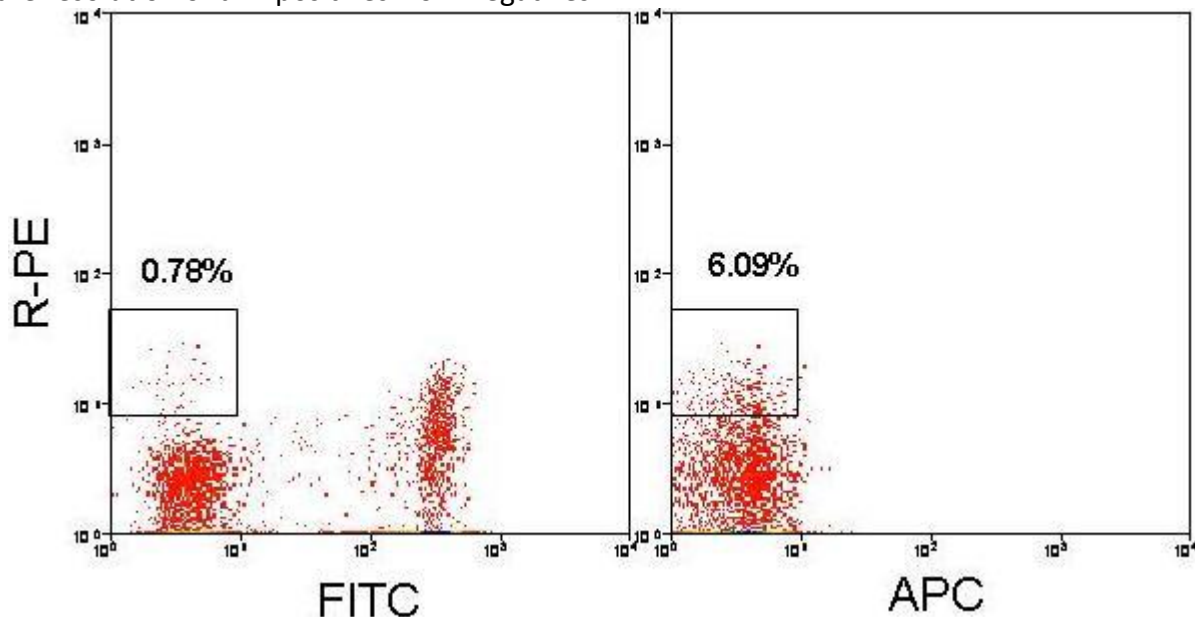


PE axis (arrow).

Note in the compensated histogram (right) that the medians, in the PE dimension (see boxed median values), of the FITC negatives and the positives are the same. Note that the tops of the distributions of the negative and positive populations are not the same in the PE dimension. Note that the medians of both the negatives and the positives have decreased (3.54 to 2.21 and 94.73 to 2.21). Since we are using a percent of the FITC signal to subtract from the PE value, the positives move much faster than the negatives.



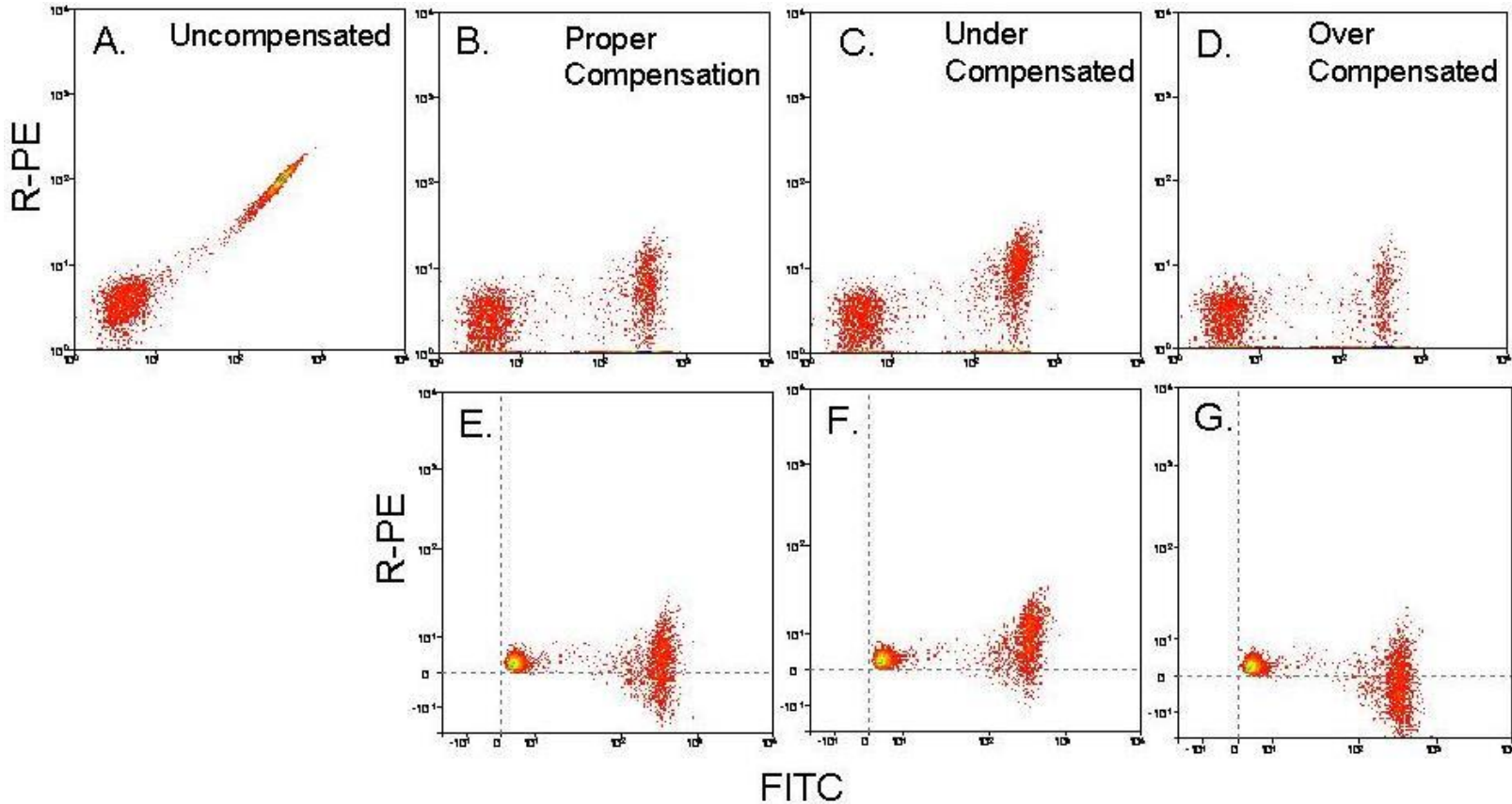
The figure above demonstrates why using simple quadrant regions for statistics is not valid for compensated data. The left most histogram shows only negative cells. Most users will apply quadrants by placing them as shown. However, doing this results in some FITC only positive cells being incorrectly scored as double positive (middle plot - arrow). If one adjusted the quadrant line positions by looking only at the FITC single color control so that all the FITC positive were also PE negative then dim PE only positive cells would be partially (or completely) scored as negative (see right figure - arrow). As is shown, the FITC positive distribution in the PE dimension is different from the negatives. This derives from compensation introducing errors or more precisely amplifying errors already existing in the data. These areas derive from photon counting statistics, log binning errors, and measurement errors. While flow cytometers measure fluorescence quite accurately they cannot do so exactly. The errors increase as the wavelength of the fluorochrome increases and as the degree of spillover into other detectors increases. This small error is increased by applying compensation and thus the data "spreads" (see Roederer for details). This spread can affect the resolution of dim positives from negatives.



The figure above and left shows how this spread can affect the determination of dim positives. On the left is shown a plot of FITC vs PE and the region shows a small dim population (0.78%). However, if we view the same data but look at APC vs PE we see 6.09% dim PE positives. What has happened is that the spread in the PE dimension of the FITC positives becomes the spread of the APC negatives in the PE dimension. Thus, some of the FITC only positive cells (brighter in the PE dimension) are scored as PE positive. This can be visualized in the right plot. If we did not know that these PE dim positives existed we likely would have underestimated them as we would have misplaced the boundary that separated PE negative from PE positive if we had only looked at the APC vs PE histogram. The ability to resolve dim populations decreases as we increase the number of fluorochromes in the assay. In general, one should try to avoid fluorochrome combinations where a bright fluorochrome can spill over significantly into a channel where we are attempting to detect the dim population. These situations make it particularly important that investigators perform additional controls called **FMO** (Fluorescence Minus One) controls. In these controls the investigator prepares samples in which each fluorescent reagent is included except one. Thus, for a 4-color stain (FITC, PE, APC, APC-Cy7) the FITC FMO control would contain the PE, APC, and APC-Cy7 reagents but not the FITC reagent. This is repeated for each fluorescent reagent. The FMO controls are then used to set the negative - positive discrimination point.

The bottom line is to avoid spillover from bright populations into channels requiring high sensitivity. This problem can be countered by using the high spillover fluorochrome on a separate population from that where you need high sensitivity.

The figure below shows uncompensated, properly compensated, under compensated and over compensated views of the same data. The compensated data are shown for both log views (B-D) and Logicle/HyperLog views (E-G). Note that the Logicle/HyperLog transformations do not change the data but are just another way of viewing the data.



The "Logicle" and "HyperLog" transforms for compensated data. The use of logarithmic amplification and data display is valuable to flow cytometry in that it permits the ready visualization of populations that can differ quite substantially in fluorescence intensity and increasing standard deviations. However, these displays present some issues when it comes to compensated data. Compensation of data, because it is a subtractive process (more accurately when discussing digital compensation it is a correction), can lead to zero and negative values for data. However, these values are undefined in log space and, thus, computer programs plot these values as 1 - i.e they accumulate on the histogram display axes. Many users will tend to overcompensate data displayed this way as the data is not normally distributed, as it should be, and medians by eye appear in an improper position. This is in part due to the fact that data compressed on the axis is typically not visible (depends on the size of the display) and, thus, is not weighted properly to our eye (see plot B in figure to right). The Logicle (developed by Dave Parks and Wayne Moore at Stanford University) and the HyperLog (developed by Bruce Bagwell at Verity Software House) transforms allow zero and negative values. They smoothly transform the data to transition from log space to linear space and display negative values that likewise transition from linear to log space. Thus, as the data is compensated it

maintains its normal distribution display such that the user may more easily visualize population central tendencies (medians) (see plot E above). In the Logicle transform the transform is also weighted/optimized to the amount of compensation needed in a particular channel and this can be seen in that the two log axes of a Logicle plot (e.g. FITC vs PE as a good example) are not identical in terms of the spacing of the log decades (not shown). Beckman-Coulter's version of Logicle, VisiComp, has removed this automatic weighting but provides it manually but unfortunately it is incorrectly not parameter specific but the same for all parameters.

The Logicle and HyperLog transforms, while different, are very similar. Three major software providers utilize the Logicle transform - FACSDiva (BD), FlowJo (TreeStar) (Diva and FlowJo call Logicle "Biexponential") and Summit (VisiComp) (Beckman-Coulter). Only Verity Software House products (e.g. WinList) use the HyperLog transform.

Autocompensation - Computer Assisted Compensation.

Compensation becomes increasingly difficult to perform manually as the number of fluorescence parameters increases. When two colors are involved, compensation only requires two compensation adjustments and can be visualized on a single bivariate plot. When 3 colors are involved, 6 adjustments are required (i.e. potential compensation adjustments = $n \times (n-1)$ where n =#of colors) and these require 3 bivariate plots to be visualized (# bivariate plots to show all combinations of fluorochromes = $(n \times (n-1))/2$). Note that not all fluorochrome pairs will require compensation but it is sometimes hard to work this out. This increased complexity can be managed by having computer algorithms work out the compensation coefficients. Summit, FlowJo, WinList, and FACSDiva (as well as others) all provide this capacity. All three require that the user provide single color controls so that the software can work out the compensation spillovers. Minimum numbers of events in the positive and negative populations are required for the software to perform the compensation. Autocompensation also requires bright positive populations for proper compensation. For more see the references listed above.

Compensation on analog cytometers.

Before the advent of digital signal processing compensation had to be performed using analog electronic circuits. Thus, compensation had to be performed prior to acquiring data for the samples. The general approach, however, was still followed - establish detector voltages, gate on a single population of cells, run single color controls, adjust compensation while single color controls are running. When running samples on these type of machines, in our facility the FACSCalibur, we recommend that this procedure still be followed. However, we do also recommend that the compensation adjustments on the machine be performed to somewhat under compensate the data. The final compensation adjustments can then be made off-line using mathematical compensation as is done on the "digital" machines. The only requirement is that the data must be under compensated and the user must be very careful to not overcompensate using the analog compensation adjustments.

Why do we need bright positives in the single color controls?

Proper compensation settings will compensate correctly across the entire fluorescence range. However, adjustment by using dim positives - whether compensating manually or by software algorithms - may result in improper compensation settings. This is a result of simply being able to accurately judge the correct compensation. The dimmer the positives the more error there is likely to be in the compensation setting. Adjustment using very bright positives will make it easier since there is less error in the adjustment. The software algorithms, which find the compensation coefficient by finding the slope of a line through the

positive population, will also more accurately determine the slope the brighter the positive population in the single color control (i.e. the longer the line to calculate the slope).

APC/PE tandem spillover issues.

A couple special situations need noting. When using PE and APC tandems, especially APC-Cy7 and PE-Cy7, one must be careful if trying to simultaneously stain - with the tandem - a relatively large population that stains brightly and then staining for a dim relatively small population in the parent channel of the tandem. For PE-Cy7 the parent channel is PE and for APC-Cy7 the APC is the parent channel. It can be very hard to resolve dim populations in the parent channel reliably under these circumstances. The reasons are twofold. First, data spread following compensation will affect the ability to resolve dim positives. Secondly, it has been found that certain cells become positive in the parent channel of the tandem dye. This effect is dependent on the tandem dye breakdown but also appears to be specific cells. The explanation for this is currently not clear. Regardless, false dim small positive populations will appear in the parent channel. This population is completely dependent on the presence of the tandem dye. If it is left out the positive population in the parent channel disappears. For a more complete discussion see Joe Trotter's paper on the subject (also in a BD HotLines).