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.