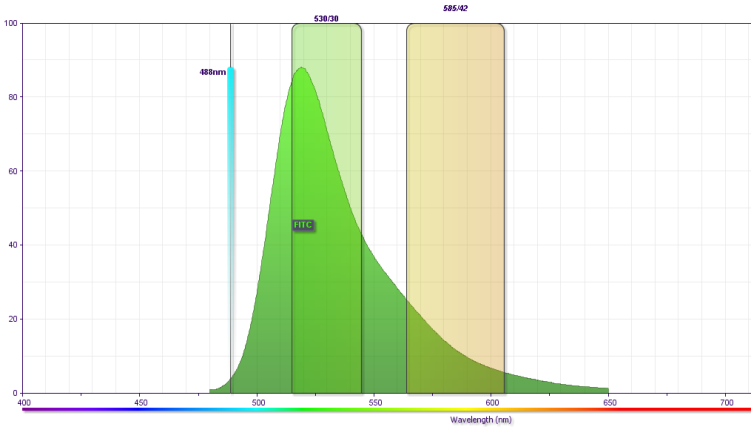
Compensation Beads

UWCCC Flow Cytometry Laboratory
https://cancer.wisc.edu/research/resources/flow/
1111 Highland Ave
7016 WIMR
Madison, WI 53705
608.263.0313

Fluorescence Spillover

Fluorescence emission peaks are wide and may have long tails that cross into longer wavelength filters causing false signal in the downstream detector. The below figure is the classic example of the FITC emission tail falling into the PE detector on the Calibur. The more colors you run, the more complicated the spillover profiles become and the more difficult it is to determine true signal from fluorescent spillover.



Why Use Compensation Beads?

Compensation beads offer a variety of benefits. The most obvious of these is that they allow you to save more of your cells for your experimental conditions rather than controls. Additionally, compensation beads stain more brightly and uniformly than cells, allowing for easy compensation of colors that may be only on rare events in the cells sample. Importantly, compensation beads can be used with the same antibodies you use for your experiment ensuring a perfect fluorochrome match.

3 Rules for Compensation

Compensation uses single stained controls to account for fluorescence spillover and is critical for obtaining good multicolor flow cytometry data. For the most accurate compensation, there are three basic rules that must be followed:

1. The compensation control must **be as least as bright** as the stained sample.
2. The negative and positive populations must have **matched autofluorescence**: cells to cells, beads to beads, etc
3. The fluorochrome used for the control must **exactly match the fluorochrome** used in the experiment: FITC cannot be used instead of GFP, tandem dyes must be from the same vial as the experimental stain.

What Compensation Beads are Available?

Most major suppliers of flow cytometry reagents offer their own compensation beads. The following table is a summary of some of our favorites.

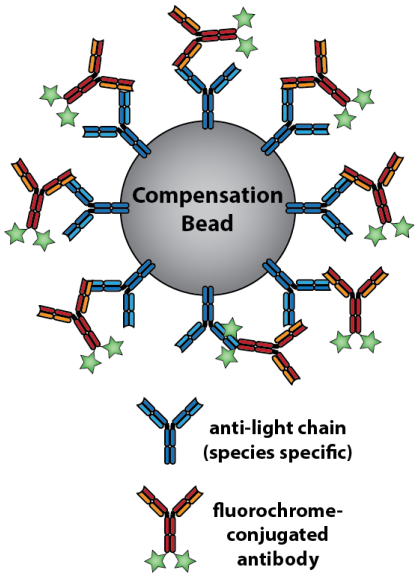
Catalog Number	Vendor	Name	Species Reactivity (advertised)	Notes
552843	BD	CompBeads	Anti-Mouse κ	
552844	BD	CompBeads	Anti-rat κ	
552845	BD	CompBeads	Anti-rat/hamster κ	
560497	BD	CompBeads Plus	Anti-Mouse κ	Very Bright
560499	BD	CompBeads Plus	Anti-rat κ	
01-1111-42	eBioscience	OneComp	Anti-Mouse κ, anti-rat κ, anti-Syrian/Armenian hamster	Not good with violet-excited fluorochromes
01-2222-42	eBioscience	UltraComp	Anti-Mouse κ, anti-rat κ, anti-Syrian/Armenian hamster	Have also shown some rabbit binding
B22804	Beckman Coulter	VersaComp	Anti-Mouse, anti-rat, anti-Syrian/Armenian hamster, anti-rabbit	Low resolution with some antibodies

Compensation with a Live/Dead Dye or Mismatched Autofluorescence

Compensation beads are not designed to bind live/dead dyes. To properly compensate these dyes, you can buy special amine-reactive beads for use with fixable live/dead or use single-stained cells. Either way you choose, you will have one compensation control with a different background autofluorescence. Rather than violating Compensation Rule 2, the Diva software is set up to handle this with the addition of an extra gate. When running compensation controls, the software automatically generates a P1 gate to identify a population in a plot of forward vs side scatter and a P2 interval gate on the positive histogram. Generally, the software uses the data in the unstained tube for a negative control, but this can be over-ridden by the addition of a P3 interval gate on the negative population in each compensation tube. If the negative P3 gate is defined in all samples, a universal negative does not need to be acquired at all. See the illustrations on the next page of P3 gates drawn to identify negative populations for beads and cells. If you have only one compensation sample that differs from the others, you can draw a P3 gate on the outlier and collect a universal negative for the others rather than drawing P3 on each sample.

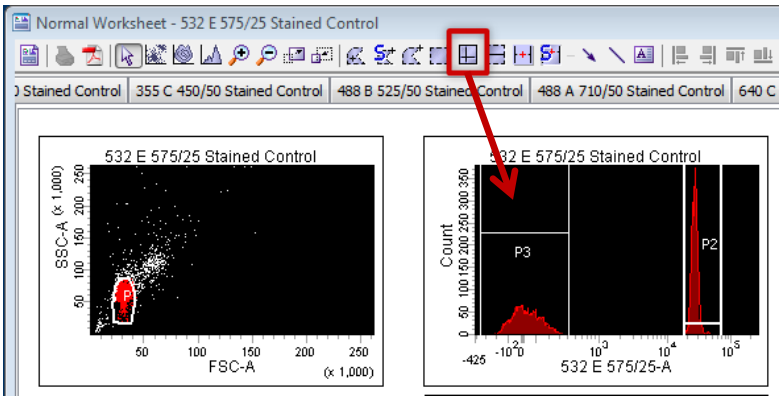
How do Compensation Beads Work?

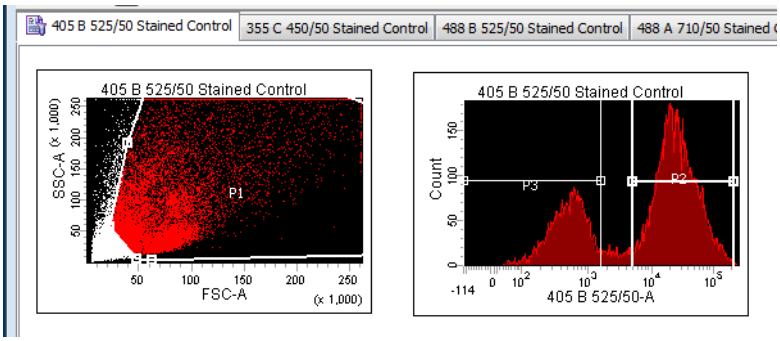
Compensation beads are small particles (typically polystyrene) that are pre-coated with antibodies recognizing species-specific antibody light chains. Special care must be taken to ensure that the compensation beads you choose will bind to the species in which your fluorochrome-conjugated antibody was raised.



Preparing Compensation Beads

In a 5 mL flow tube, add the same volume of your antibody as you use for your stain. Add one drop of positive comp beads and one drop of negative comp beads, or one drop of the all-in-one comp beads. Gently vortex the tubes and allow them to sit for a few minutes, then add ~300 µL of PBS or flow wash buffer and they're ready to go. In our experience, comp beads do not need to be washed.





Compensation with cells

Different cell populations have different autofluorescence levels. When using cells for compensation, you must choose the same cell subset for the negative and the positive. For example, the macrophage subset cannot be used as the negative population for a lymphocyte antigen or vice versa because the autofluorescences will be different.

Help! My antigen is brighter on my cells than on the comp beads!

Some very bright antigens such as CD8 may stain more brightly on cells than on the basic comp beads. The CompBeads Plus from BD have a larger particle size than any of the other beads which gives them a greater antibody binding capacity. If your antibody was raised in either mouse or rat, try CompBeads Plus for extra brightness. See the below image for a comparison of comp bead mouse-anti-human binding capacity.