# Compensation in Flow Cytometry

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These guidelines work in connection with FACS Core Facility Guidelines for

- Panel Design in Flow Cytometry (6)
- Controls in Flow Cytometry (7)
- Titration of Antibodies in Flow Cytometry (8)

Compensation must be considered when ever you analyze more than one fluorochrome.
Compensation is the mathematics that compensate for fluorochrome A emitting light into fluorochrome B's filter/wavelength area. And vice versa.
It gets complicated when working with multi-stain flow cytometry.
You must always use automated compensation. Do never – ever – perform any manual compensation!

If you need only 2-4 different stains it may be possible to avoid compensation, if you choose the right fluorochromes. Ask the FACS Core Facility.

To give the software a fair chance to calculate the best compensation matrix, the flowcytometrist must provide sufficient compensation samples.
Compensation samples are single stained samples containing a negative and a positive population.
You can use cells or you can use beads with an antibody capturing antibody on the surface. You can use cells for one antibody/fluorochrome and beads for another, if that is the best solution for you.

Requirements for compensation controls:

1) The fluorochrome must be exactly the same for the compensation control as for the stained sample.
   (You can NOT use a FITC stained compensation control while analyzing GFP stained cells or AF647 on the compensation control while analyzing APC stained cells).
   For tandem dyes you need antibody from the very same vial for the compensation control and the experimental sample.
2) The negative and positive population must have the same autofluorescence.
   Otherwise you will get an over or under compensation.
   This point is easy to achieve using compensation beads with negative and positive beads in the same tube.
   Using cells, you might have a challenge if you work with a heterogeneous cell population.
   One example could be mononuclear cells: Staining with a monocyte marker will provide positive monocytes and negative lymphocytes. These two populations have very different autofluorescence. This is no go.

3) Median fluorescence of the positive population of the compensation control must be at least as high as for the stained sample. If not, try adding more antibody to your compensation control. If this is not enough, use compensation beads with a higher binding capacity (e.g. CompBead Plus from BD (2))
4) Fluorescence intensity for experimental samples as well as for compensation controls must fall within the linear part of the scale for the given instrument.
5) Process the compensation controls in parallel with your experimental samples to give the fluorochromes the same treatment (e.g. permeabilization, fixation)
6) It is crucial to stick to your fluorescence PMT-voltages. The voltages must be the same for compensation controls and experimental samples.

### People ask us: Should we use cells or beads for compensation?

Most often we will recommend using beads (1-5). Beads have several advantages:

1) You save your precious sample for analysis.
2) Some antigens, e.g. activation antigens, could be dimly expressed on your cells, providing a poor compensation control, while the beads will provide a bright stain for compensation.
3) Beads fluorescence has a low CV, thus providing a more precise calculation. This is important in multi-parameter flow cytometry.
4) No worries concerning same autofluorescence of negative and positive populations (except for lot numbers, if the beads comes in two vials)

Then why use cells for compensation?

5) For transfected cells expressing a fluorescent protein you need (bright) positive cells and mock transfected negative cells for the compensation sample.
6) If your viability stain is 7-AAD, you need live and dead cells stained with 7-AAD for compensation. Here it is impossible to meet the “Same-Autofluorescence-Condition”. Therefore a better solution is to exchange 7-AAD with a Fixable live-dead stain and use beads for compensation.
7) Economy can speak for using cells. But be careful excluding beads which is a corner stone for providing correct multi-stain flow cytometry data.

### Choosing the right compensation beads:

1) Buy compensation beads that bind your antibodies (species, isotype, kappa/lambda).
2) Beads with a higher autofluorescence and binding capacity are designed for more autofluorescent cells and will also work well with antibodies targeting abundantly expressed antigens (2).
3) Special amine-reactive beads (3) can be used to compensate fixable live/dead dyes. These dyes do not bind to ordinary compensation beads.
4) There are special beads for the violet laser (4).

## Performing the compensation

1) Before using the compensation wizard, check that your compensation samples fulfill the requirements for compensation controls, and that the gating is OK. If not, you have to correct these conditions, before you can proceed.
2) Use the compensation wizard on the instrument to run the compensation controls. A matrix will be calucated and you can now watch your compensated samples while you collect data. You can always remove the calculated matrix again if you wish so.
3) You may chose to run compensation controls as single stained samples and calculate the matrix in a software like FlowJo.

**Questions?** Ask the FACS Core staff

## References/links

1) eBioscience, OneComp eBeads. Theese beads work well with mononuclear cells but is not the best choice for violet exited fluorochromes.
Binds antibodies from rat, mouse and hamster and are light chain independent
https://www.thermofisher.com/order/catalog/product/01-1111-42#/01-1111-42
2) Becton Dickinson, BD Compbeads PLUS,. Has a higher autofluorescence and binding capacity. #560497 binds mouse Ig, kappa #552845 binds rat and hamster Ig kappa
https://www.bdbiosciences.com/us/reagents/research/instrument-setup-maintenance/compensation-particles/anti-mouse-ig-negative-control-bsa-compensation-plus-75-m-particles-set/p/560497
Be aware not to mix lot-numbers, as these beads come in two vials – negative beads and positive beads.
3) ThermoFisher, Amine Reactive Compensation. Beads for fixable live/dead dyes
https://www.thermofisher.com/order/catalog/product/A10346#/A10346
Be aware to use the same lotnumber for negative and positive stain, as these beads come in two vials – negative beads and positive beads.
4) Thermo Fisher, Ultra Comp eBeads. Designed for the violet laser and works also with UV, 488, 561 and 433nm lasers as well
https://www.thermofisher.com/order/catalog/product/01-2222-42#/01-2222-42
5) Thermo Fisher about compensation beads:
https://www.thermofisher.com/dk/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-calibration/flow-cytometry-compensation-tools.html
6) FACS Core Facility Guidelines for Panel Design in Flow Cytometry
7) FACS Core Facility Guidelines for Controls in Flow Cytometry
8) FACS Core Facility Guidelines for Titration of Antibodies in Flow Cytometry