INTRODUCTION

Single stained CD4-PE sample:
A single fluorochrome can be detected in more than one channel, creating spillover.
COMPENSATION DEFINITION

• The process of subtracting the spectral spillover from a given fluorochrome from all neighboring channels where it is also detected

• The process for obtaining the measurement of a single reagent in the presence of two or more reagents
  
  - It is necessary because of the fact that the emission spectra of the fluorochromes we use, overlap with each other

COMPENSATING FOR SPILLOVER

Compensation is a mathematical subtraction to correct for spectral overlap

A488\text{true} = A488\text{measured} - \% 1

PE\text{true} = PE\text{measured} - 30 \%

http://www.dmr.com/compensation/
COMPENSATION: WHY AND HOW?

Why?
- Bad compensation is the most common source of data error in multicolor flow cytometry experiments
- If correct, you will get a more accurate discrimination of your population of interest
- If incorrect, interpreting data becomes extremely difficult or impossible
- Reviewers expect perfectly compensated samples

How?
- Setting the PMT voltages correct (use unstained cells and stained samples)
- Use single stain controls for each fluorochrome in the panel
- Use the autocompensation software in Diva or FlowjoX

COMPENSATION: THE RULES

There are only 3 rules to follow:

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
**COMPENSATION: RULE NR.1**

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

- Select single stain sample with the brightest fluorescence
- Dimness is relatively irrelevant, only brightest matters
- Compensation is only about estimating the slope:
  - The centers of the pos and neg populations are aligned by matching the median fluorescence intensities (MFI)
  - A sophisticated algorithm calculates the ratio of difference in MFI’s (off the spillover channel and the primary channel)
  - Small absolute errors in the position of the negative control become irrelevant as the positive controls become brighter.

**COMPENSATION: RULE NR.2**

**Background fluorescence should be the same for the pos and neg control**

- Any carrier for binding fluorochromes can be used for single stain comp controls (cells or beads), but the positive and the negative carrier of a parameter must have the same autofluorescence
- Why?
  - Compensation is a subtraction algorithm
  - This means that if the pos and the neg population have the same autofluorescence, then the autofluorescence contribution to the compensation spillover calculation will be zero
- Implies that you can make your matrix with beads and apply that matrix to cells!
COMPENSATION: RULE NR.3

Compensation controls MUST match the exact experimental fluorochrome

- Each fluorochrome has a unique emission profile
- FITC and AF488 not the same, different spillovers
- Rule is even more strict when you work with tandems
  - Each lot of tandem is unique and should require its own single stain
  - Different tandem lots will have different conjugation ratios, e.g. more Cy7 conjugated to PE or less

COMPENSATION SAMPLES

- You basically have 2 options:
  - Single stains on antibody capturing beads
  - Single stains on cells

BEADS

CELLS

TO BEADS OR NOT TO BEADS?

Advantages

• No need to waste precious cells
• Markers that are poorly expressed on the cells, will give strong signal on beads
• When the population of interest is rare or when the antibody has a low affinity for the receptor
• Most of the time, the beads will give you the strongest signal for any given parameter

Advantages

• You can compensate with the antibodies used in the experiment and not a CD4 substitute
• They are more precise
  ▶ Cells have a larger variance in background fluorescence, this results in a higher error in the computation of the compensation coefficients
  ▶ The autofluorescence of cells causes error in the matrix calculation
  ▶ Beads have a smaller error in the distribution of background fluorescence, so spillover computation is more precise
• They are relatively cheap (we can dilute them up to 10 times)
• Easy to use!
• People reuse old matrices, not so good
TO BEADS OR NOT TO BEADS?

Disadvantages

- Beads can’t be used with dyes such as PI, DAPI, CFSE,…
  - Amine reactive viability dyes will work with some beads (Arc beads)
  - You can use cells for your L/D stain and for proliferation markers while using UltraComps for the rest of the normal Ab-stained compensation controls
- Fluorescent proteins don’t work with it
  - GFP and mCherry beads are available now via Clontech
  - [http://www.clontech.com/FR/Products/Fluorescent_Proteins_and_Reporters/Flow_Cytometer_Calibration_Beads/AcGFP_and_mCherry](http://www.clontech.com/FR/Products/Fluorescent_Proteins_and_Reporters/Flow_Cytometer_Calibration_Beads/AcGFP_and_mCherry)
- They do not bind all ABs and sometimes the signal is not as bright as it would be with your cells
- Beads are not cells - sometimes problems with very big cells
- If you do permeabilization/fixation you have to double check that tandem dyes don’t react different on the beads than they would react on your cells

Which beads to use?

- There are a lot of compensation beads one the market
  - Comp beads Becton Dickinson
  - OneComp and UltraComp eBeads Ebioscience
  - ABC beads Thermofisher
  - Versacomp Beckman Coulter
  - Sphero COMPtrol Spherotech
- We were looking for the easiest solution for the flow core
- We did some tests with the Comp beads, the Onecomp and the UltraComp ebeads
- The ULTRAcomp eBeads gave the best results and were the easiest to use
TO BEADS OR NOT TO BEADS?

Why the ULTRACOMP\textsuperscript{\textregistered}ps?

- One drop needed (can be diluted 10 times with PBS)
  - The beads contain a population of 50\% capturing beads and 50\% non-capturing beads which will result in respectively the pos and the neg population
- They capture mouse, rat and hamster antibodies of IgG and IgM classes, independent of light chain
- Work with all lasers (355, 405, 488, 532, 561, 633nm)
- They are optimised to work with the violet laser

ULTRACOMP E BEADS

Website Q&A:

http://eu.ebioscience.com/resources/faq/flow-cytometry-faq.htm#OneComp-eBeads
ULTRACOMP E BEADS

IL-13 staining on cells
- High expression
- Positive control
- Qc to identify positive populations

IL-13 staining on CerComp eBeads
- Viable populations
- 90% viability
- Can use to identify positive populations
PROPOSED DIVA WORKFLOW

- Include beads in your experimental setup (next to your single cell stains)
- Run CS&T beads
- Run unstained cells and single cell stains and check the voltages (all events on scale)
- Make sure that each stain is most dominant in its own channel
- Create Application settings to link your exp with the CS&T (advisable)
- Record compensation controls but use UltraComp ebeads instead of cells
- Use autocomp in DIVA to create a matrix
- Record also single cell stains to be sure
- Record the samples or sort them
- Export samples as .fcs (3.0) files
- Analysis with Flowjo X
- Check spillover matrix
- Gating strategy and batch analysis

EXTRA TIPS

- Treat your compensation controls in the same manner as your samples
  - Exposure to light, fix / permeabilisation buffers may alter the fluorochrome
- Measure enough events:
  - 5000 for beads
  - 20000 to 30000 for cells
- Always use a live/dead cell stain
  - Use your Live/Dead stain in combination with the dump channel, e.g. DAPI + CD3/CD19-PacificBlue
- Use your TIME parameter to check if your flow cytometer is working good
- Titrate your antibodies!
- Play around with your gating strategy ----> backgating!
- Use Fluorescence Minus One (FMO) controls
- Isotype controls : highly debatable
RESOURCES

- http://flowjo.typepad.com/the_daily_dongle/tech_support/
- http://ucflow.blogspot.be/
- Google!

Compensation goal

For a given labeled/fluorescent or radioactive signal of a population, it should be the same as a negative population when viewed in the other channels.