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MetroFlow Meeting
Princeton, NJ

Fluorescence Compensation Handout - how to set up your experiment…

1) SINGLE STAINS – to set compensation

Which Cells?

You can use any cells for your single stained controls as long as you follow this rule: The positive and negative population for each single stain (ie, PE+ and PE–) MUST have the same autofluorescence.

Which Reagents?

Any reagent can serve to stain your single stained control, provided it is labeled with the same fluorochrome as your experimental sample. In addition, the single stains should be at least as bright as the experimental sample. For example, you might want to substitute a CD8 PE for a CD34 PE.

Caveats:

Just because GFP, CFSE and FITC are detected in the same channel (FL1), it does not mean you can use them interchangeably when setting up your compensation. GFP, CFSE and FITC are unique flourochromes and have different spillover into other channels.

Tandem dyes (such as Cy7PE, Cy7APC etc.) are manufactured by linking two fluorochromes. The amount of spillover into other colors depends on how they are manufactured. You cannot assume that all Cy7PE reagents are the same. Therefore you cannot substitute tandem dye reagents.

2) FMO (Fluorescence Minus One) – to set gates

In multicolor experiments, it is not possible to set gates based on an entirely unstained or fully isotype stained control. Remember, a control is defined as changing ONE condition at a time! Fluorescence Minus One (FMO) controls leave out one reagent at a time (the opposite of single stain controls!).

3) EXPERIMENTAL Controls – scientific question

Experimental controls help you answer the scientific question being asked. They are NOT meant to set the compensation or to determine where to draw your gates. Experimental controls allow a comparison of the sample from one experimental condition to another. For instance, if you are studying a cell line that has been drug treated, then you need to compare it to the same cell line that has not undergone treatment (sham treated).

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Example staining setup of a 3 color experiment:

Tube #	Description	FL1	FL2	FL3
1	*Experimental Sample*	CD3 FITC	CD4 PE	CD8 Cy5PE
2	*Compenstaion Controls* (Single stains – one for each fluorochrome used in the experiment)	CD3 FITC	-	-
3		-	CD4 PE	-
4		-	-	CD8 Cy5PE
5	*Gating Controls* (FMO – leave out one fluorochrome at a time)	-*	CD4 PE	CD8 Cy5PE
6		CD3 FITC	-	CD8 Cy5PE
7		CD3 FITC	CD4 PE	-
8	*Experimental Controls* (fully stain healthy or untreated samples to compare to experimental sample)	CD3 FITC	CD4 PE	CD8 Cy5PE
9		CD3 FITC	CD4 PE	CD8 Cy5PE
10		CD3 FITC	CD4 PE	CD8 Cy5PE

* no stain added or add isotype matched control stain.

References:

- Mario Roederer's Compensation Web Page - www.drmr.com/compensation
- Purdue Cytometry Mailing list – www.cyto.purdue.edu
- Practical Flow Cytometry 4th Edition. H. Shapiro. Wiley-Liss, 2003.
- Flow Cytometry: First Principles. 2nd Edition, A. Givan, Wiley-Liss, 2001
- Methods in Cell Biology: v.40,41, 63, 64 Darzynkiewicz, Robinson & Crissman, Academic Press, 1994, (Vol 63,64, 2000).
- In Living Color: Protocols in Flow Cytometry and Cell Sorting. R. Diamond & S. DeMaggio. Springer-Verlog, 2000.
- Current Protocols in Cytometry, Wiley-Liss. Available on-line and on CD.
- Ask the experts!