

Guideline for Proper Compensation in Flow Cytometry

- 1) What is compensation?
- 2) How to perform compensation?
- 3) How to minimize compensation?
- 4) Mistakes to avoid when compensating samples

1) What is compensation?

Spectral overlap occurs when the excitation and / or emission spectra of two or more fluorophores (and /or autofluorescence) in a specimen overlap making it difficult to isolate the activity of one fluorophore alone. It usually happens with long emission spectrum and also due to the use of bandpass and dichroic filters to separate fluorescence emission from the excitation light source.

The goal of compensation is to remove the spillover fluorescence of a particular fluorophores from the "wrong" channel (secondary fluorochromes). Each detector of a cytometer collects light from multiple sources: principally from the primary fluorochrome, but some light from other fluorochromes as well. The process of compensation is the correction for the light emitted by these secondary fluorochromes.

2) How to perform compensation?

Color compensation must be performed to correct spectral overlap during multicolor flow experiments. The goal of compensation is to correctly quantify each dye with a particular cell is labeled. This is done by abstracting a portion of one detector's signal from another,

leaving only the desired signal. Compensation usually is done step by step. The steps should be taken with every experiment where the cell types differ, where the reagents differ, or where any instrument settings change.

-First step: running an unstained control as well as cells that are singly-stained with the fluorescent probes. The stained cells must have the same autofluorescence as do the unstained cells in the compensation tube. In other words, using a fluorescence probe specific to monocytes and lining them up with lymphocytes, whose autofluorescence is significantly lower than monocytes, will fail to yield proper compensation.

-Second step: this step is necessary to adjust the PMT voltages for each desired color running single stained compensation controls. The PMT voltages must be set high enough to guarantee that the negative population is off the axis in every channel.

-Third step: set up an analysis gate to include only the cells with identical autofluorescence (e.g., a lymphocytes gate). An analysis gate is also set to include all of the negative cells and all of the positive cells.

-Fourth step: the centers of the positive and negative cell populations are aligned by matching the median fluorescences.

3) *How to minimize compensation?*