Miltenyi Biotec

Flow cytometry: Compensation

Introduction

The use of multiple fluorochrome-conjugated antibodies in a flow cytometry experiment allows the simultaneous analysis of many cell subpopulations in a single sample. A vast number of fluorochromes with different excitation and emission properties is available. However, fluorochromes can have spectral overlaps, and fluorescence emitted from a single fluorochrome into two channels can compromise the detection of fluorescence emitted by another fluorochrome into one of these channels. This needs to be accounted for during analysis. A method called fluorescence compensation enables the clear distinction of the signals emitted by these fluorochromes, thus allowing for the unambiguous detection of the cell populations.

Fluorescence compensation guidelines

To set the compensation correctly control samples must fulfill the following requirements.

1) **Compensation control sample must be stained with one color only.**
   When a cell population emits fluorescence into two channels simultaneously, this can have two reasons: 1) The cell population expresses a marker that is stained with a fluorochrome-conjugated antibody, and fluorescence spillover from the fluorochrome is detected in a second channel. 2) The cell population expresses two markers that are stained with different fluorochromes emitting into these two channels. To exclude that double-positive cells are measured during compensation, the compensation control should be stained with a single color only.
2) **Samples must contain a negative and a positive cell population for median comparison.**
   To set compensation correctly, the median fluorescence intensities (MFIs) of negative and positive cells in the spillover channel have to be compared. After adjusting the compensation factor, the MFIs should be around the same value.
3) **Negative and positive cells must have the same level of autofluorescence.**
   If there is a difference in autofluorescence, samples might be overcompensated in the experiment. Compensation is a powerful method for correcting spectral overlap, but it is not appropriate for adapting differences in autofluorescence.
4) **Surrogate markers can be used for compensation (e.g. for rare cell applications).**
   It is not necessary to stain the same marker in control sample and experimental sample. Different markers can be used for compensation. This is particularly important when the positive cells are rare and proper compensation would require the acquisition of a large number of cells.
5) **Use bright markers for setting compensation.**
   If an alternative marker is used for setting compensation, it is crucial that the marker in the compensation control is at least as bright as the marker in the experimental sample. Otherwise compensation might not be adjusted properly. In general, dim compensation controls are not suitable for determining precise compensation values.
6) **Compensation control samples must contain the same fluorochrome as the experimental sample.**
   While it is not that important to use exactly the same cells in the compensation control samples, it is vitally important to use exactly the same fluorochrome. **FITC, for example, should not be used to compensate for GFP.** Different shapes of fluorochrome emission spectra result in differences in the magnitude of spectral overlap and therefore require different compensation.

7) **Tandem fluorochrome conjugates require lot-specific compensation.**
Tandem fluorochromes are indispensable for multicolor applications. Their large Stokes shift expands the variety of fluorescence channels that can be used in an experiment. However, the saturation of the donor fluorochromes with acceptor molecules can differ from lot to lot. This can result in differences in the emission behavior of the donor fluorochrome (fig. 1). Therefore, different compensation values may be required for different antibody lots. This means that exactly the same antibody conjugate lot has to be used for compensation samples and experimental samples.

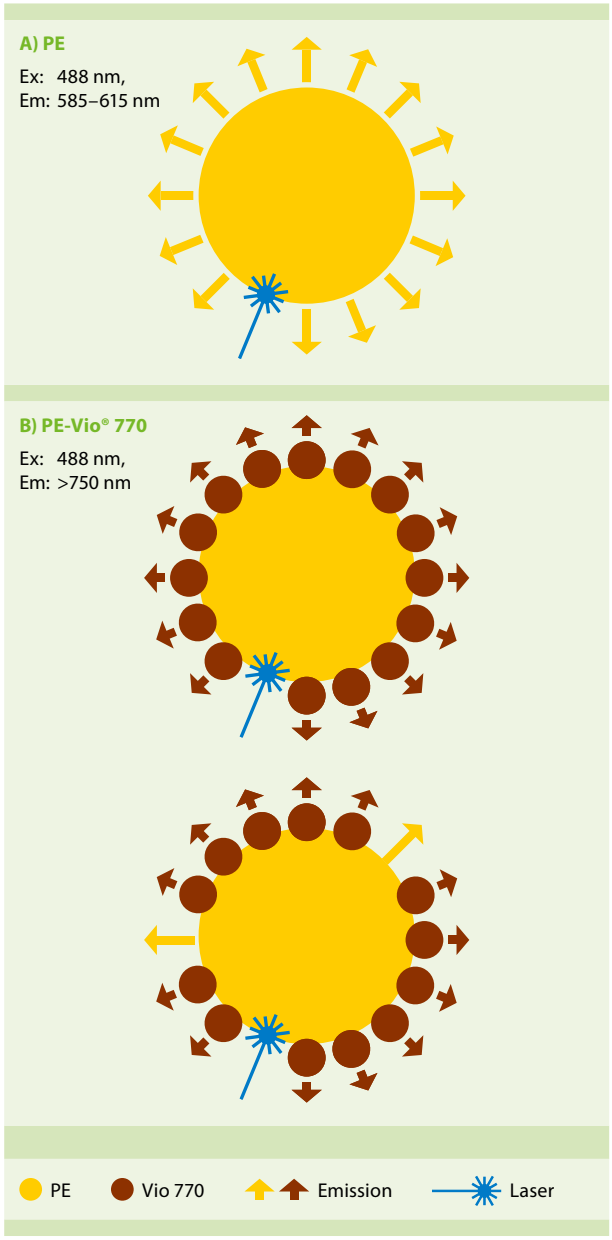

Figure 1: Schematic of fluorescence emission by a single fluorochrome (A) and tandem conjugates (B). Different saturation levels of the donor fluorochrome (i.e. PE) with acceptor molecules (i.e. Vio 770) lead to different emission properties of the tandem conjugate.

8) **Compensation beads can be used instead of cells.**
Compensation beads are "artificial cells" that can be loaded with fluorochrome-antibody conjugates. They can then be used to set compensation. They fulfill many of the requirements outlined above and are therefore universal and excellent compensation controls: negative and positive beads have the same autofluorescence (3), the fluorescence emission of these beads is very bright (5), the capture beads can be loaded with the same fluorochrome conjugate lot used in the experiment, thus enabling lot-specific compensation (7).

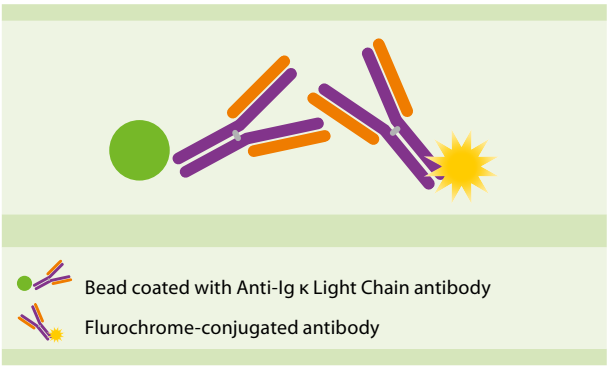

Figure 2: Schematic of fluorochrome-conjugated antibody binding to compensation bead.

A number of kits are available from Miltenyi Biotec that enable straightforward compensation. The kits are based on beads coated with antibodies against the kappa light chain of the labeling antibodies. Therefore, the beads can bind to the labeling antibodies and mimic cells that are positive for the fluorochromes conjugated to the antibody (fig. 2).

The kits also contain beads that are not coated with antibody, thus resembling negative cells. The beads allow for lot-specific compensation of tandem fluorochromes as they can be loaded with exactly the same lot. An example of compensation with a MACS® Comp Bead Kit is shown in figure 3. The following kits are available:

- MACS Comp Bead Kit, anti-mouse Igκ for capturing of antibodies of murine origin (# 130-097-900)
- MACS Comp Bead Kit, anti-human Igκ for capturing of antibodies of human origin (# 130-104-187)
- MACS Comp Bead Kit, anti-REA for capturing of antibodies from the REAfinity™ Product Line (# 130-104-693)

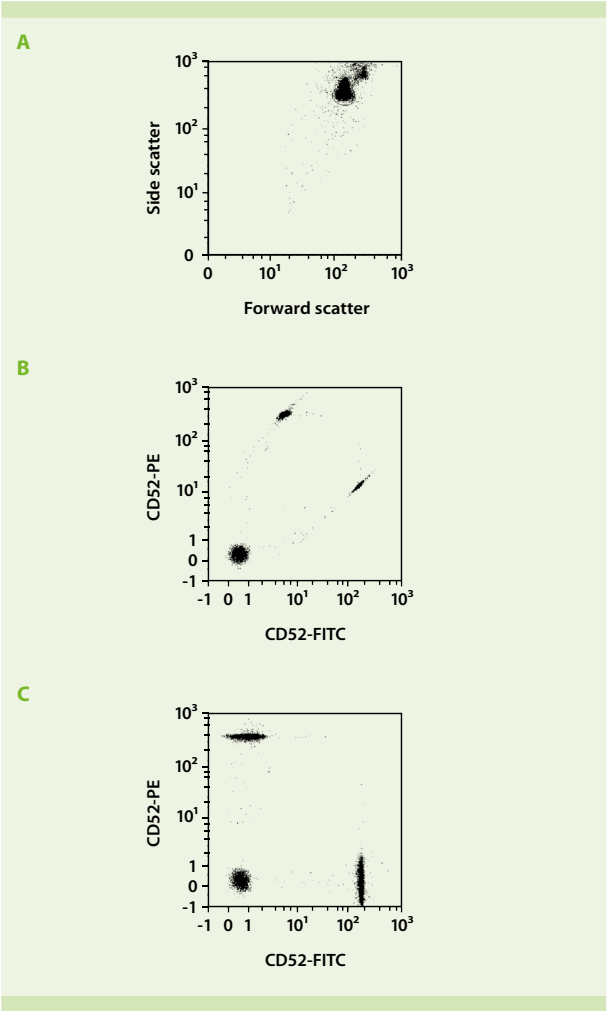

Figure 3: Example of a compensation using the MACS® Comp Bead Kit – anti-REA. The MACS Comp Beads – anti-REA were labeled with antibodies conjugated to FITC and PE and analyzed. Gating was performed on single bead events (A). MACS Comp Beads are shown before (B) and after (C) compensation using the Compensation Multicolor program with the MACSQuant® Analyzer.

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