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Compensation in Multicolor Flow Cytometry

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• **Key terms**
flow cytometry; compensation; antobody capture beads; immunophenotyping; multicolor flow cytometry

Multicolor (or polychromatic) flow cytometry is a powerful technology that allows researchers and clinicians to perform complex cellular analysis quickly and efficiently by analyzing up to 20 fluorescent parameters simultaneously. This technique enables the identification of even rare cell subpopulations with characteristic pattern of antigen expression (1). In particular, this technology was proved to be crucial in identifying functionally homogeneous subsets of cells within the enormously complex immune system and also contributes to the deeper understanding of the pathogenesis of certain immunological diseases (2).

When more than one fluorescent dye is applied simultaneously in flow cytometry detection problems may arise. In general, the emission spectra of the fluorescent dyes are inherently wide because the emitted photons result from transitions of the electrons from the first excited state to different vibrational levels of the ground state. In addition, for sensitivity reasons rather wide spectral ranges of the emitted photons are detected in the flow cytometers. Thus, the signal of a fluorescent dye may bleed into a channel applied for the detection of another dye. The simplest example to illustrate the effect of spectral overlap is fluorescein isothiocyanate (FITC) and R-phycoerythrin (R-PE). Both dyes are excited by a 488-nm laser, and the emitted photons are detected by photomultiplier tubes equipped with a 530/30-nm bandpass filter and a 585/42-nm bandpass filter for FITC and PE, respectively. As it is shown in Figure 1, FITC can be detected in the green channel (FITC channel); however, about 15% of the emitted photons spillover into the yellow channel (R-PE channel) of the flow cytometer. On the other hand, about 2% of the photons emitted by R-PE is detected in the green channel (FITC channel).

What is the consequence of the spectral spillover in case of two-color flow cytometry applying the above fluorophores and filter sets? In a model experiment, two cell surface antigens were stained with FITC and R-PE-conjugated antibodies: 25% of the cells were negative for both antigens, 25% were double positive, and 25–25% were positive for only one of the antigens. As it is shown in Figure 2A, because of the significant spillover of FITC fluorescence into the R-PE channel, the FITC single-positive cells exhibited a relatively high fluorescence intensity in the R-PE channel. Consequently, the double-positive and FITC-positive cell populations colocalized in the upper right quadrant of the dot plot and the two populations could not be discriminated (Fig. 2A).

The aim of multicolor flow cytometry is to properly quantify the fluorescence intensity of each dye with which a particular cell is labeled and to correctly identify each cell populations with distinct antigen expression patterns. To achieve this goal, we have to remove the spillover fluorescence of a particular probe from the "wrong" channel(s) applying a method referred as compensation. As the amount of the spillover fluorescence is a linear function of the fluorescence intensity at the same photomultiplier tube voltage(s) and filter set(s), introducing a spillover coefficient for each channel is sufficient for the flow cytometry software to calculate the compensated data. In the previous simulation, two coefficients are needed, one which represents the spillover of FITC into the R-PE channel and another which represents the spillover of R-PE into the FITC channel. Mathematically, compensation of FITC from R-PE simply subtracts a fraction of the FITC signal from the R-PE signal. As an example, if the amount of yellow fluorescein signal in the R-PE channel is 15% of the green fluorescein signal in the FITC channel (i.e., 15% compensation), then we can exactly determine the "true" (or pure) PE fluorescence of a cell, even in the presence of FITC fluorescence, as follows (3):

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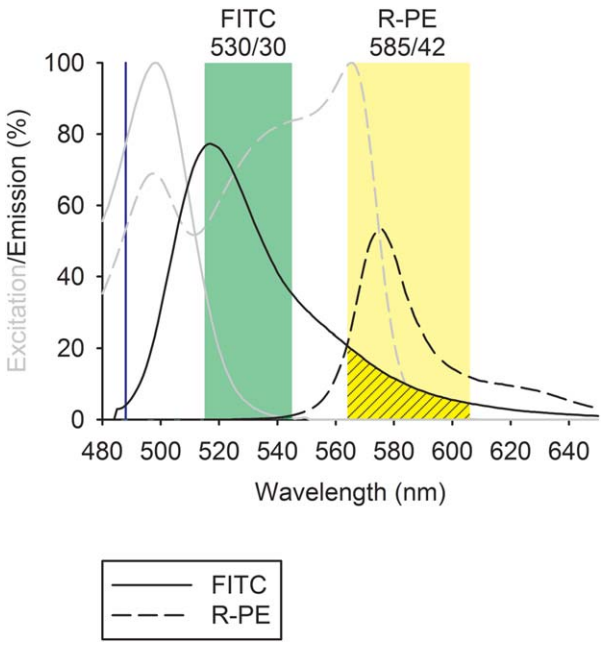

Figure 1. Excitation and emission spectra of FITC and R-PE. Colored bands demonstrate the selected spectral ranges in the green (FITC) and yellow (R-PE) detectors. Cross-hatched area represents the spillover fluorescence. Source: Life Technologies.

$$\mathrm{FLI}_{\mathrm{R\text{-}PE}}^{\mathrm{R\text{-}PE\ channel}} = \frac{\mathrm{FLI}_{\mathrm{total}}^{\mathrm{R\text{-}PE\ channel}} - 0.15 \times \mathrm{FLI}_{\mathrm{total}}^{\mathrm{FITC\ channel}}}{1-(0.15 \times 0.02)}.$$

This process is identical for correcting for R-PE fluorescence appearing in the FITC channel. For instance, if the amount of (green) PE signal in the fluorescein channel is 2% of the (yellow) signal in the PE channel (i.e., 2% compensation), then we can exactly determine the true FITC fluorescence of a cell as follows (3):

$$\mathrm{FLI}_{\mathrm{FITC}}^{\mathrm{FITC\ channel}} = \frac{\mathrm{FLI}_{\mathrm{total}}^{\mathrm{FITC\ channel}} - 0.02 \times \mathrm{FLI}_{\mathrm{total}}^{\mathrm{R\text{-}PE\ channel}}}{1-(0.15 \times 0.02)}.$$

By applying the above spillover coefficients, we could identify four subpopulations as shown in Figure 2B.

Correct compensation is even more critical and also more difficult to carry out when one or more subpopulations are small. In this case, lack of compensation leads to the fusion of the double-positive and FITC-positive cell populations (Fig. 2C). After proper compensation, we could clearly discriminate the FITC single-positive cells and the double-positive ones (Fig. 2D).

Calculation of the compensated data, especially when numerous fluorescent channels are used, requires complex matrix calculations (for details see Refs. 3–6); however, if the spillover coefficients are known, these are done by the computer software. Unfortunately, experimental determination of the spillover coefficients is sometimes difficult.

The spillover coefficients can be determined using appropriate compensation controls prepared for all fluorophores. These control samples usually include an unstained and a single-stained sample, usually mixed together. Using the unstained sample, the autofluorescence of the cells can be determined in each fluorescent channel, while measuring the single-stained sample, the spillover coefficients can be set manually in all channels where compensation is needed. By applying proper compensation coefficients, the fluorescence of the dye is measured only in the detector, which is selected for the dye, whereas in the other channels, only the autofluorescence is measured, because the fluorescence intensity resulting from the spillover of the dye is subtracted from the fluorescence intensity of the cells.

In general, an ideal compensation control contains adequate number of cells, and the labeled cells emit high fluorescence intensity, which allows the discrimination of the positive population from the negative population or unstained cells. In case of multicolor measurement, single-stained compensation controls originating from the same cell line or sample have to be used as it was demonstrated previously (7,8). In case of high enough number of cells is available, multistained compensation controls can improve the compensation accuracy as presented by Sugár et al. (4).

Unfortunately, as the number of the measured parameters increases, dimmer fluorochromes have to be included in the applied antibody panel, and consequently, it is less likely that the positive and negative populations can be separated well enough in each channel for proper compensation. Moreover in many cases, including leukocyte immunophenotyping, the ratio of the positive cells is only a few percent, and because the samples are often collected from living donors and patients, they do not contain enough positive cells for proper compensation.

If compensation controls cannot be prepared from the same cells, alternative compensation controls should be found. The most important rule is that the background fluorescence of the unstained and single-stained samples has to be equal, because differences in the background fluorescence lead to incorrect compensation. We can use a cell line expressing the antigen of interest at high level as a compensation control. In this case, the same antibody–fluorochrome conjugate has to be used for compensation and measurement. Alternatively, compensation could be carried out using another antigen expressed at high level, for example, CD45 or CD8 in case of leukocytes (3). Of course, the antibody used for compensation should be conjugated with the same fluorochrome applied in case of the antibody used for the antigen of interest. In the latter case, the cells can be obtained from freshly prepared PBMCs of a healthy volunteer.

Beads offer a good alternative for compensation controls. Because of their high uniformity, they provide very sharp peaks with small coefficient of variation. In some cases, internally stained fluorescent beads also work well (9); however, unfortunately, their usage is limited because only a few fluorochromes are available in this form.

The application of antibody capture beads provides more flexibility in compensation, because they are coated with an antibody-reactive (in most cases IgG reactive) layer capable of binding any antibody, which is used in the experiment. As uncoated beads have identical background fluorescence to the coated ones, they can serve as negative control, whereas the

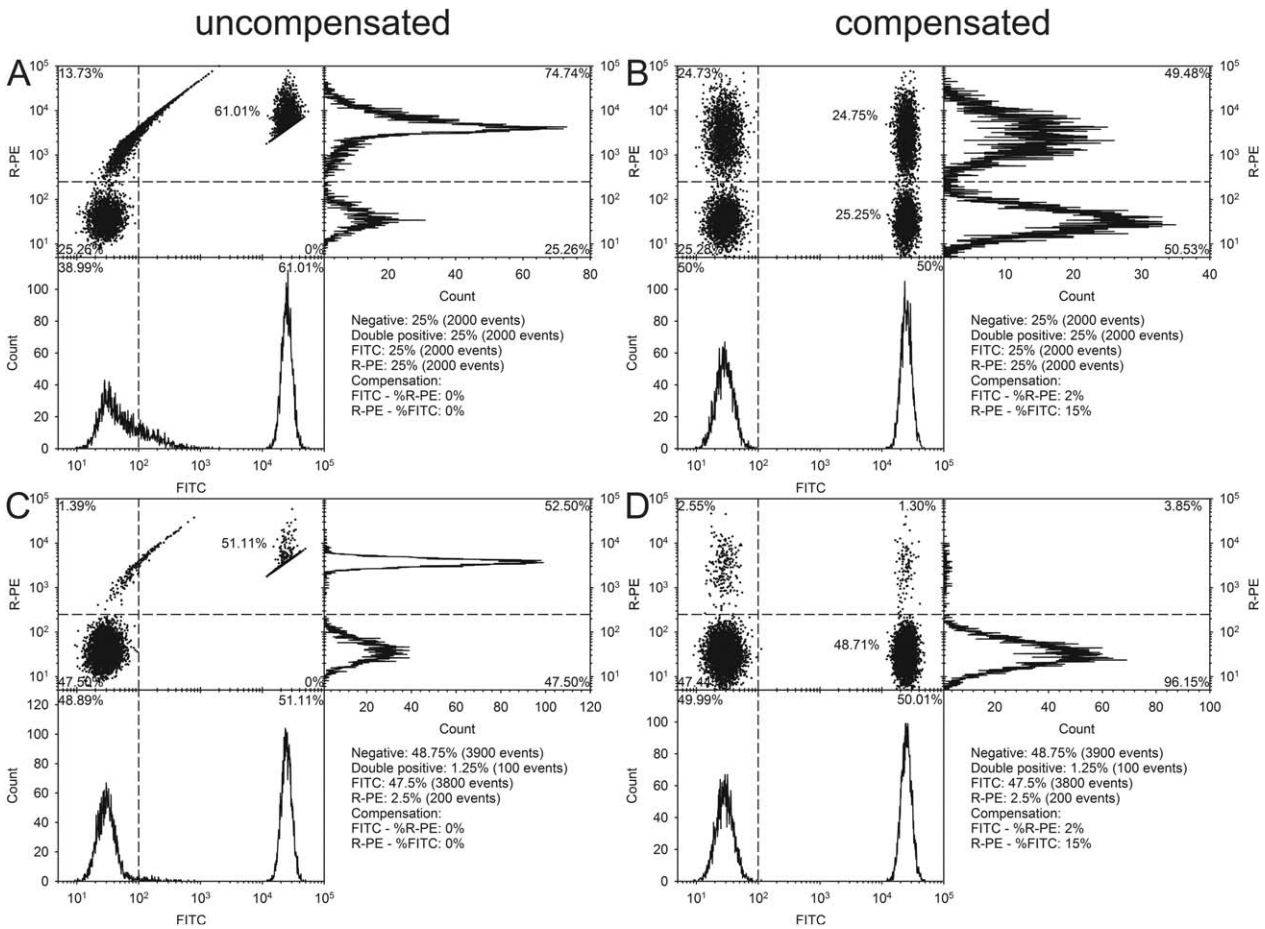

Figure 2. Simulation of the results of a two-color flow cytometry experiment using cell populations with heterogeneous labeling with FITC and R-PE without (**A** and **C**) and with (**B** and **D**) applying compensation. In Panels A and B, the ratio of the negative, single-positive cells, and double-positive cells was identical, whereas in Panels C and D, the ratio of the R-PE-positive and double-positive cells was very low. The exact parameters of the simulation are shown in each panels, and the measured percentages can be seen nearby the corresponding dot plots and histograms.

coated beads provide high signal by capturing large amount of antibodies, and thus by using them together, the compensation setup can be easily carried out.

In the current issue of Cytometry Part A, Byrd et al. (page 1038) present a compensation method using antibody capture beads, which is enable to detect 10 fluorescent parameters simultaneously in human primary lymphocytes. They showed that antibody capture beads, despite their smaller size, have similar background fluorescence to the lymphocytes and provide a bright, discrete positive population for all used antibodies, making the compensation setup easy. They also set up a compensation method using single-stained PBMCs and found that the compensation matrices generated with beads and PBMCs are not significantly different from each other, thus the application of antibody capture beads is a reasonable alternative of PBMCs in the compensation procedure. It was also demonstrated that, owing to the broad antibody specificity of the beads, they can be applied with antibodies from different species, belonging to various isotypes or even reacting with intracellular epitopes. In previous studies, using antibody capture beads for compensation immune cell subsets could be accurately determined in human (10) or in rhesus macaques (11) by staining up to 12 cell surface antigens. Rare events like low cell number subpopulations can also be measured, as reported by Zimmerlin et al. (1). In addition, this work also demonstrates that internally stained fluorescent beads and antibody capture beads can be applied in combination.

Nowadays, multicolor flow cytometry has become a routine method of clinical diagnostics and research. To precisely quantify, the ratio of subpopulations of the immune or other cells is essential for the proper diagnosis, and this quantification cannot be done without appropriate compensation. The performance of the flow cytometers in research and diagnostics is continuously growing. Clinical cytometers are capable of measuring up to 10 fluorescent parameters, whereas the top research cytometers can record data from 20 fluorescent channels. To be able to take advantage of the huge number of channels in multicolor experiments and the well-designed antibody panels, the spectral spillovers of the used dyes have to be properly compensated. By applying good compensation

controls such as antibody capture beads and accurate automatic compensation methods, which are now included in many flow cytometry software, we can increase the accuracy of the measurements and the number of the simultaneously detected parameters.

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