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10 Tips for the design of multi-color flow panels

1 Familiarize yourself with your flow cytometer

It is important to understand the capabilities of your flow cytometer. Most new cytometers have three or more lasers, selected from ultraviolet (355 nm), violet (405 nm), blue (488 nm), yellow (561 nm), and red (640 nm). In addition to the lasers, there are specific filters and detectors that determine the configuration. These components define how many colors can be detected simultaneously (for new cytometers this can be > 17). Regular calibration using fluorescent beads and cleaning will allow you to get the most out of your flow cytometer.

2 Prepare your sample correctly

Poorly prepared or unhealthy cells will lead to suboptimal results due to inaccurate gating and increased background autofluorescence. It is also important to treat the cells gently and avoid vortexing when making cell suspensions. During staining, cells should preferably be kept on ice (if the experiment permits). To reduce the formation of aggregates, which can block the flow cell, high cell densities should be avoided. The addition of DNAseI or EDTA as well as filtering can also help to reduce aggregates.

3 Remove the dead cells

Dead cells can lead to false positives due to autofluorescence and increased non-specific antibody binding. They cannot always be removed by FSC and SSC gating so it is best to use a live/dead exclusion marker. Propidium Iodide (ReadiDrop™ Propidium Iodide) or 7AAD (ReadiDrop™ 7AAD) are commonly used to identify the dead cells, but they cannot be used with fixed cells. Fortunately, there is a range of live/dead cell reagents such as Vivafix™, which can be used with live or fixed cells.

4 Optimize your staining protocol

Non-specific binding of antibodies due to charge or protein-protein interactions can lead to false positives. This can be reduced by including BSA or unconjugated antibody in your staining buffer to block these interactions. Furthermore, many cells (e.g. B cells, NK cells and macrophages) have Fc gamma receptors on their cell surface which can bind to the Fc region of your antibody resulting in non-specific staining. These can be blocked by the addition of FcR blocking reagents or the use of F(ab)2 fragments which do not contain an Fc gamma portion. High antibody concentrations can also increase non-specific binding; therefore all antibodies should be properly titrated to maximize the signal to noise ratio. Using appropriate antibody concentrations is critical for multicolor flow.

5 Decide if and when isotype controls are to be included

You may not wish to use isotype controls[1], but if you do, make sure you have included other appropriate controls, which we will mention later. Isotype controls have been developed for surface staining to determine what is specific. The correct isotype control is an antibody generated against an irrelevant antigen (e.g. KLH) of the same antibody subclass, with the same conjugated fluorophore, that is obtained from the same supplier as your test antibody and used at the same concentration. Their purpose is to ensure the observed staining is due to specific antibody binding rather than an artefact, to exclude non-specific binding to Fc gamma receptors and to exclude non-specific binding of the antibody or the fluorophore (such as PE) to cellular components.

6 Intracellular staining controls, permeabilization and potential problems

Intracellular staining can be more problematic than cell surface staining and in this case, isotype controls may not be appropriate. It is good practice to have several controls in order to be able to accurately determine your positive population. These can include an unstained sample, a negative sample stained with antibody that does not express the antigen of interest, or a known positive sample. There are several permeabilization protocols that use commercial reagents (e.g. Leucoperm™) alone or in conjunction with methanol, or with mild detergents such as saponin. Different methods could improve the staining, but some conjugates need to be avoided (e.g. PE when using methanol). Finally, avoid using biotin and FITC in intracellular panels if possible. Endogenous biotin has to be blocked with unconjugated streptavidin and FITC can bind non-specifically through electrostatic interactions.

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7 Build effective multicolor panels

When the emission spectra of two fluorophores overlap, spill over of one fluorophore into the detection channel of the other might be observed (e.g. FITC and PE). This can make it difficult to identify discrete populations without the use of spill over correction by a technique called compensation. Where possible, the spill over can be minimized by selecting fluorophores that have little or no overlap, however this is not always possible when using several fluorophores. A spectrum viewer can assist in obtaining the best fit emission profiles. Using fluorophores with little or no overlap for cell subset markers (e.g. T cell subsets) and closely overlapping fluorophores for mutually exclusive markers (such as CD3 and CD19) can prevent reduced sensitivity due to fluorescence spread.

8 Multicolor panel controls

For multicolor panels single stained compensation controls are essential for calculating compensation values. Compensation values can also be determined using antibody binding beads labeled with your proposed multicolor panel. Finally, fluorescence minus one (FMO) controls can determine fluorescence spread, gating boundaries and help avoid reduced sensitivity.

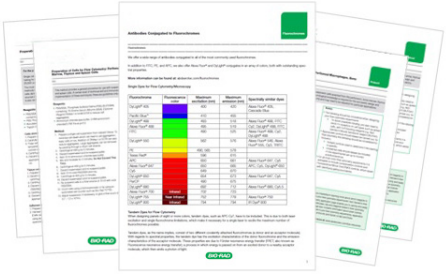
9 Know your biology

Fluorophores with the brightest stain index (such as PE) are best used for cells with the lowest antigen expression or the smallest subset, whereas dimmer fluorophores are more suitable for more highly expressed antigens. However, when using several colors, compromises may have to be made due to availability of fluorophores, antibodies and flow cytometer configuration. Tandem dyes can complicate the process due to the spillover fluorescence of the donor fluorophore into many other fluorophore channels. Staining index tables are available to assist with multicolor panel design to minimize this.

10 Treat your fluorophores with care

As you increase the number of colors in your panel, you will have to use tandem dyes such as PE-Alexa Fluor® 647 or APC-Cy7, which increase the emission range from a single laser. However they are prone to degradation by light and cellular biological activity resulting in uncoupling and fluorescence emission at two wavelengths, leading to false positives. The brightness of tandem dyes may be reduced by fixation and permeabilization, so ideally, these steps should be as mild as possible. Compensation values should be obtained from samples treated in an identical manner to account for this.

[1]For further reading see : Herzenberg L et al. Nat Imm 2006, Maecker HT and Trotter J. Cytometry Part A 2006



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