Panel Design

General Information

Multicolor flow cytometry is used for the simultaneous measurement of several fluorescence parameters of individual cells (multiplexing). It offers many advantages, including the correlation of cell data with multiple analytes and the precise definition and identification of cell populations. In addition, combining multiple colors in one experiment means that fewer samples are needed, sample volumes are smaller and throughput increases. However, as the number of colors and antigens detected increases, so does the complexity of the experiment and therefore the need for optimization and controls.

How to Build a Multicolor Flow Cytometry Panel

Creating a multicolor flow cytometry panel is one of the most difficult processes when working with multicolor flow cytometry. The following points should be considered when developing a multicolor flow cytometry panel:

1. Determine the scientific question you want to answer and perform antigen classification
    a. Rank your antigens according to **expression** (if known)
    b. Check your antigens for **co-expression**
2. Check the optical **configuration** of the available flow cytometer. You must define exactly which fluorophores you can or cannot use on your available flow cytometers.
3. Use an online **spectra viewer** to obtain information about excitation and emission of the fluorochromes you can use as well as spectral overlaps.
4. The **spillover-spreading error** has a significant impact on the quality of your mutlicolor immunofluorescence experiments. It depends on spectral overlap, fluorophore brightness as well as antigen density. Follow the rules below to design a panel to minimize the known spillover error. A spillover spreading matrix (SSM) generated from the flow cytometer can be used for optimal fluorochrome selection. In addition, all antibodies and dyes should be titrated to minimize spillover error.
    a. Low expressed antigens and markers of special interest should be matched with bright fluorochromes and receiving minimal spreading error to enhance sensitivity. However, low expressed or rarely expressed markers can be assigned to fluorophores that cause a lot of spreading error since spreading will then be only an issue for a small number of cells.
    b. Highly expressed antigens should be matched with dim fluorochromes that cause minimal spreading error.
    c. **Fluorochrome brightness** is determined by the intensity differences between stained and unstained cells, which are determined by the background fluorescence (e.g. due to non-specific staining, cellular autofluorescence and device noise) and the intensity distribution of the unstained cell population. In addition, it also depends on the antibody, antigen, device configuration, cell type and other factors. However, staining index tables can be used as a general guide.
    d. If you need to use fluorochromes with significant spectral overlap or spreading error, use them for different subpopulations or mutually exclusive markers. For co-expressed markers, assign fluorophores with minimal spread into each other.

e. Assign fluorophores that cause a lot of spreading error to a dump channel (one or more antibodies that remove cells that will not be analyzed, such as lineage). In this case, no cells of interest will have the offending fluorophore(s) and spreading can be ignored.

» **Be aware that tandem fluorochromes may be less stable and have a shorter life-time compared to non-tandem fluorochromes.**

Panel Validation

Before running experimental samples, check that your panel is working by following the steps described below:

1) To ensure that you work in the linear range for all detectors and maximize the signal to noise ratio you must first **titrate** your antibodies, viability stain and other stains. Additionally, unspecific antibody binding may be reduced by adding a blocking agent (e.g. with BSA, FcR blocking reagents). Consider saturation titration versus separating titration.

» **TIPP: Remember to include negative cells to detect non-specific staining.**

2) To exclude spillover effects from one channel to another, **compensation** must be performed. To do this, it is necessary to prepare and run both unstained control cells and single stained controls together with your multi-stained samples.
3) A **viability stain** may be added to these individual validation stains, which enables validation only on living cells, as dead cells can contribute to false positive staining.
4) Additionally, Fluorescence minus one (**FMO**) controls can be included to determine fluorescence spread, gating boundaries and help avoid issues with sensitivity.
5) After compensation, create histogram overlays of blank, FMO, single stain and multi stain cell samples for each fluorochrome.

   Ideally, blank and FMO are equally bright, and single stain and multistain are equally bright. This overlay reveals data spread as well as the over- or undercompensation. It can also reveal antibody/fluorochrome cross-reactions.

   Once the panel has been validated, it is not usually necessary to include FMOs for each marker - only for those where FMO is required to accurately determine the threshold for positive staining (such as continuously expressed antigens).

Useful Resources

Cytek® FSP™ offers a **Webinar Series** on Panel Design, which is highly recommended:

https://cytekbio.com/blogs/spectrolearn

Several **Spectra Viewer** are available online. Here are some examples:

https://cloud.cytekbio.com/spectrum/cloudspectrumviewer

https://www.bio-rad-antibodies.com/spectraviewer.html

https://www.bdbiosciences.com/en-at/resources/bd-spectrum-viewer

https://www.beckman.at/flow-cytometry/fluorescence-spectrum-analyzer

https://www.biolegend.com/en-us/spectra-analyzer

Several online tools can help with **Panel Design**. The tool's algorithms take into account most of the aspects mentioned above. Examples of free online tools are listed here:

- https://app.fluorofinder.com
- https://cloud.cytekbio.com/panelbuilder/panels

TIPP: highly recommended - create a free log in!

https://www.thermofisher.com/order/panel-builder/#!/

https://www.biolegend.com/en-us/panel-selector

https://www.miltenyibiotec.com/AT-en/resources/tools/flow-cytometry-panel-builder.html

IF YOU HAVE ADDITIONAL QUESTIONS, PLEASE CONTACT THE CYTOMETRY CORE FACILITY AT ZMF-CF-Zytometrie@jku.at