



Panel Design in Flow Cytometry

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This guideline work in connection with the FACS Core Facility Guidelines

Titration in Flow Cytometry (13)

Controls in Flow Cytometry (14)

Compensation in Flow Cytometry (12)

A big challenge in multicolor flow cytometry is setting up and validating a panel of antibodies conjugated to fluorochromes that gives you clear and correct results.

You can find good descriptions at several vendor homepages (e.g. ref 1, 3 and 4)

Designing your panel, several issues have to be acknowledged:

- 1) Know your hypothesis. Which markers do you need to answer your scientific question?
- 2) Rank your antigens according to expression (if you know). Low expressed antigens should be matched with bright fluorochromes to enhance sensitivity. Highly expressed antigens should be matched with dim fluorochromes. Abundancies for some antigens are listed in reference (1).
- 3) Fluorochrome brightness is dependent on instrument configuration.
We have calculated the brightness of fluorochromes or stain index for our LSRFortessa (2) for certain PMT-V settings.
- 4) Choose only fluorochromes which can be detected on the flow cytometer you have available (3 and 14).
- 5) Use one or more of the online "spectra viewers" to gain information about excitation and emission of the fluorochromes (5, 6, 7 and 8).
- 6) Try to minimize spectral overlap. If you need to use fluorochromes with considerable spectral overlap, use them for different subpopulations.
- 7) Dim markers and markers of special interest should be placed in channels that do not receive much spectral overlap.
- 8) Always include a viability stain in your panel, as dead cells can contribute with false positive staining.
- 9) Block before adding antibodies to your samples to avoid Fc binding of your antibody (9, 10)
- 10) Be aware that tandem fluorochromes may be less stable and have a shorter life-time compared to non-tandem fluorochromes.

You can find an overview showing lasers and detectors for all the core facility's instruments on our homepage (14).



We highly recommend the site FluoroFinder (3) for setting up a multicolor panel. You can create a free log in and relate to Aarhus FACS Core Facility. Then you can choose any of our flow cytometers. The lasers and filters on this specific flow cytometer will show up in the table where you choose antibodies and stains for your panel. The table will only provide fluorochrome options that comply with the selected flow cytometer. FluoroFinder cooperate with many antibody companies and you will find a huge number of antibodies, live-dead stains and other dyes at the site.

When you have created a panel in FluoroFinder, you can use the button “Core Facility Review”. This will send your panel setup to the FACS Core Facility and we will give you feed back before you buy any reagents.

For our LSRFortessa, we have made suggestions for best fluorochromes to choose together (2).

Validating your panel

Before you run experimental samples, verify that your panel works and that you can find all cell populations of interest.

Be sure to work within the linear range for all detectors – for negative populations as well as for positive.

- 1) First thing to do is to titrate (12) your antibodies, viability stain and other stains. You need of course positive cells for your titrations, but also negative cells to be able to reveal unspecific staining.
- 2) Prepare and run the following samples:
 - a. Compensation samples. Single stained beads or cells (11)
 - b. Unstained (blank) control cells
 - c. Single stained samples. If you use single stained cells for compensation, you already have these samples. However, if you use beads for compensation, make single stained cell samples for validation of the panel. These validation single stains could be added viability stain, allowing you a validation on only live cells.
 - d. All-stained sample (multi-stained)
 - e. Fluorescence minus one (FMO) controls (13).
Although FMO control for every fluorochrome is quite expensive, it will give you a perfect overview for validating the quality of your panel. It will, in the end, save you time and money.
- 3) Set up your data for validation:
 - a. Compensation samples: Gate on singlets. Perform the compensation (11) and set up the compensation samples in a fluorescence NxN plot. You can overlay uncompensated and compensated samples in FlowJo and in NovoExpress.
 - b. If point a. is done for compensation beads, make a new NxN plot for the compensated single stained cell samples. Exclude debris and gate on singlets. If you have included a viability marker, exclude dead cells (except from your viability marker sample).



- If a. and b. looks good, go on to c. and d. (any banana shape reveals a problem)
- c. Set up the multistain sample in a NxN plot.
 - d. Make histogram overlays of blank, FMO, single stain and multi stain cell samples for each fluorochrome.
In the perfect world, the blank and the FMO will be equally bright, also the single stain and the multistain will be equally bright. This overlay will reveal data spread as well as over or under compensation. It may also reveal antibody/fluorochrome cross reaction.
 - e. The FACS Core Facility will appreciate discussing the data with you 😊
Do never hesitate to ask us questions.

Once the panel is validated, it is typically not necessary to include FMO's for every marker – only for those where FMO is required to accurately determine the threshold for positive staining.

References

- 1) <https://www.biolegend.com/en-us/multicolor-staining>
- 2) <https://facs.au.dk/links/> (Choose “Example of lymphocyte experiment on the LSRFortessa”)
- 3) <https://app.fluorofinder.com/panels/new>
- 4) <https://www.thermofisher.com/dk/en/home/references/newsletters-and-journals/bioprobables-journal-of-cell-biology-applications/bioprobables-71/bioprobables-71-flow-cytometry-panel-design.html>
- 5) <https://www.biolegend.com/en-us/spectra-analyzer>
- 6) <https://www.bdbiosciences.com/en-us/applications/research-applications/multicolor-flow-cytometry/product-selection-tools/spectrum-viewer>
- 7) <https://www.thermofisher.com/dk/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html>
- 8) <https://fluorofinder.com/spectra-viewer/>
- 9) <https://www.ncbi.nlm.nih.gov/pubmed/27731950>
- 10) <https://onlinelibrary.wiley.com/doi/full/10.1002/cyto.a.20969>
- 11) <https://www.ncbi.nlm.nih.gov/pubmed/27731950>
- 12) FACS Core Facility Guidelines, Compensation in Flow Cytometry
- 13) FACS Core Facility Guidelines, Titration in Flow Cytometry
- 14) FACS Core Facility Guidelines, Controls in Flow Cytometry
- 15) <https://facs.au.dk/links/>