# Multicolor Flow from Start To Finish: A Quick Reference Guide

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https://cancer.wisc.edu/research/resources/flow/

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## Multicolor Flow

Designing and optimizing a multicolor flow experiment from the ground up takes a significant amount of front-end work to plan and optimize. This development time will save you time and precious sample in the long run!

## A Day in the Library Will Save You a Month at the Bench

1. Define your scientific question. What's the hypothesis and corresponding null hypothesis?
2. Perform a literature search for similar published flow analyses.
   a. What markers and antibody clones are being used by others to identify your populations of interest?
   b. Is there an OMIP (Optimized Multicolor Immunofluorescence Panel) in Cytometry A you could consult?
   c. Use a critical eye! Not all published flow is good flow.
3. Plan your biological controls.
4. Think about what statistical analyses you'll need to do at the end. There is a Biostats Shared Resource that can help.

## Theoretical Panel Design

Once you have your list of markers, start matching them up with detectors based on availability and design best practices.

1. Dim/rare marker antibodies should be conjugated to bright fluorochromes.
2. Minimize spectral overlap
   a. Fluorochrome choice
   b. If you must use fluors with large spectral overlaps, put them on mutually exclusive markers.
3. Use your resources
   a. Literature
   b. Spectraviewers
   c. FluoroFinder® (https://uwflow.fluorofinder.com)
   d. Your core staff!
4. Include an appropriate viability dye. These come in many colors for live or fixed cells.

## Useful References

Optimizing a Multicolor Immunophenotyping Assay. Mahnke and Roederer. Clin. Lab. Med. 27 (2007) 469-485 (available Flowdata/0 Flow References/Multicolor Optimization)

Designing, Planning, and Performing Polychromatic Flow Cytometry Experiments. Pratip Chattopadhyay. PDF of lecture slides on the flow server Flowdata/0 Flow References/Polychromatic Flow Pratip C

Flow Cytometry Controls, Instrument Setup, and the Determination of Positivity. Maecker and Trotter. Cytometry Part A 69A:1037-1042 (2006) (available Flowdata/0 Flow References/Cyt69A-06(FCSetup))

OMIPS: Flowdata/0 OMIPs

Cytometry A: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1552-4930

www.chromocyte.com/educate/

**Biostatistics Consulting Drop-in Clinic**
Biostatistics offers a drop-in clinic for UWCCC members *Monday and Wednesday afternoons.*

| Location: | 6153 WIMR |
|---|---|
| Time: | 2-5pm |
| Phone: | (608) 265-8477 |
| Mondays: | Tom Havighurst |
| Wednesdays: | Derek Norton |

http://www.uwhealth.org/uw-carbone-cancer-center/for-researchers/shared-resources/27875

## Panel Optimization and Testing

Order your reagents to optimize and test your shiny new panel. It's time to get in the lab and try it out!

1. Titrate your antibodies. This is a critical step to ensure optimal staining.
    a. Titrate your antibodies on the cell type you'll be using for your real experiment, if possible. Titrations are easier to interpret if you have some positive and negative cells in the same tube so that you can properly assess background.
    b. Titrate antibodies one at a time, each in its own tube, not all at once.
    c. Try some concentrations above and below the manufacturer's recommended amount. For example: if the manufacturer says to use 5 µL, try 1, 2.5, 5, 7.5, and 10 µL.
    d. Adding a viability dye to the titrations can improve signal by removing background/non-specific uptake caused by dead and dying cells.
2. Test your full panel using the antibody concentrations determined by your titrations.
    a. Run all FMO controls (Fluorescence/Full minus one)
    b. Make sure your compensation controls are appropriate.
        i. Fluorochromes must match exactly (FITC≠Alexa488, Tandem dyes must be from the same lot and vial.)
        ii. Comps must be *at least* as bright as your samples.
        iii. Positive and negative particles must be the same type. Beads to beads, cells to cells.
        iv. Collect enough events. We recommend 20,000 events for beads and 50,000+ events for cell-based comps.
    c. Run your test sample(s). Collect enough events.
5. Analyze the data from the test run to identify problem areas.
    a. Does the compensation look good?
        i. Recompensate in FlowJo. Diva is more prone to artifacts and mistakes.
        ii. Look for angled or hooked populations.
    b. Are your populations of interest well separated?
    c. Are there any markers where spillover spreading is interfering?
        i. Use your FMOs!
6. If there's a problem area, go back and fix it.
    a. Swap antibody clones or colors
    b. Re-titrate anything new
    c. Adjust voltages
7. If it looks good, standardize and go for your big experiment!

## Useful References


https://www.chromocyte.com/educate/Beginners-Guide-Training-Resources/Tips-for-Good-Flow-Cytometry-Experiments/Antibody-Titration-The-Key-to-Customizing-your-Panel-Design

http://ucflow.blogspot.com/2009/06/antibody-titrations.html

Titration of Fluorochrome-Conjugated Antibodies for Labeling Cell Surface Markers on Live Cells. Hulspas. Current Protocols in Cytometry 6.29.1-6.29.9 (2010)

Titering Antibodies. Stewart and Stewart. Current Protocols in Cytometry 4.1.1-4.1.13 (1997)

http://ucflow.blogspot.com/2011/12/10-steps-to-successful-flow-cytometry.html

http://expertcytometry.com/when-to-use-and-not-use-flow-cytometry-isotype-controls/

Rare-Event Analysis in Flow Cytometry. Donnenberg and Donnenberg. Clin. Lab. Med. 27 627-652 (2007) (available Flowdata/0 Flow References/Rare Event Donnenbergs)