Flow Basics 2.3: Panel Design

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Laura K Johnston, PhD
Scientific Associate Director
Cytometry and Antibody Technology Facility
University of Chicago
The Flow Basics 2.0 Series

2.1: Basic Cell Staining Protocol
2.2: Optimizing the Basic Cell Staining Protocol
2.3: Panel Design
2.4: Experimental Design – Choosing Controls
2.5: Instrument Setup and Compensation

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Flow Basics 2.3: Panel Design
Components of Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Know cytometer configuration
3. Assign the fluorophores to the markers
4. Test the panel on cells
Components of Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Know cytometer configuration
3. Assign the fluorophores to the markers
4. Test the panel on cells
For successful panel design, gather information beforehand

1. Decide on markers for the panel, determine antibody availability

2. Identify antigen co-expression

3. Identify expression pattern/Classify antigens (primary, secondary, tertiary)

4. Decide on a rough gating strategy
Decide on the Number of Markers

• How many markers are needed?
  • Are there any markers that would be nice to have, but not necessary?

• If many markers are needed, do they need to be in the same panel?
  • Two smaller panels will be easier than one large panel, but more cells and reagents will be needed for two panels
Panel Difficulty

- The larger the panel, the harder it is to design.

- For a “larger” panel (about 10+ markers), the difficulty level also depends on antigen co-expression:
  - Panels looking at a variety of cell types are easier to design (less co-expression).
  - Panels looking at many markers on a single cell type are harder to design (more co-expression).
Decide on Markers – Antibody Clones and Fluorophore Availability

- Once markers have been decided:
  - Determine if a specific clone is preferred
  - Create a list of fluorophores available for each marker (BioLegend, BD, Miltenyi, and ThermoFisher/eBioscience are the vendors we use the most) – I like FluoroFinder’s antibody search tool

Two different clones for the same antibody can produce very different staining patterns:
Identify Antigen co-expression

- What markers are you using to define/identify populations?
  - Helps identify markers that are mutually exclusive
- What markers characterize these populations once you have identified them (such as activation status)?
  - Characterization markers are usually co-expressed
- What markers are on a lot of cell types? (example: CD45, CD3 in whole blood)
- What markers are on a small number of cell types? (example: CD56, CD25 in whole blood)
Classify Antigens

- **Primary**: on or off (examples: CD3, CD4, CD45)

- **Secondary**: continuum (examples: CD45RA, HLA-DR)

- **Tertiary**: low or unknown expression (examples: FoxP3, ICOS)

  - If you are unsure about expression, you can check the data provided on vendor websites for your antibody of interest (BioLegend, BD, eBioscience/Thermofisher, Miltenyi)
Determine Antigen Density/Expression Level

- As part of classifying the antigens in your panel, you should also be aware of the expression level.
- Usually a general idea of high, low, or unknown is sufficient
- If a marker is secondary (continuum) and you are interested in high and low expressing cells, you can treat it as low expression

<table>
<thead>
<tr>
<th>Cell</th>
<th>Antigen</th>
<th>Molecules per Cell</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cell</td>
<td>CD4</td>
<td>100,000</td>
<td>Streit, 2000, Cytometry 10:201</td>
</tr>
<tr>
<td>CD4+ B cell</td>
<td>CD4</td>
<td>5,000</td>
<td>Wyman, et al. 2000, J Immunol 165:1071</td>
</tr>
<tr>
<td>B cell</td>
<td>CD19</td>
<td>100,000</td>
<td>Streit, 2000, Pathobiology 68:57</td>
</tr>
<tr>
<td>Macrophage</td>
<td>CD11b</td>
<td>100,000</td>
<td>Abernethy, et al. 1997, J Immunol 158:256</td>
</tr>
</tbody>
</table>

Disclosures: While these numbers are published data, actual numbers can vary significantly depending on factors such as antibody clone used, patient, method of molecule number calculation, flow cytometry and fluorochromes used. Biolegend recommends that these numbers only be used as relative indicators of high, intermediate, or low expression of proteins on cell subtypes. We also recommend verifying our product data sheets to view actual fluorescence data for fluorochrome-tagged antibodies.

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Flow Basics 2.3: Panel Design
Decide on a rough gating strategy

• This can still be helpful even if you are planning on using algorithms to analyze your data (tSNE, FlowSOM, etc.)

• Examples:
  • Schematic
  • Published data

• Tip: It can also be useful to identify a single plot that is critical and will be in the final figure. Plan to put those fluorophores on different lasers so that the plot looks great!
  • Example: looking at CD4/CD8 ratio – don’t put CD4 and CD8 on the same laser (PE and PE-Cy7)
Gating Strategy Example: Schematic
Gating Strategy Example: Published Data

• Note how this gives information about antigen classification and co-expression!

Steps for Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Know cytometer configuration
3. Assign the fluorophores to the markers
4. Test the panel on cells
Know Cytometer Configuration

• Use the tools specifically made for the instrument you plan to use (if possible)
  • Tools for our instruments can be found here: https://voices.uchicago.edu/ucflow/panel-design/
  • If you don’t have an instrument-specific tool, look for a general brightness index. Spillover can be determined in spectra analyzers or Fluorofinder.com has general instrument configurations
Choose a Flow Cytometer

• How many antigens do I want to detect?
• What instrument am I planning on using?
  • At our facility, we label our instruments as as “number of lasers-number of maximum parameters”. So the X20 5-18 has 5 lasers and 18 detectors.
  • Instrument configurations for our facility can be found [here](#).
  • Note: for the X20, the instrument is configured to use either BV711 or BV650. Please talk to the staff if you would like to learn how to check or swap the filters, or if you need a special configuration.
Choose a Flow Cytometer

- Our “Benchtop analyzer available fluorophores” tool is a quick guide to our instrument configurations

<table>
<thead>
<tr>
<th></th>
<th>Fortessa X20</th>
<th>Fortessa 4-15</th>
<th>Fortessa 4-15 HTS</th>
<th>Attune NxT</th>
<th>LSR 4-12</th>
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<tbody>
<tr>
<td>UV</td>
<td>BUV396</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>UV</td>
<td>DAPI</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>BUV496</td>
<td>X</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>UV</td>
<td>BUV737</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>BUV805</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Violet</td>
<td>BV421</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Violet</td>
<td>BV510</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Violet</td>
<td>BV605</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Violet</td>
<td>BV650</td>
<td>X</td>
<td>X (SWAP FILTER WITH BV711)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Violet</td>
<td>BV711</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Violet</td>
<td>BV786</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Blue</td>
<td>FITC</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Blue</td>
<td>PerCP-Cy5.5</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Yellow/Green</td>
<td>PE</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Yellow/Green</td>
<td>PE-Dazzle595</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Yellow/Green</td>
<td>PE-Cy5</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Yellow/Green</td>
<td>PE-Cy5.5</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Yellow/Green</td>
<td>PE-Cy7</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>APC</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Red</td>
<td>APCR700</td>
<td>X</td>
<td></td>
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</tr>
<tr>
<td>Red</td>
<td>APC-Cy7</td>
<td>X</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Choose fluorophores based on cytometer configuration and panel size

- The best way to start building a panel is to assign one fluorophore per laser.
  - Example: a 4-marker panel on a standard 4-laser instrument would be BV421, FITC, PE, and APC
- As you increase the number of markers, continue to spread the fluorophores out on the lasers
  - Example: an 8-marker panel would have 2 fluorophores on each laser.
  - Bad example: for a 5-marker panel, do not put all 5 markers on the violet laser
- Leave the fluorophores that spill into many detectors for last – don’t use them if you don’t have to
Why are fluorophores with high spillover annoying to use?

• More spillover means more compensation.
• More compensation means more time on the instrument, more time calculating/checking compensation, more chance of error (for beginners)
• Depending on the panel more spillover could mean more spreading error: the data has more variation instead of being a small, tight population

• Flow cytometry is hard enough, always take the easy route!
Fluorochrome Assignment Tools

• Our “Benchtop analyzer fluorophore equivalence table” tool is a quick guide to fluorophores that are detected by the same filter
• How to use: Pick one fluorophore/dye per row when designing a panel

<table>
<thead>
<tr>
<th>Laser</th>
<th>Laser Filter</th>
<th>Fluorophore</th>
<th>Dye/Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>379/28</td>
<td>BUV396</td>
<td>DAPI</td>
</tr>
<tr>
<td>UV</td>
<td>450/50</td>
<td>DAPI</td>
<td>Hoechst</td>
</tr>
<tr>
<td>UV</td>
<td>515/30</td>
<td>BUV496</td>
<td>BFP</td>
</tr>
<tr>
<td>UV</td>
<td>740/35</td>
<td>BUV737</td>
<td>Zombie Violet</td>
</tr>
<tr>
<td>UV</td>
<td>820/60</td>
<td>BUV805</td>
<td>Zombie Aqua</td>
</tr>
<tr>
<td>Violet</td>
<td>450/50</td>
<td>BV421</td>
<td>Sytox Blue</td>
</tr>
<tr>
<td>Violet</td>
<td>525/50</td>
<td>BV510</td>
<td>Zombie Violet</td>
</tr>
<tr>
<td>Violet</td>
<td>560/20</td>
<td>BV560</td>
<td>CFP</td>
</tr>
<tr>
<td>Violet</td>
<td>660/20</td>
<td>BV650</td>
<td>AmCyan</td>
</tr>
<tr>
<td>Violet</td>
<td>710/50</td>
<td>BV711</td>
<td>Zombie Aqua</td>
</tr>
<tr>
<td>Violet</td>
<td>780/60</td>
<td>BV786</td>
<td>CFP</td>
</tr>
<tr>
<td>Blue</td>
<td>530/30</td>
<td>FITC</td>
<td>GFP</td>
</tr>
<tr>
<td>Blue</td>
<td>640/40</td>
<td>PerCP-Cy5.5</td>
<td>Sytox Green</td>
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<tr>
<td>Yellow/Green</td>
<td>588/15 PE</td>
<td>AF555</td>
<td>Propidium Iodide</td>
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<tr>
<td>Yellow/Green</td>
<td>610/20 PE-Dazzle595</td>
<td>PE-CF594</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>Yellow/Green</td>
<td>780/60 PE-Cy7</td>
<td>PE-Vio770</td>
<td>PE-Texas Red</td>
</tr>
<tr>
<td>Red</td>
<td>670/30</td>
<td>APC</td>
<td>PE-Vio615</td>
</tr>
<tr>
<td>Red</td>
<td>710/50</td>
<td>APCR700</td>
<td>mCherry</td>
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<td>Red</td>
<td>780/60</td>
<td>APC-Cy7</td>
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<td></td>
<td></td>
<td>7-AAD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zombie Red</td>
</tr>
</tbody>
</table>

Fortessa X20
Fluorochrome Assignment Tools

- A brightness index specifically generated for each of our Fortessas can be found on our website.
Steps for Successful Panel Design

1. Gather information beforehand about the markers and experiment
2. Use the tools available for panel design
3. Assign the fluorophores to the markers
4. Test the panel on cells
Fluorochrome assignment

1. Start with limiting reagents
   • Make note of any fluorescent proteins (GFP, mCherry, etc.)
   • Assign very rare markers to the only fluorophore options available (e.g. marker X is only available in PE)

2. Assign the remaining markers – be aware of co-expression
   • Utilize all of the information gathered previously

3. Assign viability dye
Fluorochrome Assignment: Fluor Brightness vs. Antigen Density

• Assign bright fluorophores to low expressed markers and dim fluorophores to highly expressed markers

• Low expressed markers should not be put on dim fluors – highly likely to not be able to resolve the positive population

• High expressed markers can be put on bright fluors if the bright fluors don’t need to be saved for low expressed markers
Fluorochrome assignment: Co-Expression

• For co-expressed markers, assign fluorophores with minimal spillover into each other

• Be cautious of using and assigning fluorophores with lots of spillover

• If there is an extremely important biaxial plot (aka a plot that will be put in a final figure), consider assigning two fluorophores that have no spillover. This will produce the best-looking and therefore most convincing data.
What fluorophores have high spillover and should be avoided if possible?

- **PerCP-Cy5.5** and **PE/Dazzle 594** spill into many channels and we recommend using them only when needed (such as a 10 color panel, not a 4 color panel)
  - Using both together can be very challenging – choose one!

- **PE-Cy5** and **PE-Cy5.5** are not commonly used and not great fluorophores (lots of spillover), so we don’t recommend using them

- **PerCP** is not a stable fluorophore, we recommend using PerCP-Cy5.5 instead
How to deal with fluorophores with high spillover

• Assign them to a dump channel (one or more antibodies that remove cells that will not be analyzed). In this case, no cells of interest will have the offending fluorophore(s) and spillover can be ignored.

• Assign them to a low expressed or rarely expressed marker. In this case, the spillover will only be an issue for a small number of cells.

• Assign them to a marker that is on a cell subset that does not co-express any other markers in the panel. For example, assign it to a neutrophil marker when all other makers in the panel define lymphocytes and monocytes.
Other Notes About Fluorophores

- Some fluorophores have brighter or better alternatives (use brightness charts)
  - Example: AF488 is more stable than FITC, BV421 is much brighter than pacific blue, APCR700 is brighter than AF700
- The UV laser is dim and reagents are expensive, so we recommend that you only use it if you have to
How to utilize primary/secondary/tertiary information

• Tertiary markers must be placed on bright fluorophores
  • Placing a tertiary marker on a dim fluorophore will likely produce suboptimal results (unable to separate positive cells from negative cells)

• Secondary markers will benefit from moderate-bright fluorophores in order to resolve the dimmest population
  • Placing a secondary marker on a dim fluorophore will certainly resolve the high-expressing population, but it may be challenging to distinguish the low-expressing cells from the negative cells

• Primary markers can be placed on dim fluorophores
  • They can also be placed on bright markers, but those are often used up by secondary and tertiary markers
Fluorochrome assignment: Method 1

• Write out each marker you want in your panel (don’t forget live/dead!) in a vertical list

• Next to each marker, list available fluorophores
  • You may try to utilize antibodies that your lab already has to minimize purchasing of new antibodies
  • Search BioLegend, eBioscience, Miltenyi, and BD for available antibodies

• Use your puzzle-solving skills to assign fluorophores to your markers. Remember there is no perfect panel!
  • Assign markers based on antigen classification, antigen co-expression and gating strategy

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Flow Basics 2.3: Panel Design
### Fluorochrome assignment: Method 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>AF488</th>
<th>PE</th>
<th>APC-Cy7</th>
<th>Pacific Blue</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>AF488</td>
<td>PE</td>
<td>APC-Cy7</td>
<td>Pacific Blue</td>
</tr>
<tr>
<td>CD4</td>
<td>PE-Cy7</td>
<td>APC-Cy7</td>
<td>Pacific Blue</td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>PerCP-Cy5.5</td>
<td>PE-Cy7</td>
<td>APC</td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>PE-Cy7</td>
<td>FITC</td>
<td>PE</td>
<td></td>
</tr>
<tr>
<td>Ly6G</td>
<td>APC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>AF488</td>
<td>AF647</td>
<td>APC-Cy7</td>
<td></td>
</tr>
<tr>
<td>Viability</td>
<td>PI</td>
<td>Live/Dead Aqua</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fluorochrome assignment: Method 2

- All of our instruments can be found here: [https://app.fluorofinder.com/uchicago](https://app.fluorofinder.com/uchicago)
- Under “select markers” enter your markers of interest and target species (the website is a bit slow)
  - Viability stains and fluorescent proteins can be added on the next page
  - If you are using a fluorescent protein, add a marker name on this page
  - If you would like to use a dump channel, just enter one of the antibodies
  - You can go back to check the cytometer configuration at the top of the screen if you want
- Select continue, then select products
- Click a fluorophore to select a specific product
  - Note: We recommend choosing antibodies from BioLegend, eBioscience, BD, or Miltenyi
- As you select products, you will not be able to select interfering products
  - For dump channels you can purchase multiple antibodies in one color
  - At the top of the page, select viability dyes or fluorescent proteins if needed
- Click “show spectra viewer” at the top to assess spillover (see next slide)
- Hit continue or print or export as an excel sheet
- Feel free to have someone at the CAT facility check over your panel before you purchase antibodies or run your experiment!!
FluoroFinder’s Panel Spectra Viewer

Select Products

Panel Professor  Save Panel  Go Back  Continue

LASER/ FILTER  MARKER  COLOR  % FILTER
561 575/26  CD19  BRILLIANT VIOLET 605
561 575/28  HLA DR  ALEXA FLUOR 647
561 575/26  CD14  PERCP-CY5.5
561 575/26  CD16  PE-DAZZLE 594  15%
561 575/26  CD3  PE  69%
661 575/26  CD4  PE-CY7  29%

Order Markers by Fluorochrome Availability  Limit to Core Facility Recommended Colors

Markers 1 - 8 of 15
FluoroFinder’s Panel Spectra Viewer

• After assigning fluorophores in a panel, FluoroFinder has a tool to determine predicted spillover

• For each laser and filter set, assess if the spillover is acceptable

• In this example the 561 575/26 filter is assigned to CD3
  • CD4 is 29% and coexpressed with CD3 – move this marker
  • CD16 is 15% but not coexpressed, no need to move
Final Notes

• Check the panel on test samples before running a full experiment
  • Can you resolve your populations of interest?

• Don’t forget to titrate antibodies before testing the panel!
Stay Tuned for the Rest of the Flow Basics 2.0 Series

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- 2.2: Optimizing the Basic Cell Staining Protocol
- 2.3: Panel Design
- 2.4: Experimental Design – Choosing Controls
- 2.5: Instrument Setup and Compensation