Panel Quality Check

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Laura K Johnston, PhD
Scientific Associate Director
Cytometry and Antibody Technology Facility
University of Chicago
Now that you have completed training, how do you start troubleshooting a panel?
Steps for running a large panel on the aurora

1. Design a panel using the aurora-specific tools

2. Titrate antibodies
   - Be especially careful of titrating the fixable viability dyes!
     - Cytek recommends Biolegend’s Zombie NIR or ThermoFisher’s Live/dead blue
     - Read more about titrating viability dyes on our blog [here](#)

3. Perform a quality check on the panel
   - Keep going through the slides to find out more

3.5. Determine if compensation beads or cells should be used to unmix the sample properly
   - Remember, beads can give unmixing errors!
Recommendation for panel design

• If you are new to the aurora, **don’t start with your 20+ color panel.** It’s too much.
  • Instead, Design your full panel. Select about 10 of your markers to start and do a Panel Quality Check (discussed in later slides)
    • Bring both cells and comp beads for reference controls
    • Then test your full panel and do another Panel QC

• Once you are more comfortable with the aurora, you can test your larger panels from the beginning

• Remember all the troubleshooting for reference controls: antibody/reagent titration, beads vs. cells, fixatives and other buffers
Steps for running a large panel on the aurora

4. After all troubleshooting is completed, you can run your experiment
   • Remember reference controls should be run every time samples are run for best results
     • If this is challenging, contact Laura to discuss other options
Why is the panel quality check so important?

• Remember that panel design is theoretical
• The harder the panel, the harder it is to predict all of the spreading errors
  • Harder panels are larger and/or have higher numbers of co-expressed molecules
• Panel QC tells us if there are any issues with the panel so we can determine if markers need to be moved to other fluorophores
Why is the panel quality check so important?

- Panel QC is critical for:
  - Panels with high complexity index (see how the value jumps from 9.3 for 26 colors vs 29.75 for 30 colors)
    - Find out your panel’s complexity index here: https://spectrum.cytekbio.com/
  - Panels that look at many co-expressed markers on one subset of cells

### Complexity Index
- Combined index
- A smaller complexity index indicates less overall experimental spread

**Examples (5 laser)**
- 10 colors: 2.5
- 20 colors: 7.3
- 26 colors: 9.3
- 30 colors: 29.75
Examples of performing a quality check on your panel to determine if spreading is an issue
Methods for QC

• **Option 1:** compare single stain to multicolor stain to determine if resolution is the same
  - If the staining pattern in multicolor stain is different than single stain, we can conclude that another color in the panel is spreading and causing potential issues

• **Option 2:** compare a smaller fraction of your panel to the entire panel to determine if all major populations can be defined
  - Ex: 24-color panel. Also stain a tube with 12 of the 24 markers and make sure that you can find the same populations in both tubes

• I recommend both options, but these slides will show you how to do option 1
What is needed for a panel QC experiment (option 1)?

• To perform a panel QC, the fully stained sample and reference controls need to have exactly the same staining conditions
  • Same tissue
  • Same antibodies, antibody concentration
    • Note if you are using less than 2 µL for reference controls I recommend diluting the antibody 1:2-1:10 first and pipetting a larger volume (5 µL instead of 0.5 µL)
  • Same incubation time/temp
  • Same cell concentration
  • SAME EVERYTHING!

• In order to make conclusions from a panel QC, we are going to assume that everything is the same, except some tubes have one antibody and one tube has all of the antibodies.
What if you need compensation beads to unmix the data?

- I recommend doing a full set of ref controls on cells and a second full set on compensation beads (except the viability dye won’t work well on beads)
- Unmix the data with compensation beads, and use the cells reference controls to apply any compensation if needed
Before analyzing the panel QC, always check for correct unmixing

- Panel QC can only be performed if there are no unmixing errors

Examples of correct unmixing:

Examples of unmixing errors:

Slides from Cytek
Steps for panel QC

1. Make a histogram of unstained, single stained cells and full stained cells for each fluorophore

2. Identify fluorophores where the intensity of the full stained cells are brighter than the single stained cells
   • Assuming you used the same cells for your single stain and full stain and there are no unmixing errors, the main reason for the full stain being brighter is that there is spreading from another fluorophore in the panel

3. After identifying problematic fluorophores, investigate further with single stains, FMOs, bivariate plots

4. Determine if a marker needs to be moved to another fluorophore
Make a histogram of unstained, single stained cells and full stained cells for each fluorophore

- **FlowJo tips:**
  1. In FlowJo, create a group containing all single stain controls
  2. In the layout, create a histogram overlaying unstained, full stained, and one single stain
  3. Right click the unstained tube in the histogram legend and select “set as control”. The text will become italicized.
  4. Repeat to set the full stain as control
  5. Create a batch report – make sure the single stain group is selected
  6. After the batch report is complete, change the x axis to the corresponding fluorophore of the single stain (you have to select all items and “ungroup” before you can change the axis)
1. Make a histogram of unstained, single stained cells and full stained cells for each fluorophore

For each plot: 1. is the full stain brighter than the single stain? 2. Is the staining pattern different?

Unstained
Full Stained
Single Stained

Some intensity, same staining pattern. OK!

Some intensity, different staining pattern, need a closer look

Panel Quality Check

Cytometry and Antibody Technology Facility
Out of all 10 markers, there are 3 where the full stain is brighter than the single stain or the staining pattern looks different.

**Single Stains**
- Zombie NIR looks fine! Dead cells can be gated.
- PerCP-Cy5.5 high cells are fine, but losing resolution of dim cells.
- Lots of spreading in SB436, losing some resolution.

**Fully Stained**
Once you have discovered an issue with resolution there are two options:

1. Move the fluorophore of the positive marker
   • In this example, move CD11b from PerCP-Cy5.5 to a different fluor and move CD19 from SB436 to a different fluor

2. Move the fluorophore that is causing the spreading

• How do you quickly identify the fluorophore causing the spreading?
  • Reference controls (single stained cells)
  • FMOs can also identify problems, but not everyone wants to stain all FMOs (and it’s requires a lot of antibodies). Reference controls are usually sufficient.
How do you use reference controls to identify the fluorophore causing the spreading?

• Use reference controls – **CELLS** – and look at the exact same plot for every tube (usually one fluorophore on x axis and SSC on y axis)
  • Compensation beads will only tell you the “worst case scenario” like the spread matrix, you want to use the cells stained with your actual antibodies of interest
  • To assess spreading, we’re going to assume that all tubes have only one fluorophore, so we should only expect one plot to show a staining pattern, while all of the others look negative. If a tube doesn’t look negative, then we can assume the fluorophore used to stain that tube is spreading into the fluorophore on the x axis
Using single stains to identify which fluorophore is causing spreading in SB436

Spreading into SB436 occurs in the BV421 and eF450 single stains.
BV421 and eFluor450 spread into SB436 – but we already knew this from the spread matrix!

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To read this table: spread of fluor in the row impacts resolution of the fluor in the column. Red means the fluor in

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Panel Quality Check

THE UNIVERSITY OF CHICAGO BIOLOGICAL SCIENCES

Cytometry and Antibody Technology Facility
Using FMOs to identify which fluorophore is causing spreading in SB436

When eF450 or BV421 is removed from the panel, the resolution of SB436 improves! Should I change my panel? If so should I move CD19, CD16, or CCR7?
Neither CD19-SB436, CD16-eF450, or CCR7-BV421 need to be moved because the panel was properly designed!

- Are these plots pretty? No.
- Am I going to make this a critically important figure in my paper? No.
- Can I gate on CD19^+ cells? Yes!
Using single stains to identify which fluorophore is causing spreading in PerCP-Cy5.5

It doesn’t look like a single fluorophore is spreading into PerCP-Cy5.5
Using FMOs to identify which fluorophore is causing spreading in PerCP-Cy5.5

This one is tricky. Something is happening with PerCP-eF710, but no single is changing the resolution of the dim population. Maybe caused by multiple fluorophores based on the spread matrix. Solution: We decided to move CD11b to another fluorophore because we are interested in the CD11b dim population and wanted to see if resolution could be improved. However it may have been ok on PerCP-Cy5.5.
What’s going on with PerCP-Cy5.5 and PerCP-eF710?

PerCP-eF710 single stain control looks correct!

For some reason in the full stain PerCP-eF710 looks “overcompensated”. That’s where the spreading is coming from when you look at PerCP-Cy5.5 vs SSC.
Spreading Example #2

Same intensity, different staining pattern, with the negative of the full stain being brighter than the negative of the single stain.

Positive and negative populations are clear in single stain dot plot.

Positive and negative populations are lost when other fluorophores are added to the tube.

Spreading leads to loss in resolution of positive population.
Spreading in BV785 is caused by APC-Cy7

Solutions: Move CD184 from BV785 to another fluor or move CD73 from APC-Cy7 to another fluor
Next Steps

• The panel QC determined that one or more markers need to be moved to a different fluorophore
  • Move the fluorophore(s) and repeat panel QC experiment until satisfied with the panel.

• The panel looked good – all populations can be resolved.
  • If you only performed the panel QC on part of the whole, proceed with a panel QC on the full panel.
  • If the full panel was checked, great! The panel is ready to use, proceed with the experiment.