Performing Spectral Unmixing
Tips for Gating Reference Controls

Gating compensation beads:

Gating cells:
Tips for Gating Reference Controls

1. Make gates small – focus on cells that express the marker
2. Gate cells that have the highest expression of the maker
Tips for Gating Reference Controls

Clean Spectral Plot for Reference Control

Bad Spectral Plot for Reference Control
Tips for Gating Reference Controls

4. The best gate positions generate the best spectrum

*best spectrum = brightest, cleanest, and only one signature
Tips for Gating Reference Controls
Gate Placement Affects Unmixing

Images from Cytek
Check Unmixing Accuracy
Unmixing errors can lead to wrong conclusions!

Data WITH Unmixing Error

Correct Unmixing

Images from Cytek
Always check unmixing accuracy – use the same method as checking compensation

- Create NxN plots using reference controls (single stained **cells**) to check for unmixing errors.
Use caution when checking unmixing accuracy on compensation beads.
Unmixing errors can be seen in fully stained samples by looking at negative populations

Examples of correct unmixing:

Examples of unmixing errors:

Images from Cytek

Cytometry and Antibody Technology Facility

Aurora Training Course
What to do about unmixing errors

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<thead>
<tr>
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<th>Unmixing Errors?</th>
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<tbody>
<tr>
<td>Single Stained Controls</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Full Stained Sample</td>
<td>YES</td>
<td>YES</td>
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See option 1 and option 2

See option 3
Fixing Unmixing Errors: Option 1

- Return to the unmixing wizard
Fixing Unmixing Errors: Option 2

- Apply manual compensation
Fixing Unmixing Errors: Option 3

• If single stained controls are correctly unmixed but full stained is not, you did not follow the rules for good reference controls. Bring better controls.
Autofluorescence extraction can improve unmixing if AF is complex

BV510 single stained cells **without** AF extraction

BV510 single stained cells **with** AF extraction
Confused about your data?

• Set up a meeting to talk to Laura!
  • LJohnston@bsd.uchicago.edu

• I can also put you in touch with a Cytek Technical Application Specialist