Compensation and Unmixing Troubleshooting

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VERSION CONTROL

NUMBER OF VERSION	DATE OF VERSION	SUMMARY OF CHANGES
V1	26-03-2026	Original version

RECORDS GENERATED BY THIS PROCEDURE

Records' Identification	Indexation	Archive Responsible
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After doing the settings on the cytometer, the compensation and unmixing must be validated with the NxN matrix of all the markers. If problems are detected with the compensation/unmixing or population resolution, the unmixing should be troubleshooted:

1. **Check Autofluorescence on Unstained Sample**
   - Confirm that the AF is mostly in the UV and V zones of the spectrum (in case there are AF peaks in the other detectors or too high in the UV and V please contact the Flow Cytometry Platform staff)
   - On the NxN check if the Unstained has high Autofluorescence (AF) and if so, extract the AF and compare the unmixing with and without AF extraction
   - If necessary, multiple AF can be extracted. For this contact the Flow Cytometry Platform Team.
2. **Check the Single-Color Controls**
   - Adjust the gate of cells or beads in the unstained and in the single-color controls to the population of interest
   - On the single-color control, adjust the gate for the positive population to take only the most positive part of the peak
   - The primary peak channel is as expected for each reference control
   - For each single color, check that the primary peak channel is in the predicted/defined detector and adjust in case it isn't
   - Confirm that the full spectrum for each reference control is as predicted in a full spectrum viewer or in the Cytek/ BD Research Cloud
   - Make sure to have at least 500, ideally 1.000 evts inside the positive population
   - Check the similarity and spreading matrix and compare with the expected (should be similar) – on the unmixing module for the Cytek Aurora system and on FlowJo for the BD FACSymphony A5 system, as well as the BDFortessa, X20 and Accuri C6 Plus Systems
   - Evaluate Tandem Degradation
3. **Check the unmixing (NxN)**

   On the NxN of the mix check:
   - Low resolution/discrimination of the population (consider re-Titrating your antibodies)
   - Compare the reference controls with the mix/ sample and check that the reference controls have the same or higher brightness as the mix/sample. If not, re-do the single-color control with a better positive control for that marker
   - Compare the % of positive population in the single stain vs in the multicolor sample. If there is a decrease in one or more markers, adjust the antibody (Ab) sequential staining protocol (some Abs may need to be added before the mix staining). Steric hindrance can only be found and troubleshooted with FMOS.
   - Evaluate your sample preparation protocol (consider re-optimizing the Protocol)
   - Evaluate the panel per se (AF and spread are the main causes of loss of resolution). If one or more antibodies are interfering with the experiment's success, consider changing the Panel and testing other fluors.
   - Tandem Degradation
   - If something weird is found that you can't identify the cause, please contact the Cytometry Platform staff to perform the troubleshooting and optimization of the experiment and the panel to get trustworthy, reproducible, high-resolution data