

# Systematic analysis of spectral mismatch between compensation particles and cell in conventional and spectral flow cytometry

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## Abstract

Compensation particles are widely utilized support reagents that are simple to use and provide a clear and bright signal for setting compensation or unmixing for multi-color flow cytometry experiments. Formulations vary between vendors, but these products generally consist of both a positive, antibody-binding beads in conjunction with negative, non-binding beads. Despite the many advantages of using compensation particles for single-color reference controls, there are instances when fluorophore-conjugated antibodies bound to compensation particles produce slightly different spectral emission signatures compared with stained cells. These differences can result in incorrect compensation and spectral unmixing and have been observed anecdotally for specific combinations of fluorophores. Since differences in spectral signature occurs at specific wavelengths and their corresponding filter sets, it can be difficult to predict compatibility. The lack of understanding of these discrepancies has led to distrust in using compensation particles and a belief that some fluorophores are incompatible with compensation particles for some experiments, but not in others. This distrust is noted especially when using compensation particles for spectral flow cytometry, where an accurate assessment of each fluorophore's spectral emission signature is critical for unmixing and interpreting multi-color experiments.

We set out to systematically explore the spectral mismatch between cells and compensation particles for both conventional and spectral flow cytometry. We evaluated fluorophores from Thermo Fisher Scientific and selected fluorophores from other vendors using UltraComp eBeads™ Plus Compensation beads. For each fluorophore, unmixing errors were identified by applying an unmixing matrix created using compensation beads to single-stained cells. A compatibility matrix was created based on which fluorophore pairings had unmixing errors. Fluorophore pairings with unmixing errors were then run on a conventional cytometer to determine if spectral unmixing errors were translatable as compensation errors on a conventional cytometer. Finally, a multiparameter panel was run on both conventional and spectral cytometers to qualitatively compare and assess the impact of spectral unmixing and compensation errors caused by compensation particles on panel performance.

This information can serve as a valuable reference and provide clearer guidance for using compensation particles on both spectral and conventional flow cytometers.

## Introduction

Invitrogen™ UltraComp eBeads™ Plus Compensation Beads are a useful tool for conventional compensation and spectral unmixing, when:

- Sample is limited
- Antibody stains a very small subset of cells
- Dim staining is expected
- Antibody does not stain a distinct population of cells
- Spectral characteristics of the dyes are not affected

UltraComp eBeads Plus compensation beads, is the second generation of the UltraComp eBeads compensation beads, which have been optimized to be more compatible with violet-laser excited fluorophores compared to the first generation. However, even with the better optimized UltraComp eBeads Plus compensation beads, there are spectral unmixing issues when used with certain fluorophore combinations. To further explore the unmixing and compensation issues when using compensation particles, UltraComp eBeads Plus compensation beads and human peripheral blood cells were stained with CD4 (clones SK3 and RPA-T4).

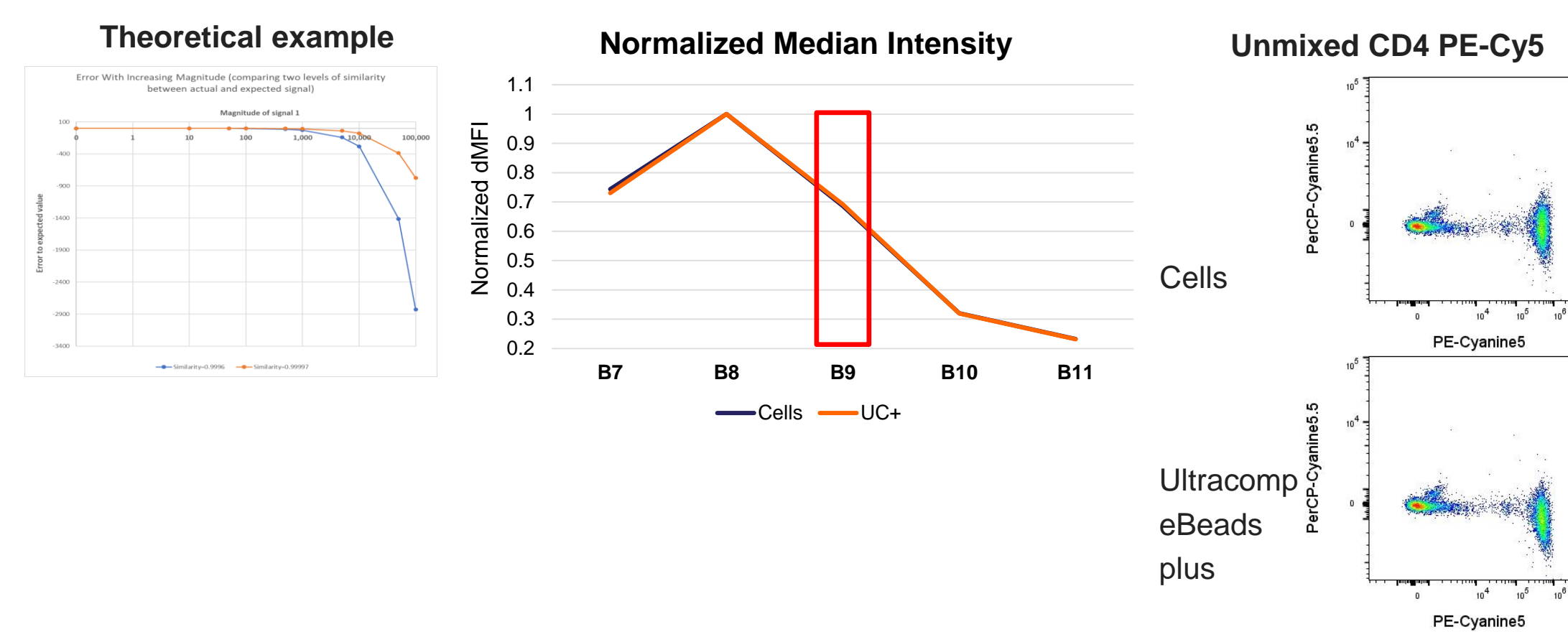
## Results

**UltraComp eBeads Plus stained spectral signatures are similar to cell-stained samples but can still lead to unmixing issues.**

Though the overlay of spectral emission signature of PE-Cyanine5 on Cells and UltraComp eBeads Plus has a similarity calculated to be 99.992%, noticeable unmixing issues can be identified. PE-Cyanine5 single-color cell-stained sample was analyzed using either a single-color stained cell sample or UltraComp eBeads plus for unmixing, other fluorophores were unmixed using cell-stained samples. Data shown are from 2-fluorophore unmixing matrix. Unmixing issues can be identified depending on fluorophores used together with PE-Cyanine5.

**Subtle changes in the spectral signature of single-color controls can lead to unmixing issues.**

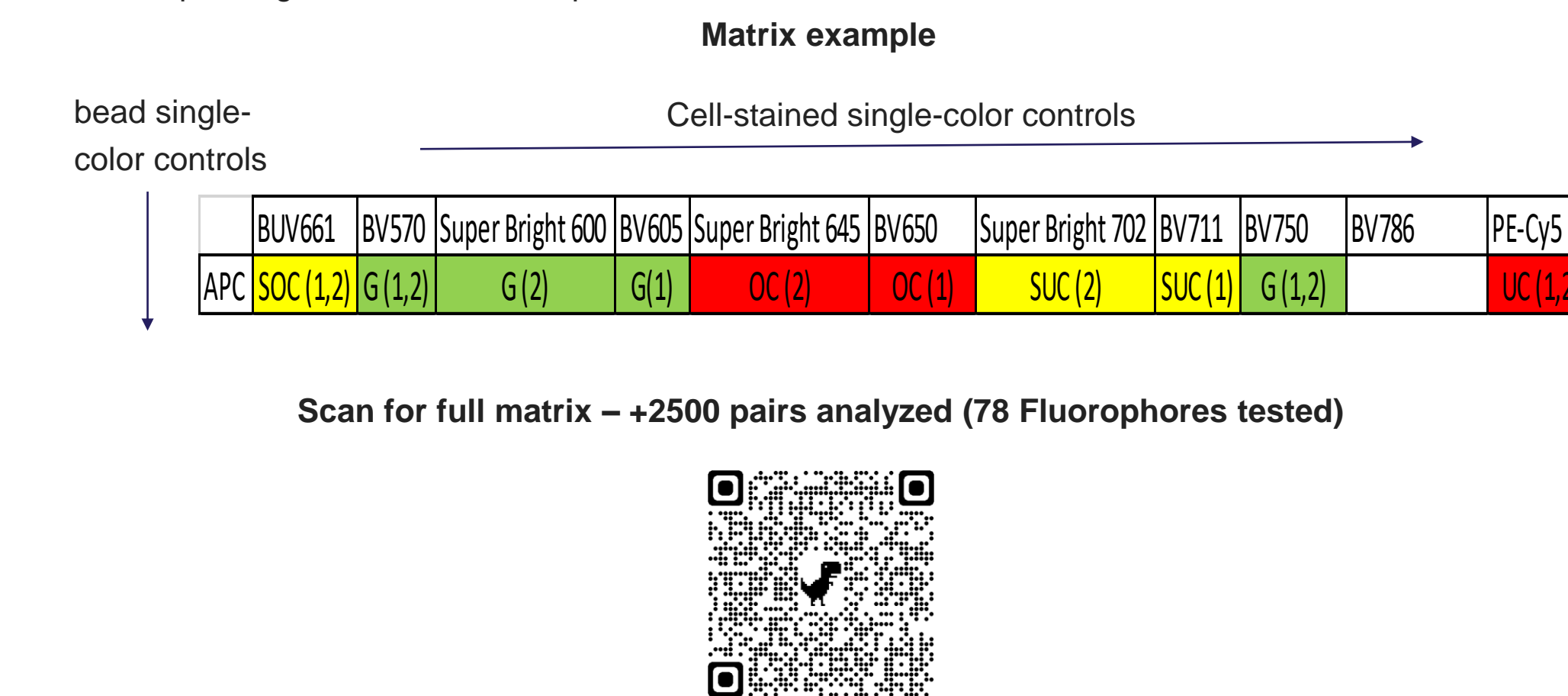
Unmixing issues may be more noticeable when the staining on the experimental sample is on a high density, bright population. The following experimental data was generated using CD4, to emphasize any unmixing issues. PE-Cyanine5 spectral signature difference between cells and UltraComp eBeads plus compensation beads in channel B9, the primary peak for PerCP-Cyanine5.5, is only 0.8%.



**Compatibility matrix created for spectral unmixing on the Cytek® Aurora using CD4.**

CD4 in a variety of fluorophores from Thermo Fisher Scientific, Biologend, and BD Biosciences were evaluated by staining with cells and UltraComp eBeads plus compensation beads. Multiple mock panels were created to enable evaluation of dye pairings in an experiment after spectral unmixing, denoted by numbers in the parenthesis, to explore a variety of fluorophore combinations. One compensation bead-stained fluorophore was used for unmixing at a time, with all other single-color controls in a mock panel used cell-stained single-color controls. After unmixing with beads, the unmixing matrix is applied to the cell-stained single-color control and evaluated.

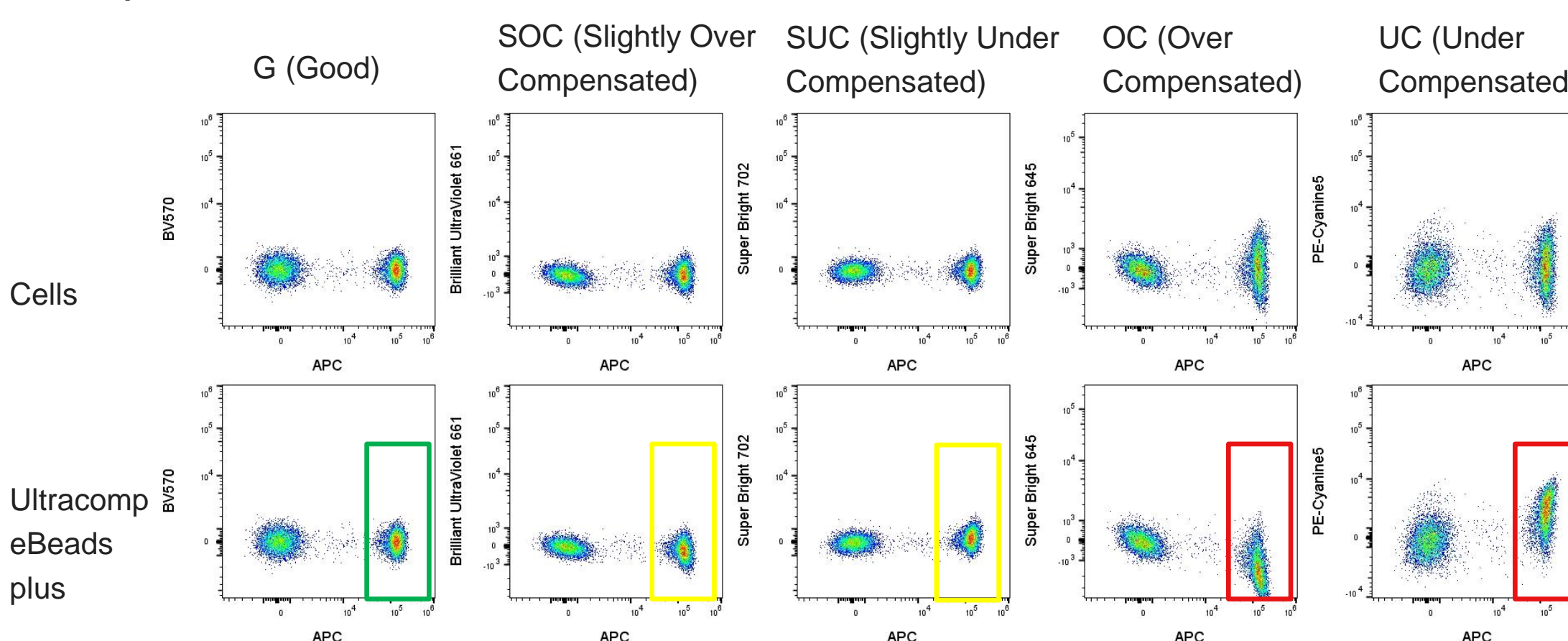
Conventional cytometry compensation verbiage is used to categorize unmixing issues. This matrix evaluates how unmixing with beads stained with a specific fluorophore may cause unmixing issues when looking at bivariate plots against another fluorophore.



**Unmixing issues were subjectively categorized into the following classifications:**

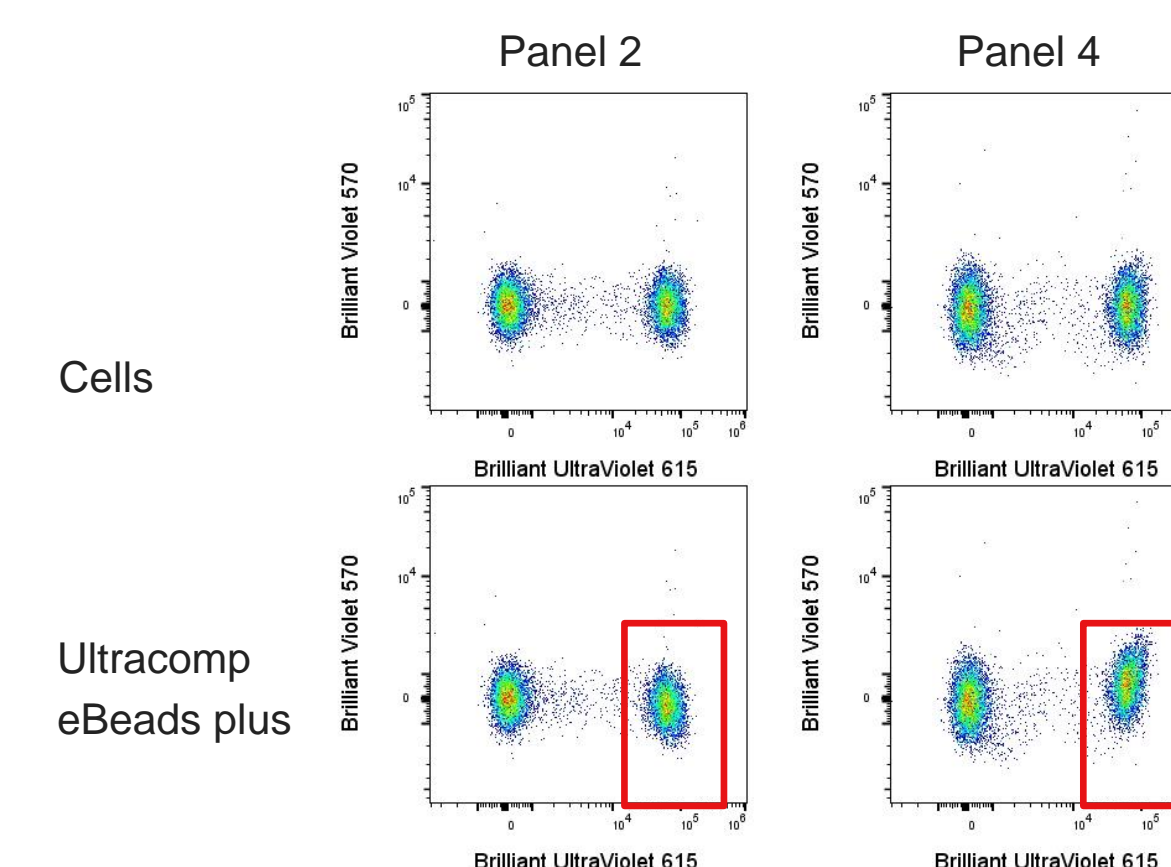
G (Good, no unmixing issue) – colored green; minor unmixing issues: SOC (slightly over-compensated), SUC (slightly under-compensated) – colored yellow; and OC (over-compensated), UC (under-compensated), - colored red. Some pairings had unmixing issues depending on the mock panel fluorophore combination and these were highlighted in orange. Pairs that have not been analyzed yet are blank, white cells, while pairs that cannot be analyzed are highlighted in blue and labeled N/A. Note, that other kinds of unmixing issues were not evaluated in these series of experiments, and not captured by this matrix.

**Examples of Classifications**



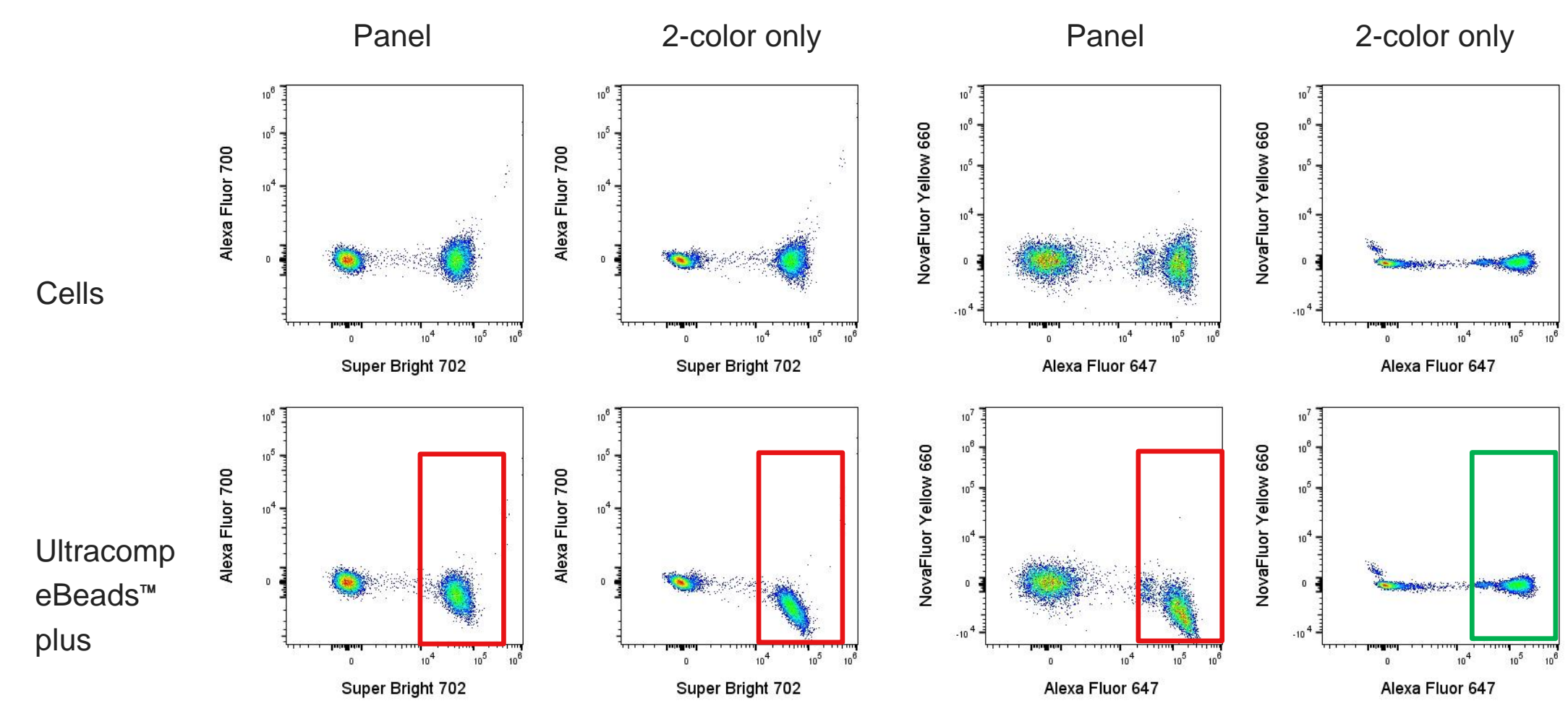
**Unmixing issues can be dependent on fluorophore combinations that are being unmixed.**

There were some unmixing issues that were identified in one mock panel and were different in another mock panel. This suggests that unmixing issues are not only isolated to a specific dye pair and based on the primary staining peaks of the fluorophores, but unmixing issues may come from influences of secondary peaks and other channels depending on the fluorophore combinations that are being used to unmix.



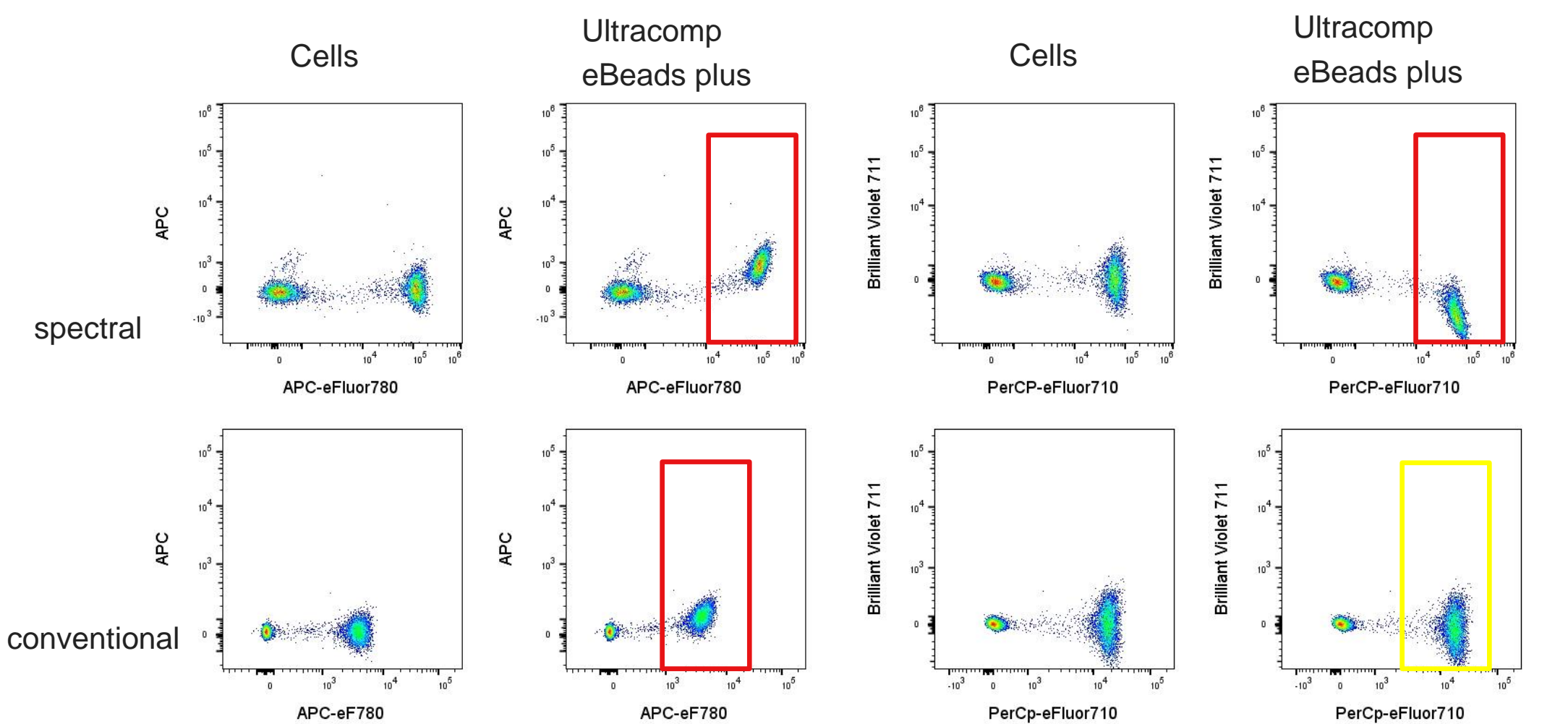
**Select fluorophore pairings, with unmixing issues, were re-analyzed in experiments of just 2 fluorophores to confirm that unmixing issues were specifically due to that specific pairing.**

Most unmixing issues were reproduced when only unmixing a pair of fluorophores using the UltraComp eBeads plus compensation beads. This suggests that difference in spectral signature of the compensation beads cause improper unmixing between the specific fluorophore pairing. However, there were also several instances where unmixing issues only occurred in multi-color mock panels, but not in fluorophore pairs. This suggests, that differences in the spectral signature of a single-color control, not directly related to unmixing of that specific fluorophore pair, can cause unmixing issues depending on the specific combinations of all the fluorophores being unmixed.



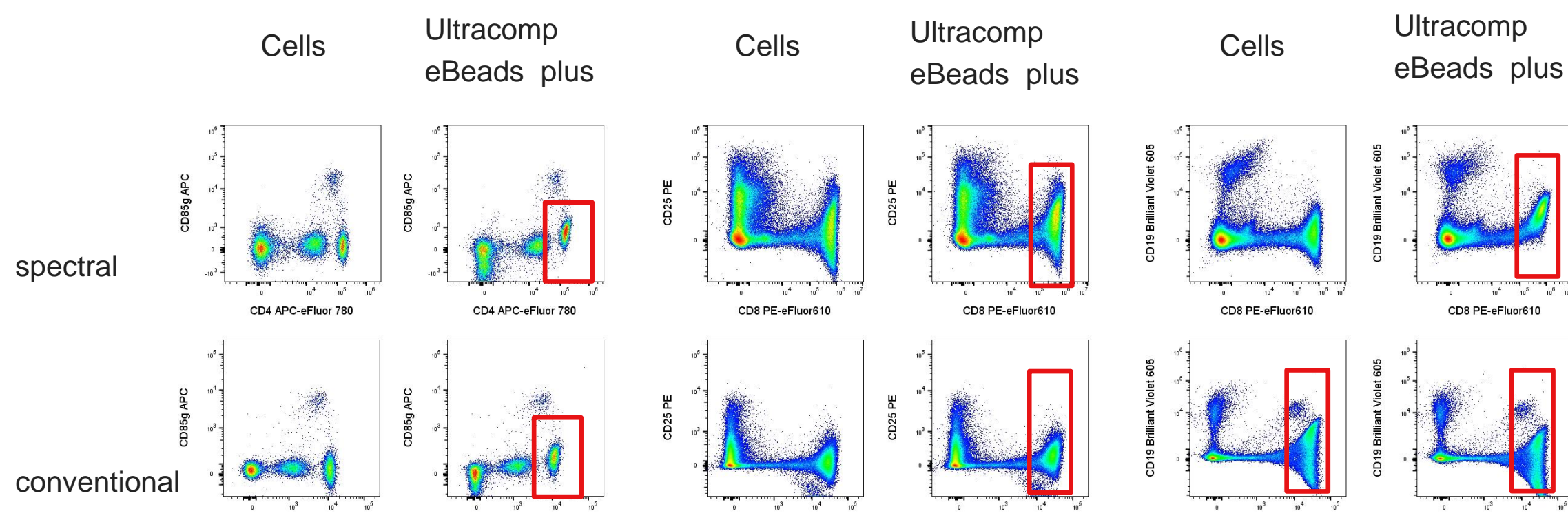
**Fluorophore pairings with unmixing issues also had issues with conventional flow cytometer compensation.**

Selected dye pairings compatible on a conventional cytometer, that had unmixing issues on a spectral cytometer, were further analyzed on a conventional flow cytometer. In 75% (n = 42) of the fluorophore pairings evaluated, incorrect compensation was also found when using compensation particles. However, there were also a few pairings where conventional compensation looked better with beads, which again suggest that non-primary peaks of a fluorophore can also cause unmixing issues.



**Multiplexed samples using compensation beads to unmix or compensate will lead to incorrect unmixing and compensation issues.**

Dye pairings with unmixing issues were specifically used to evaluate panel performance on both a spectral and a conventional instrument. In general, unmixing issues were reproducible on conventional instruments as compensation issues. It is possible that due to a conventional cytometry not being able to multiplex certain combinations of fluorophores, generally smaller panel sizes, and differences in spreading error, compensation issues with conventional flow cytometry when using compensation beads are not recognized as much as spectral flow cytometry.



## Conclusions

- The generated compatibility matrix shows that majority (>82%) of fluorophore pairings evaluated will unmix correctly when using UltraComp eBeads plus compensation beads (see generated compatibility matrix).
- Unmixing and compensation issues using compensation beads are possible in both spectral and conventional flow cytometry.
- Compatibility matrix generated is specific for UltraComp eBeads plus compensation beads using CD4, a high abundant specificity. Other compensation tools are unique and will have unique results.
- Though unmixing and compensation issues are a possibility with use of compensation beads, they remain a valuable tool especially when we have a better understanding of when these issues may occur.

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