Spectral Cytometry Reference Controls: The Search for the Perfect Particle

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BACKGROUND

One of the challenges of high dimensional spectral flow cytometry experiments is finding reference controls resulting in no unmixing errors.
The conditions for ideal reference controls were originally cited by Mario Roederer and have now become universally accepted within the flow community:

1. Negative and positive reference controls should have identical autofluorescence.
2. The reference control's positive signal should be at least as bright, preferably brighter than the cells in the assay.
3. Positive control particles should not be rare.
4. Reference controls should use exactly the same fluorochrome as in the assay, tandem dyes should have the same batch number.

Using the same cells as in the experiment is the ideal reference control, however sometimes this can be difficult if the frequency of cells expressing the target marker is too low, the cells may be valuable or limited, or the sample may be heterogenous making the autofluorescence of positive and negative controls difficult to match.

An alternative is to use surrogate particles such as antibody capture beads. However, with polystyrene bead-based controls that seemingly meet all criteria, the fluorescence signature or spectral fingerprint of some dyes can be altered when bound to the beads, resulting in unmixing errors.

Therefore, we propose a 5th condition is added to those listed above.

5th condition for reference controls:

"Particles should not alter the spectral characteristics of the dye."

IS BRIGHTER BETTER?

With the release of a new hydrogel-based reference particle from Slingshot Bioscience, we evaluated this alongside two different polystyrene capture beads to determine which performs best against cells as a spectral reference control.
A 25-Color Immunoprofiling Assay, cFluor® Reagent Kit (Cytek® Biosciences) was used to stain whole human blood. Reference controls were either cells, polystyrene antibody capture beads (SiComp) and UltraComp ebeads™ (Thermofisher) or hydrogel antibody capture particles from Slingshot Biosciences (SpectraComp®). We evaluated the level of brightness of the reference controls via Median Fluorescence Intensity (MFI). SpectraComp® controls were found to consistently produce a higher MFI than all other controls.

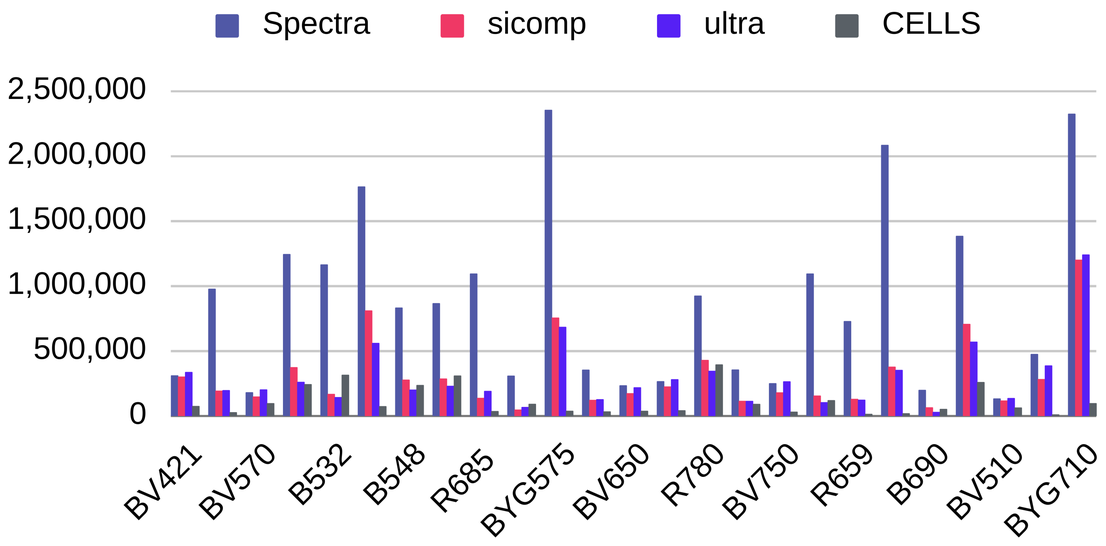


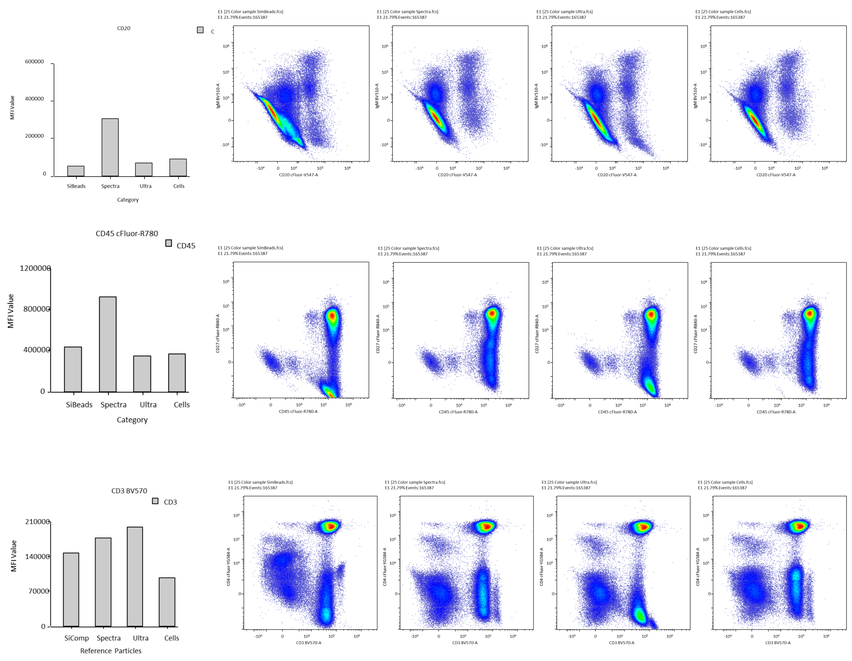
Figure 1: Median fluorescence intensity (MFI) comparison of 25-colour immunoprofiling panel with compensation controls. (A) SpectraComp®. (B) SiComp. (C) UltraComp ebeads™ . (D) Cells.

Condition #2 states that the reference control's positive signal should be at least as bright as the cells in the assay, however the SiComp beads were not as bright for 3 fluorophores (B532, BYG610, V547) and the Ultracomp ebeads™ were not as bright for 5 fluorophores (B532, B548, BYG610, V547, R780). Our hypothesis is two fold: (1) Certain beads may not be saturated with antibodies at concentrations found in the pre-optimised immunophenotyping panel, resulting in suboptimal binding and hence lower signals. (2) Polystyrene-based beads interact with certain dyes (possibly via FRET) which quenches the fluorescent signal or alters the spectral signature of the dye itself. Both these will ultimately lead to unmixing errors.

SPECTRAL UNMIXING ERRORS

Using FlowLogic Software, we analysed the data in relation to unmixing errors. A number of anomalies were found, and we will highlight three examples below.

CD20 conjugated to cFluor-V547 had lower MFI values than cells in the SiComp and Ultracomp beads. Conversely these controls also show some level of unmixing errors, which may be a result of these lower MFI values.
CD45 conjugated to cFluor R780 was found to give slightly lower MFI values for Ultracomp only. However again we found that both polystyrene-based beads show unmixing errors for this fluorophore.
Finally, for CD3 conjugated to BV570, all controls showed higher MFI values than cells, however again both polystyrene-based beads are exhibiting unmixing errors.



FlowLogic SSI Report	IgM BV510-A
Cells CD20 cFluor-V547-A	0.694
SiBeads CD20 cFluor-V547-A	-6.825
Spectra CD20 cFluor-V547-A	0.131
Ultra CD20 cFluor-V547-A	-4.833

FlowLogic SSI Report	CD27 cFluor-R840-A
Cells CD45 cFluor-R780-A	0.378
SiBeads CD45 cFluor-R780-A	-3.106
Spectra CD45 cFluor-R780-A	0.444
Ultra CD45 cFluor-R780-A	-4.703

FlowLogic SSI Report	CD4 cFluor-YG584-A
Cells CD3 BV570-A	0.095
SiBeads CD3 BV570-A	-5.771
Spectra CD3 BV570-A	0.038
Ultra CD3 BV570-A	-5.111

Figure 2: MFI comparisons, unmixed plots, and SSI (Secondary Stain Index) reports showing unmixing errors for (A) CD20 V547 vs IgM BV510. (B) CD45 R780 vs CD27 R840. (C) CD3 BV570 vs CD4 YG584.

ANALYTICAL VALIDATION

Unmixing With Cells Versus Polystyrene Beads and Hydrogel Particles

Total numbers of unmixing errors with each reference control were compared across the 25-Color Immunoprofiling Assay and normalized to a single number of total errors per control. The unmixing errors were corrected with a conventional compensation matrix, the resulting values were added together and the final figure gave an estimate of the total errors for each reference control. Both cells and SpectraComp® displayed a minimal amount of unmixing errors whereas both polystyrene-based beads showed a substantially larger amount of errors. We suspect this is due to some interaction of the polystyrene particle with the dye. Interestingly we found that Ultracomp and SpectraComp® particles shifted their negative control value between unstained and stained controls, therefore we highly recommend that universal unstained controls be included with every batch of reference controls.

Figure 3: Spectral unmixing error comparison between cells and compensation particles for use as reference controls. (A) Cells (B) SlingShot SpectraComp® particles (C) Thermofisher UltraComp eBeads™ (D) SiComp beads

Figure 4: Indicative graph showing the shift in negative values using SpectraComp® and Ultracomp eBeads™

CONCLUSION

Using single colour reference controls is essential in high dimensional cytometry, and minimising unmixing errors will ensure that the data is of the highest quality. We have shown that polystyrene-based beads are not always as bright as cells for certain fluorophores and have a higher rate of unmixing errors. Despite polystyrene particles generally being as bright or brighter than cells, they still result in significant unmixing errors, suggesting some other interaction of the bead with the dye. SpectraComp® particles satisfy all 5 conditions of the rules of compensation/unmixing as a better overall cellular mimic, therefore using these particles will ultimately improve the quality of your data.