A Comparative Study of Particle Controls for Improving Unmixing in High-Parameter Flow Cytometry

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ABSTRACT

With the ability to delve deep into the complexities of biology, high-parameter flow cytometry has revolutionized the field, paving the way for groundbreaking discoveries. However, amidst its triumphs, there are hurdles that need to be overcome, such as the accuracy of unmixing and compensation in high-parameter panels. This need for better accuracy in high-parameter panels has become increasingly apparent once users attempt to standardize flow cytometry assays. While cell-based controls have long been considered the gold standard for achieving this, the limitations become evident when dealing with rare cell types and low antigen expression. In such cases, polystyrene beads have become a gap fill. However, these introduce compensation errors and unmixing inaccuracies in multicolor samples which require re-optimization.

We postulate that the charge on polystyrene plays a role in the observed inaccuracies in multicolor cell samples. Thus, Slingshot Biosciences has developed an uncharged hydrogel-based capture particle as a third option for single stained controls. These gels are intended to improve the quality of compensation and unmixing for particular fluorophores by reducing charge-based interactions. To test these claims, we evaluated the accuracy of both gels and polystyrene based particles against single stained cells on two panels. We measure accuracy with the Secondary Stain Index (SSI), a metric for identifying compensation/unmixing errors above a threshold. This metric allows one to quantify the degree of over/under-compensation and unmixing inaccuracy on single stained cells with particular control types.

We used a panel derived from both OMIP-71 and OMIP-69, two optimized phenotyping panels, as well as CD4 conjugated antibodies on the same fluor set. To evaluate unmixing errors, we unmixed single stained cells acquired on 3 different instruments using either cells, Slingshot's CD4+ TruCytes™ (for epitope-specific hydrogel binding), anti-Fc capture polystyrene beads, or Slingshot's anti-Fc capture gels. Using this analysis of fluorophore-antibody combinations we seek to further standardize high-parameter flow cytometry by quantifying trends to overcome present challenges in reproducibility. We also hope to find additional reasons why errors are inconsistent across panels and instruments.

METHODOLOGY

Panel Design

A 25 color panel was derived from a combination of antibodies used in OMIP-71, with alternatives taken either from OMIP-69 or tested in-house on the Cytek Aurora. The panel was designed to be compatible with a BD A5SE, Cytek Aurora 5L, and SONY ID7000 5L. The equivalent fluorophore set on CD4 antibodies was purchased in tandem to assess antibody-specific differences.

Panel Optimization

Titers from publications were validated in-house using a Leukopak provided by AllCells. To verify titers, 400,000 cells were stained in 100 uL of a staining cocktail made in staining buffer (BD Pharmingen) and staining performance was compared against OMICS-Guard and IC Fixation buffer. To maintain staining performance across plates, 83000 CD4+ TruCytes®, 46 uL of HyParComp, and 50 uL of UltraComp were used for each single stained synthetic sample respectively. Single stained cells, multicolor samples, and stained synthetic particles were tested for performance in staining buffer (BD Pharmingen), OMICS-guard (BD Pharmingen), and IC Fixation Buffer (ThermoFisher).

Shipment and sample acquisition

Samples were shipped to Universities of Pennsylvania, Michigan, and Chicago in 50 uL of OMICS-guard buffer per well in a V-bottom plate. Plates were foil sealed tightly, and packaged with cardboard prior to shipment overnight on ice. BD A5SE samples were resuspended in tubes for running, the remainders were ran in the original plate. 100 uL of buffer was added to ensure adequate running volume

Panel 1 was run 1 day after shipment, and the CD4 panel was run 2 days after shipment. Parallel runs of the same stained samples were performed at Slingshot headquarters to compare differences introduced in the shipping process.

Data Analysis

Cytek Aurora data was unmixed in SpectroFlo, ID7000 data was unmixed on the SONY ID7000 software, and BD A5 data was unmixed in FlowJo using Autospill. Autofluorescence was extracted using the lymphocyte population. Each single stained control was gated for positive and negative populations in FlowJo. Positive MFI, negative rSD, and negative MFI, were exported as CSVs.

SSI was calculated on cells unmixed with either Cell, Hydrogel, UltraComp, or TruCyte single stained controls (where applicable) per instrument. All data was gated identically in FlowJo for exporting statistics. Secondary Stain Index calculations were performed using R on the exported CSVs.

Panel Design*

Fluor	BUV395	Zombie UV	BUV496	BUV615	BUV737	BUV805
Marker	CCR4	Live/dead	CCR6	NKG2D	CD161	CD3
Clone	1G1	N/A	11A9	1D11	DX12	UCHT1

Fluor	BV421	V450	BV510	BV570	BV605	BV650	BV711	BV750	BV786
Marker	CD25	CD45	CXCR3	CD4	HLA-DR	CD56	CD103	CXCR5	CD8
Clone	M-A251	HI30	G025H7	RPA-T4	G46-6	NCAM16.2	2 E 7	RF8B2	RPA-T8

Fluor	BB515	BB700	RB780
Marker	PD-1	CXCR6	CD69
Clone	M-A251	13B 1E5	FN50

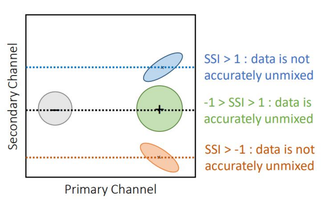
Fluor	PE	PE-CF594	PE-Cy5	PE-Cy7
Marker	CD28	CD45RO	CD95	CD7
Clone	CD28.2	UCHL1	DX2	M-T701

Fluor	APC	APC-R700	APC-Cy7
Marker	TCR γδ	CD127	CCR7
Clone	B1	HIL-7R-M21	G043H7

*CD4 set uses the same fluors on clone RPA-T4

Secondary Stain Index[1]

$$SSI = \frac{median\ ^{unmix}_{pos\ sec} - median\ ^{unmix}_{neg\ sec}}{2 \times rSD^{unmix}_{neg\ sec}}$$



RESULTS

Number of observed unmixing errors differ across instruments and antibody sets. Hydrogels exhibit fewer or equivalent errors to polystyrene on the Aurora, but more errors on the A5SE. The ID7000 has fewer errors than either and shows mixed results between panels.

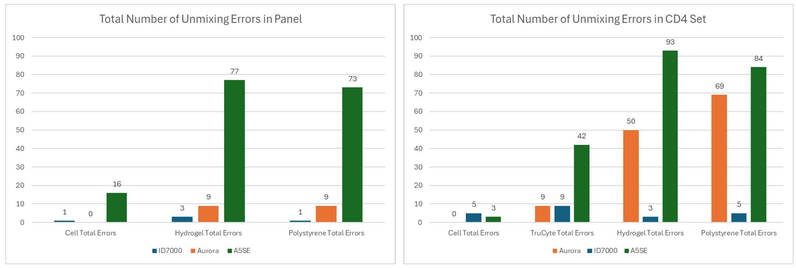


Figure 1: Total number of observed unmixing errors above threshold across all instruments unmixed with the respective particle type. Note that the A5SE experienced red laser failures during the run, thus there is no CD4 data for APC, APC-R700, and APC-Cy7 on the A5.

TruCytes® (epitope binding) particles show fewer errors compared to anti-Fc capture particles on the A5SE and Aurora, but not the ID7000. More errors are found in the far red emitters and Blue/YG laser excited fluors on the CD4 fluor set.

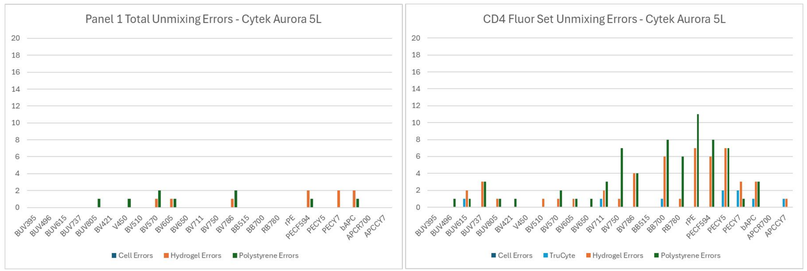


Figure 2a: Cytek Aurora data for the panel and CD4 equivalent.

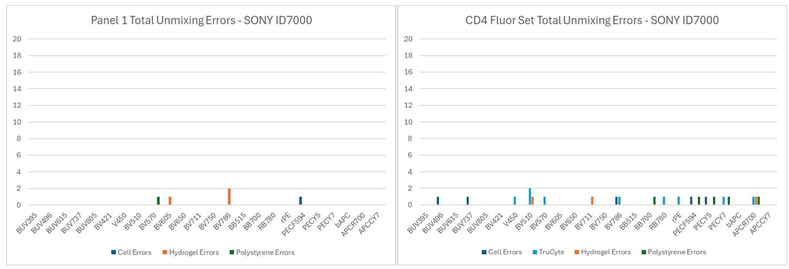


Figure 2a: SONY ID7000 data for the panel and CD4 equivalent.

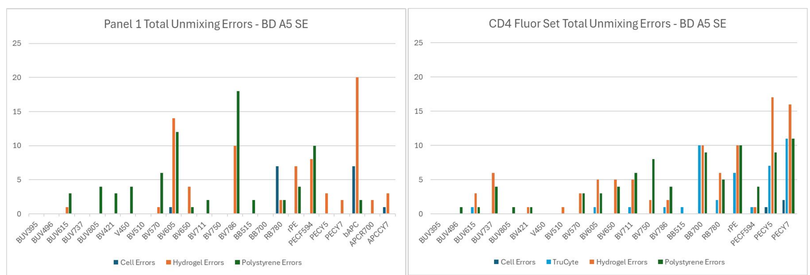


Figure 2c: BD A5SE data for the panel and CD4 equivalent. Note that the red laser failed during the CD4 set acquisition, thus CD4 APC, APC-R700, and APC-Cy7 could not be analyzed.

CONCLUSIONS

Our original aim was to elucidate the patterns associated with unmixing cells using hydrogels vs. polystyrene single stained controls due to charge. Our results found that trends can vary significantly by instrument/unmixing method, rather than particle charge. The major difference in calculation is the use of Ordinary Least Squares (OLS) on the Cytek Aurora and BD A5SE vs Weighted Least Squares (WLS) on the SONY ID7000. It is possible that this contributes to the number of errors, but data on a universal unmixing platform capable of both methods is needed.

Our results also suggest that the antibody conjugated to the fluor has some impact on the number of unmixing errors. When comparing the total error distribution between panels, we see an increase in the number of errors found on the CD4 fluor set vs. the OMIP derived panel 1. Given that CD4 is a strongly expressed marker compared to many markers in Panel 1, it is possible that the overall brightness plays a role in the calculated SSI value.

Previous results have shown instances where hydrogels show fewer SSI errors than polystyrene based particles on the Cytek Aurora (2). In the panels here, we similarly see fewer errors in BV711, BV750, PE, and PE tandems (except for 1 instance). However, this trend does not repeat across other instruments, suggesting that there may be discrepancies due to the instrument. A complication arises as the A5SE red laser failed on the day of the CD4 run, so some data could not be analyzed.

Finally, we see a major difference in the number of errors on CD4+ cells when CD4+ TruCytes® are used as single stained controls on the Aurora and A5SE. This implies that a major factor in the accuracy of a single stained control for these two datasets is the binding site, as both cells and TruCytes® bind the Fv region of the antibody instead of the Fc region.

A limitation on the study is the diversity of unmixing algorithms that prevent true head to head comparisons between particle performance. An unmixing platform that incorporates all variations of unmixing would allow better comparisons between the particles. Future studies will also look at how spectra normalize between cells and synthetics to further elaborate on where discrepancies occur and how these influence errors.

Citations

1. **Daniels, Kathy, Gardner, Rui, Morgado, Jose Mario. Secondary Stain Index, api.fccf.mskcc.org/uploads/FPI_sec_stain_index_09261cd1d9.jpg.**
2. **Dankar, Amay. Optimizing Controls for Flow Cytometry. Slingshot Biosciences, 15 November. 2023. Webinar. https://fluorofinder.com/webinars/**

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